CATABOLISM OF FOLATE IN MAN AND SYRIAN GOLDEN HAMSTER

BY

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SUMMARY

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The metabolism of a mixture of [2-^{14}C] and [3',5',7,9,-^{3}H] - folic acid has been studied in man and in Syrian golden hamster. Intact folates labelled with both ^{14}C and ^{3}H are excreted in urine. Breakdown products of folic acid labelled with ^{3}H only as folate catabolites were found in all urine samples. Small amounts of metabolites corresponding to the part of the molecule containing ^{14}C were detected as well. Folate radioactive polyglutamates were present in the liver. A small amount of the ^{14}C dose was found in the expired air. P-acetamidobenzoic acid was the major catabolite in the urine of the hamster while p-acetamidobenzoyl-L-glutamate was the minor. In man p-acetamidobenzoyl-L-glutamate was the major urinary catabolite. Following the administration of xanthopterin, allopurinol, dihydro-orotic acid and growth hormone (somatotropin), differences were seen in folate handling. The percentage of dose found in the liver was similar in all four conditions. The percentage of scission products appearing in the urine related to the liver breakdown was different. Allopurinol, dihydro-orotic acid and somatotropin greatly increased the excretion of ^{3}H - labelled fragments and apparently stimulated folate catabolism. A possible mechanism of folate breakdown in the tissues is suggested to be a simple chemical oxidative cleavage of the C_9-N_{10} bond of labile

folate derivatives produced during the normal metabolic pathways by small species such as the superoxide anion ($O_2^{\div}$) formed by these agents in vivo. Xanthopterin administration produced a slight increase in folate catabolism. Xanthopterin induced kidney hypertrophy in the hamster is not due to inhibition of folate catabolism. As known inhibitors of xanthine oxidase (allopurinol, xanthopterin) increase folate catabolism, xanthine oxidase cannot directly bring about folate catabolism. A patient with malignant disease excreted less radioactivity in urine and less folate catabolites compared with a non malignant patient. In a patient with chronic alcoholism, the excretion of urinary radioactivity was greatly increased compared to non alcoholics and showed little change in folate catabolism. The effect of a low dose of 10-formylfolate on dihydrofolate reductase was studied in man using oral administration of unlabelled 10-formylfolate and radiolabelled folate.

The appearance of some labelled folic acid in the urine indicated some in vivo inhibition of the reduction of folic acid by dihydrofolate reductase.

KEY WORDS

Folate metabolism, Folate catabolism, 10-formylfolate, Malignant disease, Chronic alcoholism

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To my husband

CONTENTS

ABBREVIATIONS:

FA = folic acid

10-CHOFA = 10-formyl folic acid

5-CH_3THF = 5-methyltetrahydrofolate

p-AcABG = p-acetamidobenzoyl-L-glutamate

p-AcABA = p-acetamidobenzoic acid

CHAPTER 1

Introduction

Contents

1.1 Folates; structures, coenzymes, interconversions and metabolic functions

Folates are a group of naturally occurring compounds widely distributed in all living organisms. They are derived from the reduction of and, addition to, the parent compound folic acid (Figure 1-1), which is composed of a pterin ring linked with a p-aminobenzoyl moiety to a glutamate residue (Turner, 1977; Blakley, 1969; Rowe, 1978, 1983). The term folic acid was introduced in 1940 to designate a nutritional factor isolated from spinach leaves which was essential for the growth of Streptococcus faecalis (Mitchell et al., 1941). Folate derivatives differ from each other in regard to the substitution of a one carbon group at position N5 or N10 or both, the state of reduction of the pterin nucleus and the number of glutamyl units (Blakley, 1969; Butterworth et al., 1963). The folates function as coenzymes in a variety of enzymatic reactions that entail the transfer of a single carbon unit in the synthesis of purines, a pyrimidine, methionine from homocysteine, and the serine-glycine conversion. Formylation and oxidation-reduction of other folate derivatives also occurs (Blakley, 1969; Hoffbrand, 1976; Chanarin, 1969, 1979; Rowe, 1978, 1983). A scheme (1) shows the metabolic pathways of folic acid and its derivatives in mammals. The major metabolic pathways of folic acid and its reduced derivatives have been established in a variety of prokaryotic and eukaryotic cells (Blakley, 1969). The folate pathway is a complex series of interlocking enzymatic reactions which generate a number of relatively unstable folate coenzymes critical for several important biosynthetic reactions.

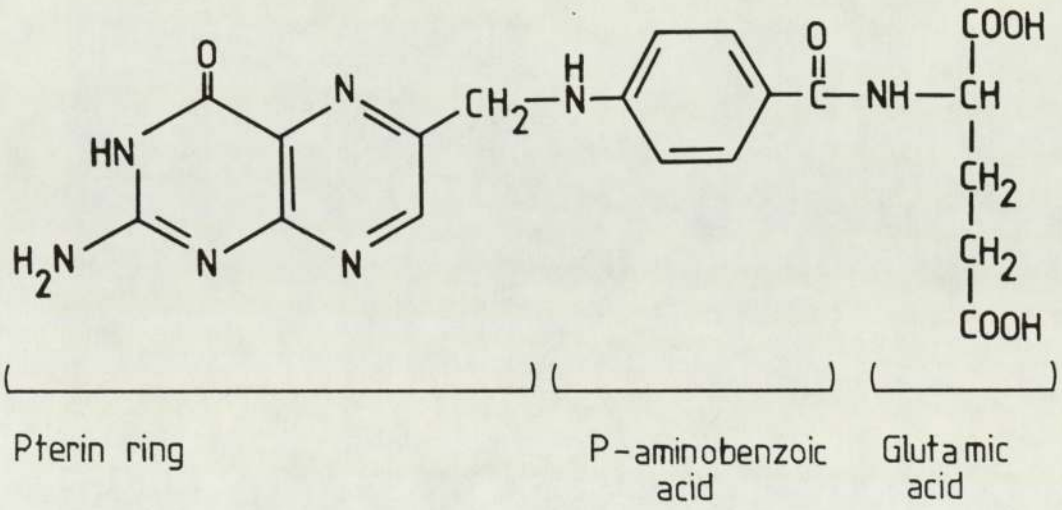
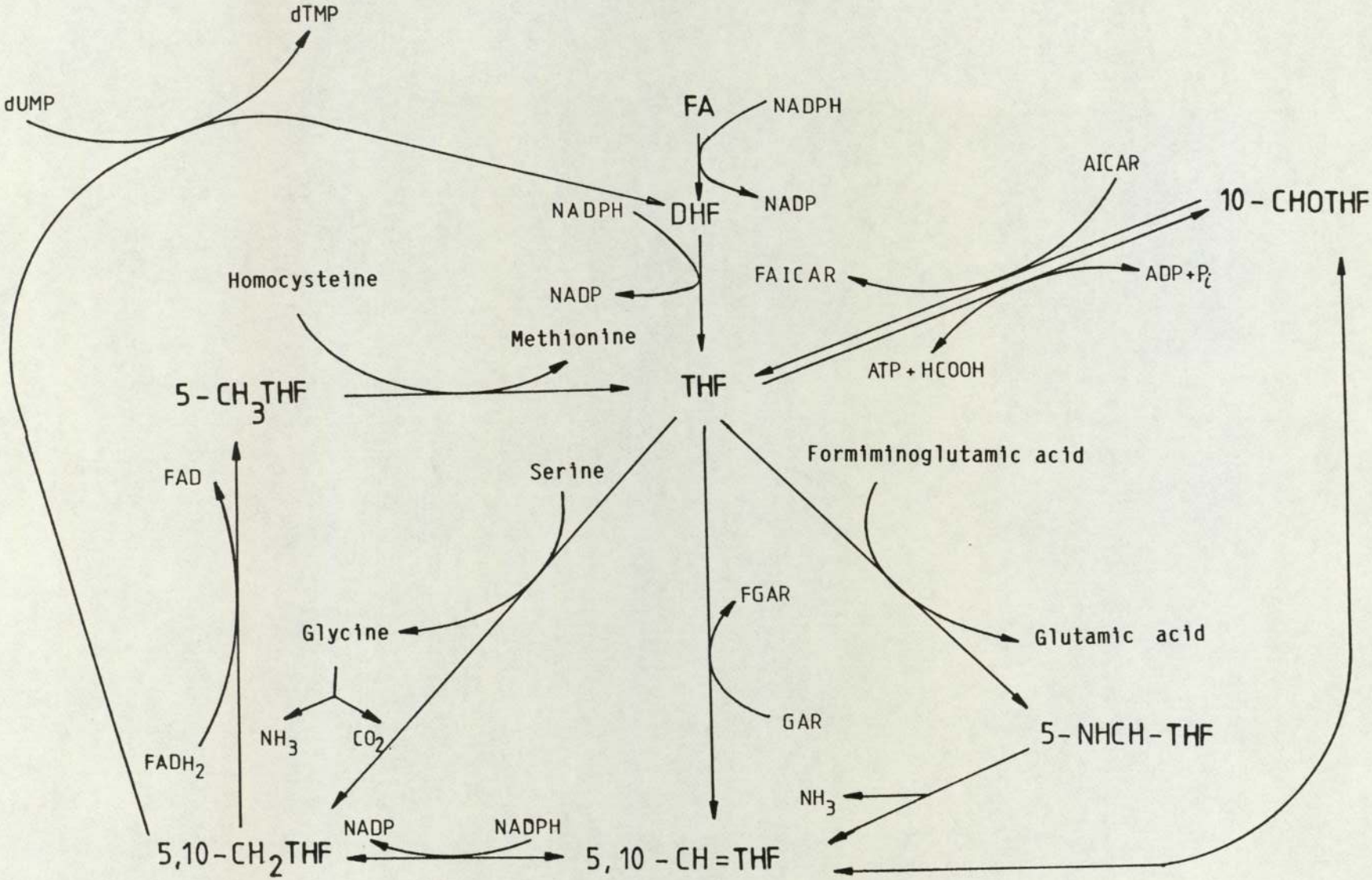


Figure 1-1 : Folic acid

ABBREVIATION

DHF	=	Dihydrofolate
THF	=	Tetrahydrofolate
5 - CH_3THF	=	5-methyltetrahydrofolate
10 - CHOTHF	=	10-Formyltetrahydrofolate
5 - NHCH-THF	=	5-Formiminotetrahydrofolate
5,10 - CH = THF	=	5,10-methenyltetrahydrofolate
5,10 - CH_2THF	=	5,10-methylenetetrahydrofolate
AICAR	=	5-amino-4-imidazole carboxamide ribonucleotide
FAICAR	=	5-formamido- 4-imidazole carboxamide ribonucleotide
GAR	=	Glycinamide ribonucleotide
FGAR	=	Formyl glycinamide ribonucleotide
dUMP	=	deoxyuridine Monophosphate
dTMP	=	deoxythymidine Monophosphate
NADP	=	Nicotinamide adenine dinucleotide phosphate
NADPH	=	Reduced _ Nicotinamide adenine dinucleotide phosphate
FAD	=	Flavin adenine dinucleotide
$FADH_2$	=	Reduced – flavine adenine dinucleotide
ADP	=	Adenosine diphosphate
ATP	=	Adenosine triphosphate



Scheme 1 : The major folate interconversion pathways

Folic acid itself is not biochemically active, but becomes so after it is reduced to 7,8-dihydrofolic acid (DHF) (Figure 1-2) and to 5,6,7,8-tetrahydrofolic acid (THF) (Figure 1-3) by the dihydrofolate reductase enzyme [5,6,7,8-THF:$NADP^+$ oxidoreductase (EC 1.5.1.3)] (DHFR) with NADPH as hydrogen donor (Chanarin, 1979; Harper, 1975) (Figure 1-4).

Dihydrofolate reductase is a key enzyme in one-carbon metabolism. It is widely distributed in most mammalian tissues, being found in liver, kidney, embryonic tissues, leukocytes of patients with leukaemia, in certain tumour cells and in various micro-organisms (Blakley, 1969; Rowe, 1983). In mammals, this enzyme is largely localized to the cytosol with a small amount of activity in the nucleus and the mitochondria.
Recently, Colman and Herbert (1980) have found this enzyme in the brain of rabbit, calf and rat. DHFR consists of a single non-aggregating polypeptide chain of around 20,000MW in all species (Futterman and Silverman, 1957; Zakrzewski, 1960; Rowe, 1978, 1983). The complete amino acid sequences of the enzymes from a number of bacterial and animal cells are known (Benkovic, 1980). DHFR - central to folate derivative flux - is the target of antimicrobial and anticancer drugs (Bertino, 1971).

DHF is formed during the synthesis of thymidylate, while THF is converted to a series of one-carbon folate derivatives, and is a key metabolic folate (McGuire et al., 1979). These interactions of THF are illustrated in Figure (1-5). THF adds a CH_2 group to form 5,10-methylenetetrahydrofolate (5,10-CH_2THF).
The 5,10-CH_2THF (Figure 1-6) forms thymidine in a reaction which is held to be the rate limiting factor for DNA synthesis in the presence of thymidylate synthetase (EC 2.1.1.6) by transfer of a

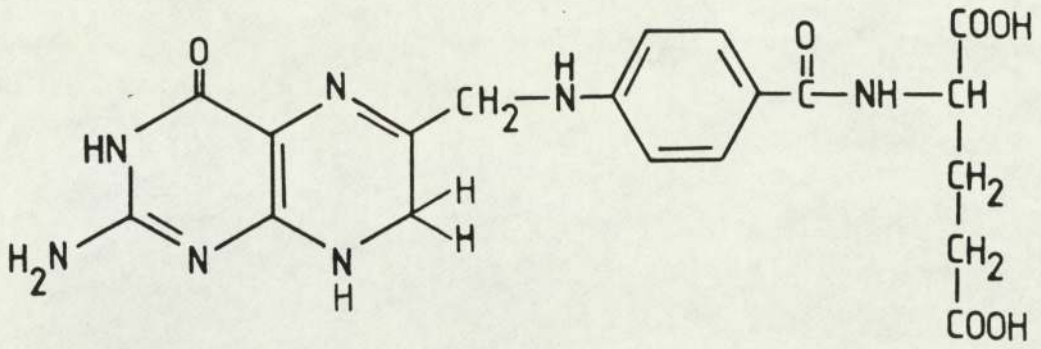


Figure 1-2 : Dihydrofolic acid

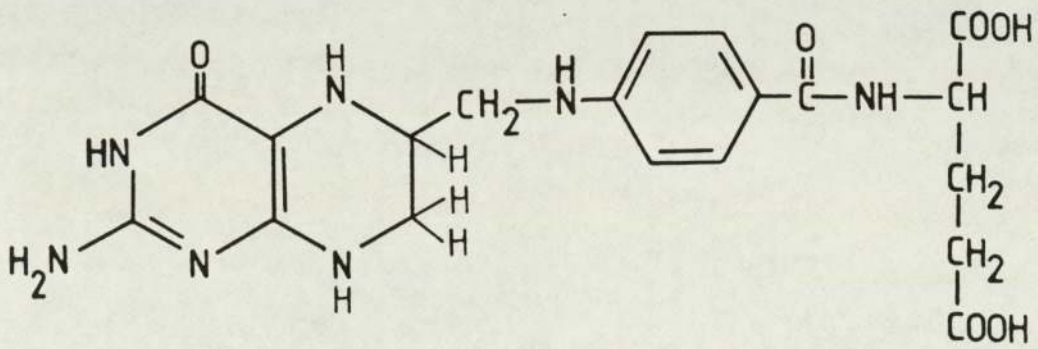


Figure 1-3 : Tetrahydrofolic acid

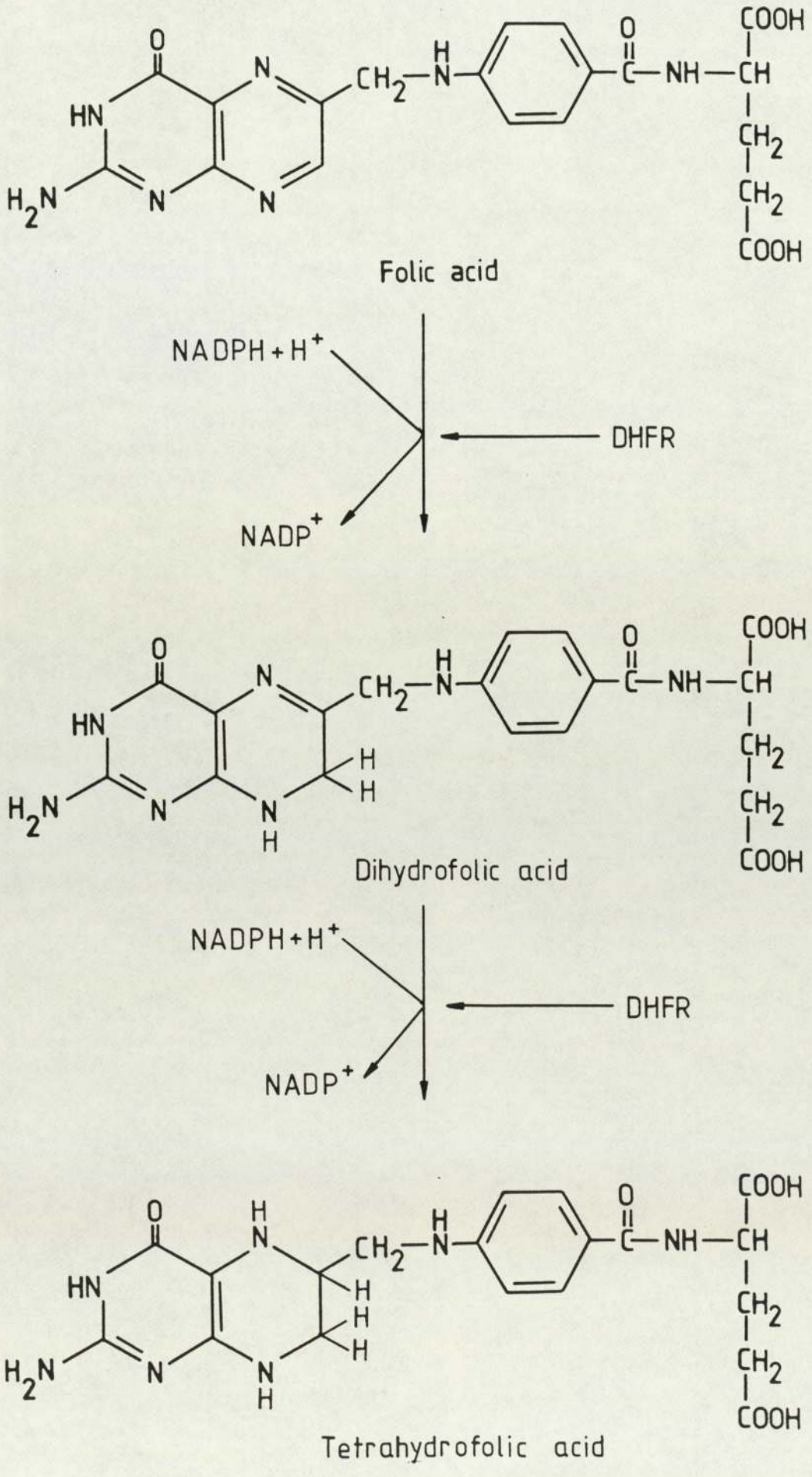


Figure 1-4 : Reduction of folic acid to its coenzymes

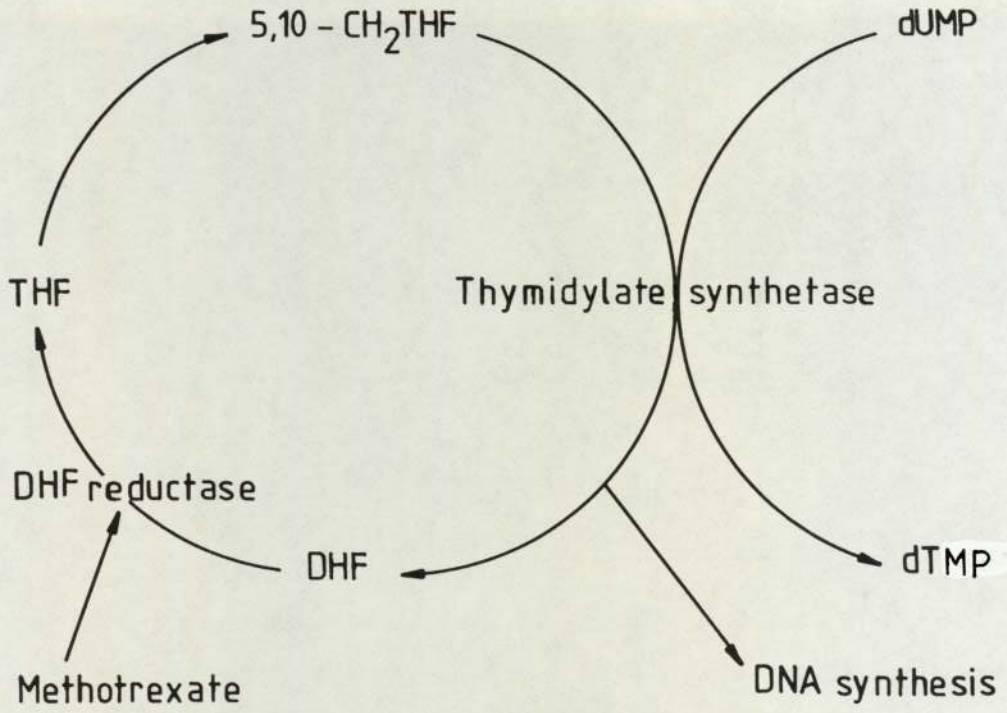


Figure 1-5 : The methylation of deoxyuridine catalyzed by thymidylate synthetase is accompanied by formation of DHF which inturn is reduced to THF by DHF reductase

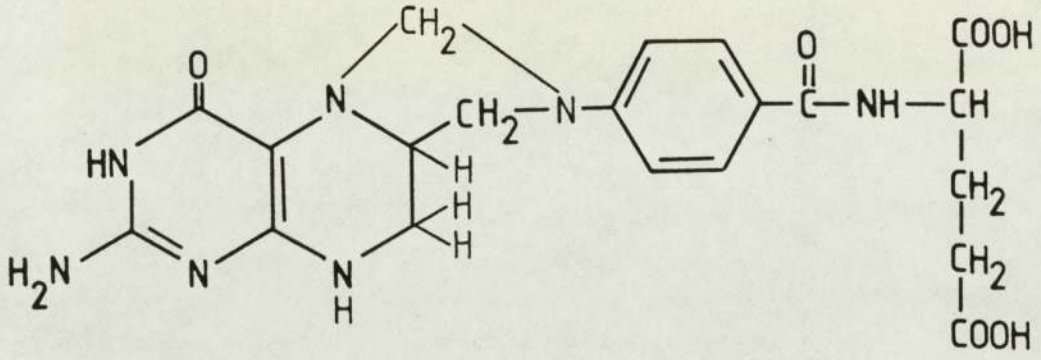


Figure 1-6 : 5,10-methylenetetrahydrofolic acid

($5,10\text{-}CH_2THF$)

methylene group to deoxyuridine (Figure 1-7) and its simultaneous reduction to CH_3 (Chanarin, 1979; Rowe, 1983). The hydrogen is derived from position 6 on the pterin ring of 5,10-CH_2THF (Pastore and Friedken, 1962) to generate DHF, which is later reduced by DHFR to THF. Thus thymidylate synthetase is coupled with DHFR to operate in sequence in the synthesis of thymidine and THF. 5,10-CH_2THF functions as the one-carbon unit carrier at the formaldehyde level of oxidation. Another sequence of reactions of THF is concerned in the origin of carbon 2 and 8 of the purine nucleus (Figure 1-8). 5,10-CH_2THF is dehydrogenated by 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) to form 5,10-methenyltetrahydrofolate (5,10-CH=THF) (Figure 1-9). Hydrolysis of 5,10-CH=THF gives 10-formyltetrahydrofolate (10-CHOTHF) via the enzyme 5,10-CH=THF cyclohydrolase (EC 3.5.4.9) (Chanarin, 1969).

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

10-Formyltetrahydrofolate (Figure 1-10), transfers formate to glycinamide ribonucleotide (GAR) using GAR transformylase (EC 2.1.2.2) (Figure 1-11) (Dev and Harvey, 1978a). 10-CHOTHF also involves the donation of a one carbon atom from 10-CHOTHF to form 5-amino-4-imidazole carboxamide ribonucleotide (AICAR) by the enzyme AICAR transformylase (EC 5.3.1.16) (Figure 1-12). Subsequently the ring is closed to form inosinic acid (Chanarin, 1969, 1979).

In each of these reactions the 10-CHOTHF loses the formyl group and is converted back to THF. Methylenetetrahydrofolate dehydrogenase and cyclohydrolase activities are present on a single protein which has been isolated from E. coli (Dev and Harvey, 1978b), pig liver (Mackenzie, 1973; Tan et al., 1977; Mackenzie and Baugh, 1980), chick liver (Caperelli, et al, 1980) and from yeast (Paukert et al., 1977).

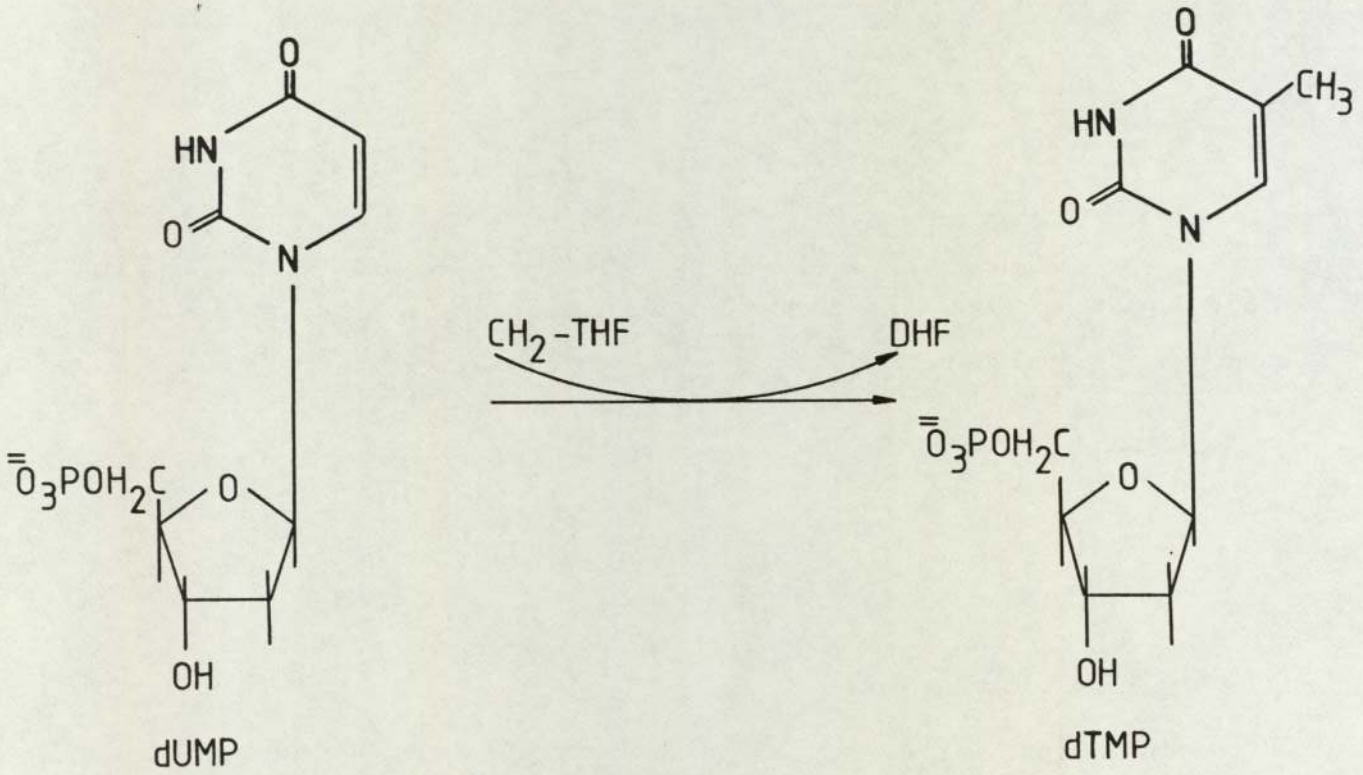


Figure 1-7 : The formation of thymidine from uridine

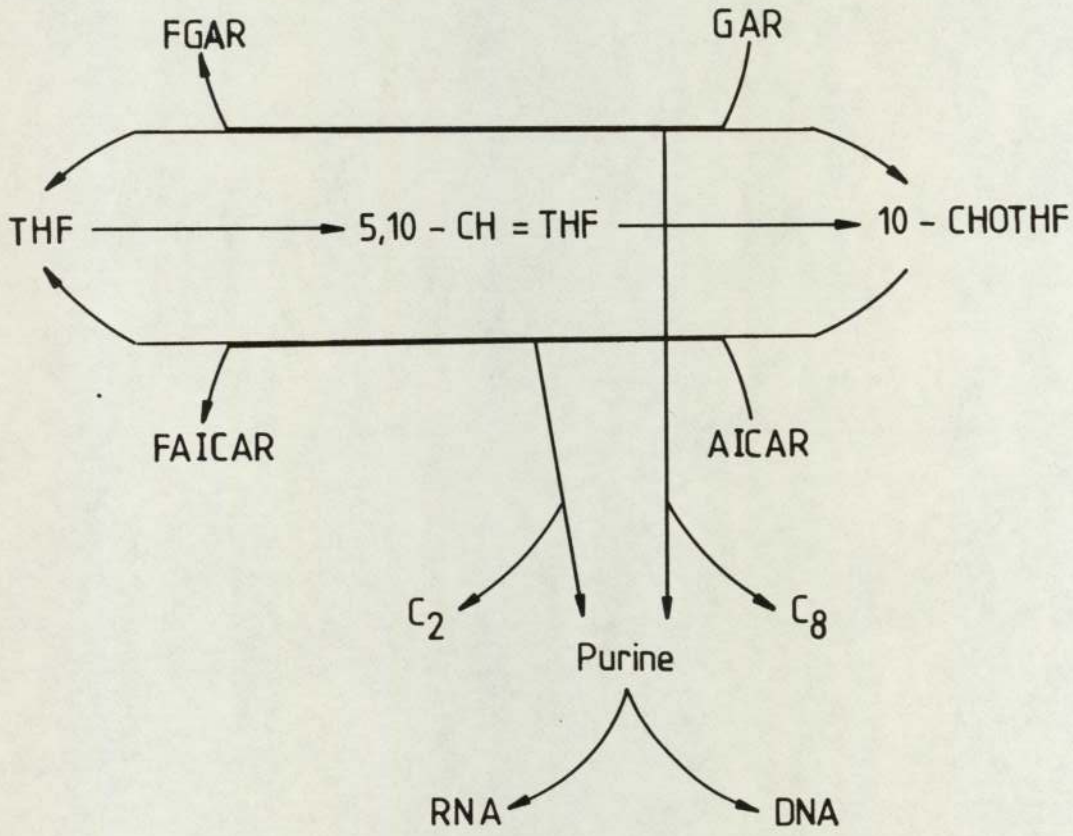


Figure 1-8 : purine biosynthesis

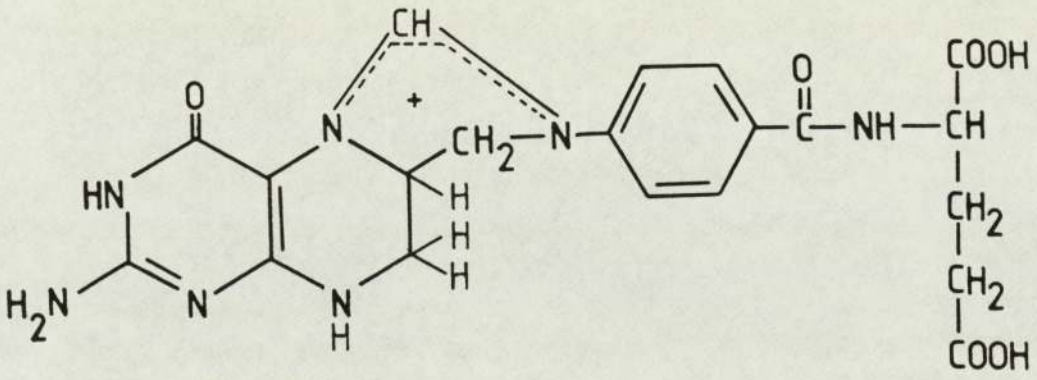


Figure 1-9 : 5,10-methenyltetrahydrofolic acid

(5,10-CH =THF)

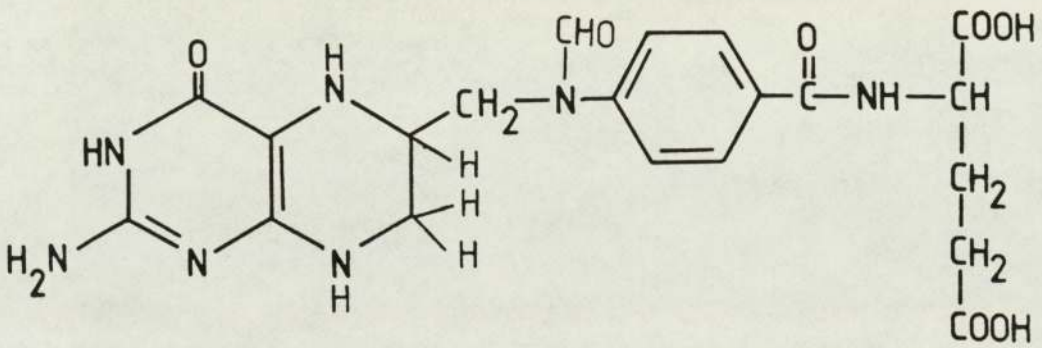


Figure 1-10 : 10-formyltetrahydrofolate (10-CHOTHF)

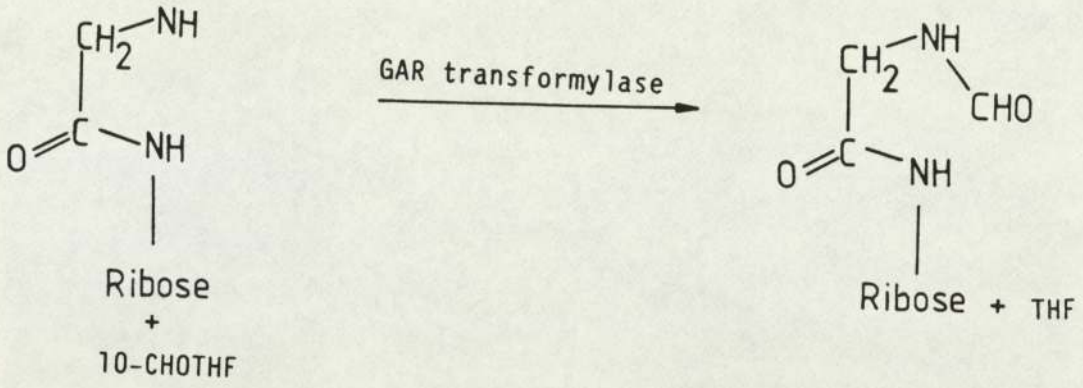


Figure 1-11 : Insertion of Carbon-8 of the purine nucleus

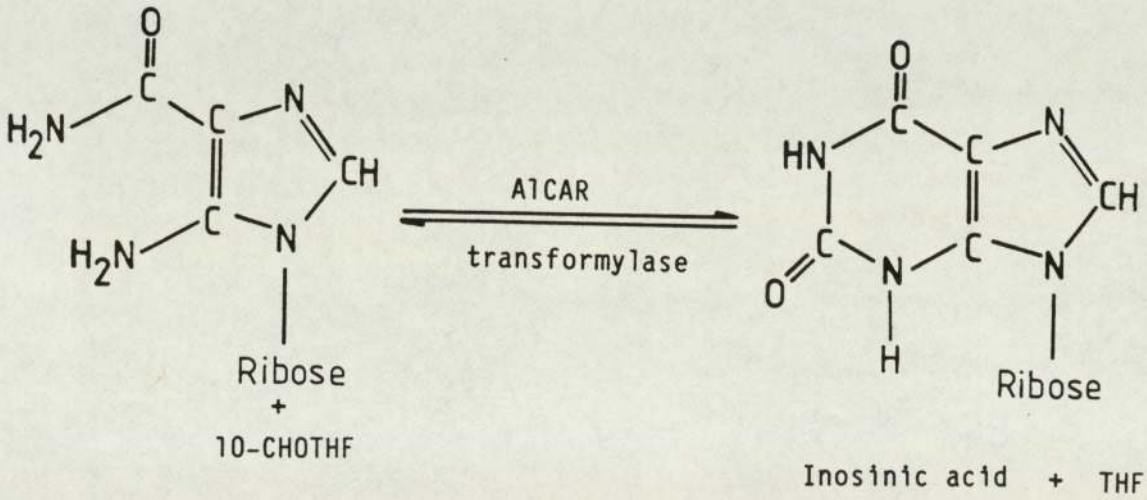


Figure 1-12 : Closure to purine nucleus by addition of Carbon-2

Although THF can be regarded as the key species in the folate metabolic cycle, other folate coenzymes such as 5-methyltetrahydrofolate (5-CH_3THF) (Figure 1-13) are widely distributed. This species is concerned with the synthesis of methionine, a reaction in which vitamin B_{12} coenzyme participates (Chanarin, 1979). The entry of this compound into THF pool involves the methylation of homocysteine to methionine (Figure 1-14) by transfer of a methyl group from 5-CH_3THF, a transfer catalyzed by the enzyme [5-methylTHF:homocysteine methyltransferase (EC 2.1.1.13)] (methionine synthetase) which contains B_{12} as coenzyme in the presence of a catalytic level of S-adenosylmethionine (SAM) and a reduced flavine-adenine dinucleotide ($FADH_2$) (Mangum and Scrimgeour, 1962; Taylor et al., 1974; Hatch, et al., 1961; Kisliuk, 1961; Chanarin, 1969; Rowe, 1978, 1983). The tetrahydrofolate regenerated from this reaction is then recycled back to 5-CH_3THF by picking up one carbon atom fragment to form 5,10-CH_2THF, and this is reduced to 5-CH_3THF (Hillman and Steinberg, 1982; Blakley, 1969). Mudd and Poole (1975), have reported that this process provided about 50% of methionine which is required in the normal metabolism in man. Methionine synthetase was found to be an active enzyme in the regulation of the metabolism of the activated C_1-units (Saur , 1979).

The interconversion of serine and glycine occurs in which the β-carbon atom of serine is transferred to THF with formation of 5,10-CH_2THF and glycine with the enzyme serine transhydroxymethylase (EC 2.1.2.1) (Blakley, 1969; Schirch et al., 1977).

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

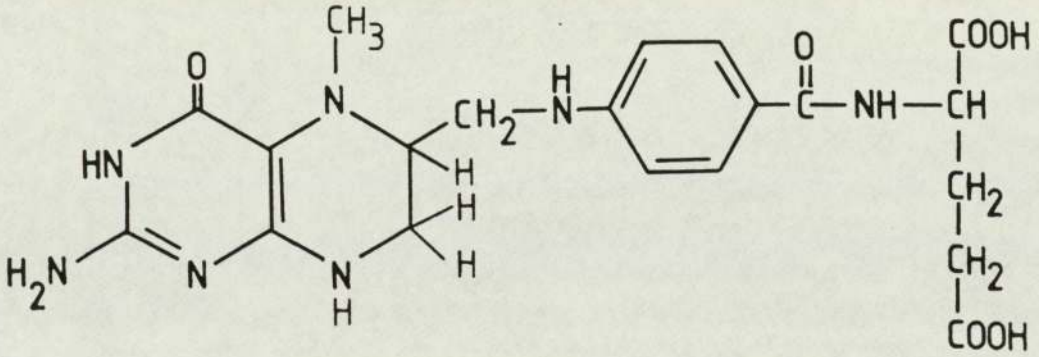
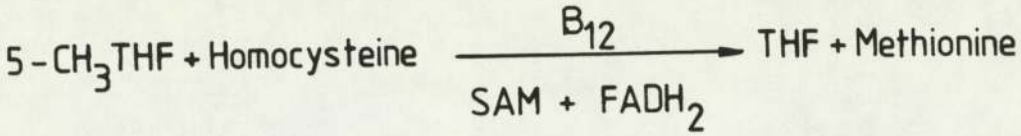
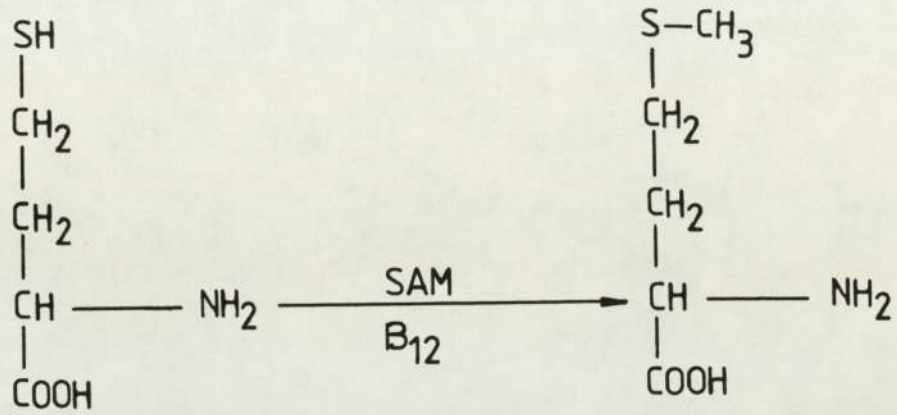


Figure 1-13 : 5- methyltetrahydrofolic acid





Homocysteine

Methionine

Figure 1-14 : Methylation of homocysteine to methionine

This reaction is a major source of the one-carbon units needed for biosynthesis (Kretchman and Price, 1969). The most important source of serine and glycine is carbohydrates or closely related compounds (Blakley, 1969; Chanarin, 1979). The reversible interconversion of serine and glycine has been demonstrated to occur in the absence of THF, but the rate of serine degradation and synthesis is reduced by about four and three orders of magnitude, respectively (Chen and Schirch, 1973).

All of these reactions require pyridoxal phosphate (Schirch and Gross, 1968; Chanarin, 1979; Ulevitch and Kallen, 1977; Schirch and Slotter, 1966). Serine transhydroxymethylase has been detected in the livers of animals and from plants (Blakley, 1969; Rowe, 1983). The bulk of this enzyme activity is in the cytosol, but, a genetically distinct enzyme is found in mitochondria (Davies and Johnston, 1973; McClain et al., 1975; Rowe, 1978, 1983). The incorporation of formate into serine by lymphocytes in cobalamin deficiency is diminished (Ellegard and Essman, 1973).

Formimino-L-glutamate (FIGLU) is an intermediate in the amino acid histidine catabolism. The degradation of FIGLU involves transfer of the formimino group to THF with the release of glutamic acid and ammonia (Miller and Waelsch, 1957; Slavik and Matoulkova, 1954). Therefore, the formyl moiety enters the one-carbon pool. 5-Formimino THF (5 CHNH-THF) (Figure 1-15) is formed from these reactions in the presence of enzyme formiminoglutamate tetrahydrofolate formiminotransferase (EC 2.1.2.5) but it is unstable, thus it is hydrolysed to 5,10-CH=THF by formiminotetra- hydrofolate cyclodeaminase (EC 4.3.1.4) (Tabor and Rabinowitz, 1956).

Formiminoglutamic acid + THF ⟶ 5-Formimino THF + glutamic acid

5-Formimino THF ⟶ 5,10-methenyl THF + NH_3

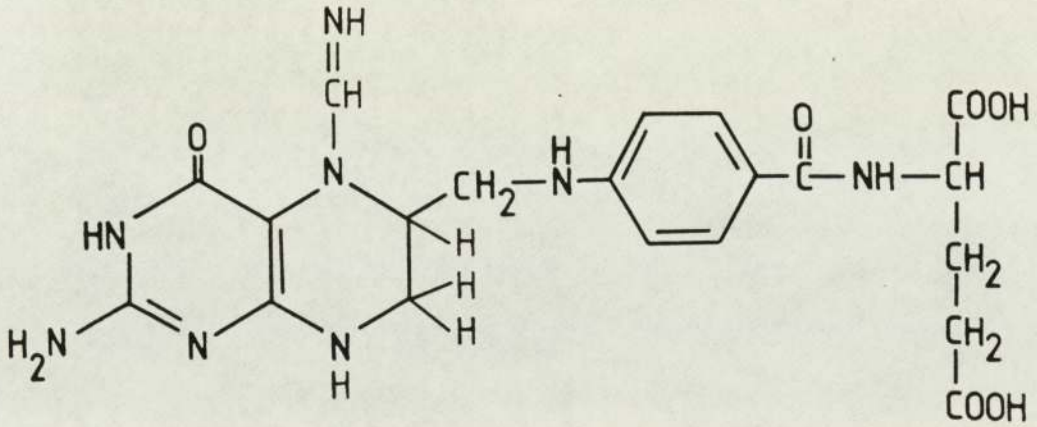


Figure 1-15 :5-Formiminotetrahydrofolic acid

The excretion of FIGLU in the urine is increased in folate deficiency, following a histidine load, reflecting a decreased availability of THF (Harper, 1975; Rowe, 1983).

1.2 Folate polyglutamates

The naturally occurring forms of folate are principally found in tissue as polyglutamates in which several additional glutamic acid residues are bound in peptide linkage to the α-carboxyl group (Hoffbrand, 1975; Connor and Blair, 1980) (Figure 1-16). The polyglutamate peptide chain is cleaved by a group of peptidases referred to as conjugase or more specifically, as α-glutamyl hydrolase (EC 3.4.22.12) (Silink and Rowe, 1975; Halsted, 1980a; Rowe 1983). These α-glutamyl carboxy peptidases have a very wide specificity with regard to the state of oxidation and substitution of the folic acid moiety (Blakley, 1969).

In mammals folate conjugases are present in high concentrations in pancreas, liver, kidney, spleen, brain (Bird, et al., 1945, 1946) and chiefly in the lysosomes. High concentrations of this enzyme are found in the mucosal cells of small intestine (Lavoie, et al., 1975) which play a vital role in the intestinal absorption of folate polyglutamates (Chanarin, 1979). The polyglutamate compounds are the principal intracellular forms of folate. The monoglutamates which are concerned with transport of folate within the body are the principal form in the extracellular fluid (Chanarin, 1979). They both have similar properties towards enzymes. The folate polyglutamates are active substrates for all the interconverting enzymes previously described (Kisliuk et al., 1974; Cheng et al., 1975; Coward et al., 1975; Blakley, 1969; Coward et al., 1974). Because of their higher intracellular concentration, they are the natural substrates and

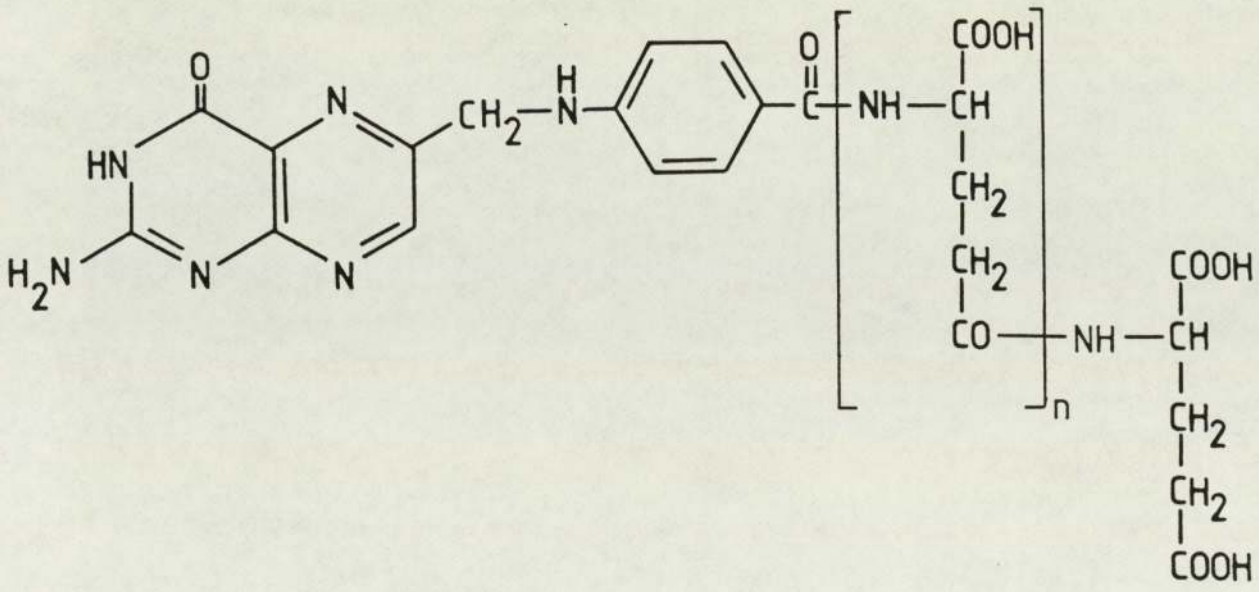


Figure 1-16 : Folic acid polyglutamates

n = 1 - 6

regulators of intracellular folate-dependent reactions (Turner, 1977). Mutant Chinese hamster ovary cells which were unable to make folate polyglutamates, were unable to grow without the addition of the end products of folate activity (glycine, adenosine and thymidine) (McBurney and Whitmore, 1974). The enzyme required for polyglutamate synthesis is pteroylpolyglutamate synthetase or ligase (PPS) (EC 6.3.1.2) which uses a reduced folate as substrate. THF and 10-CHOTHF are much superior substrates than 5-CH_3THF (Hoffbrand et al., 1977; Chanarin, 1979). PPS is found in extracts of E. coli (Griffin and Brown, 1964) and in the liver of mammals (Spronk, 1973; Gawthorne and Smith, 1973). The enzyme methyltransferase (EC 2.1.1.13) was shown to have greater activity with polyglutamates than the monoglutamates derivatives in mammals (Coward, et al., 1975) as does mammalian thymidylate synthetase (Kisliuk, et al., 1974).

In addition to being active coenzymes the folate polyglutamates may have a role in control of folate activity within the cell, thus the formyltetrahydrofolate polyglutamate was able to inhibit thymidylate synthetase (Friedkin et al., 1975). However, Kisliuk et al., (1974) demonstrated that dihydrofolate polyglutamate derivatives also had an inhibitory role. Recently, it was found that the stage of the cell cycle and the contents of the culture medium may effect the relative distribution and polyglutamate chain lengths of the various cellular folates (Hilton et al., 1979; Thomson et al., 1977).

Folate polyglutamate synthesis appears to be complete at 24 hr after oral folate doses in rat (Bates, et al., 1980).

5-CH_3THF is the major folate polyglutamate store in the liver (Shin, et al., 1972; Rowe, 1983). The half-life of polyglutamate in rat liver was 3-4 days (Thenen, et al., 1973). Studies on the rat using

both folic acid and 5-CH_3THF confirmed that both oral and parenteral folates are rapidly taken up initially by the liver and participate in an enterohepatic cycle (Steinberg et al., 1979). Under physiological circumstances probably only 5-CH_3THF enters the cell. The normal substrate for polyglutamate synthesis is either the 5-CH_3THF or THF (Chanarin, 1979).
10-Formyltetrahydrofolate is also a major folate polyglutamate derivative present in the cell. Scott and Weir (1976) have found that rat liver contains formylated polyglutamate derivatives composed of a substantial amount of 5-formyl and 10-formyltetrahydrofolate polyglutamate, in addition to the 5-CH_3THF polyglutamate derivatives. 10-CHOTHF enzymically arises via THF formylase (formate-activating enzyme) which transfers formate (HCOOH) to THF and in this form, the formyl group is active in purine synthesis. Both 5-CH_3THF and 10-CHOTHF are the major folate in plasma.

1.3 Folate oxidation and catabolism

Numerous studies have been performed to identify and quantitate the product of oxidation of folates to give detailed explanations of the stability of naturally occurring folates in food (Blakley, 1969; Chippel and Scrimgeous, 1970; Blair and Pearson, 1974; Lewis and Rowe, 1979; Maruyama et al., 1978; Reed and Archer, 1980).
Folic acid and its natural derivatives are readily oxidized under mild conditions of pH, temperature and oxygen. 5-CH_3THF is much more stable to oxidation than THF and the oxidation product 5-methyl-5,6-DHF (Figure 1-17) can be reduced back to 5-CH_3THF (Rowe, 1978) by adding ascorbate. The oxidation may also yield a pyrazino-s-triazene derivative (Jongejan et al., 1979) which is not metabolised either in rat (Kennelly et al., 1979) or in man

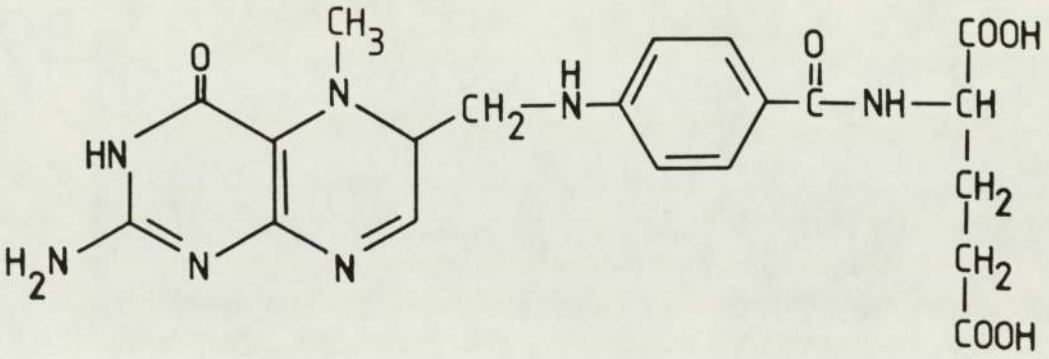


Figure 1-17 : 5-methyl-5,6-DHF

(Ratanasthien, 1975). 10-CHOTHF is oxidized by atmospheric oxygen to 10-formylfolate (10-CHOFA) (Figure 1-18) which is biologically inactive in animals and man, although it can be utilized by certain microorganisms(Chanarin, 1979).

Although Bertino and co-workers (1965) reported that this species (10-CHOFA) is not reduced by DHFR, it can enter the folate pool in the rat and has a metabolism similar to that of folic acid. This may be due to gut microflora or gut enzymes (Connor and Blair, 1979). Folic acid is alkali stable, but it is cleaved aerobically to p-aminobenzoyl-L-glutamic acid and pterin-6-carboxylic acid (Stokstad et al., 1948; Lewis and Rowe, 1979) (Figure 1-19), while its aerobic hydrolysis in acid yields 6-methyl pterin (Stokstad, et al., 1948). Tetrahydrofolate is rapidly oxidized by air with formation of p-aminobenzoyl-L-glutamic acid and a number of pterins among which xanthopterin (Figure 1-20), pterin (Figure 1-21) and folic acid have been identified as well as some dihydrofolate (Blakley, 1957; Silverman et al., 1957; Osborn and Huennekens, 1958; Zakrzewski, 1966a, 1966b; Chippel and Scrimgeour, 1970; Blair and Pearson, 1974). Dihydrofolate is oxidized to give folic acid, formaldehyde,p-aminobenzoyl-L-glutamic acid, and dihydroxanthopterin (Figure 1-22) (Chippel and Scrimgeous, 1970; Hillcoat et al., 1967). 5,10-CH_2THF is more stable to oxidation than THF, particularly in the presence of excess formaldehyde (Blakley, 1969), whereas 5,10-CH=THF at neutral or alkaline pH is converted to the unstable 10-formyltetrahydrofolate (Chanarin, 1979). It was suggested that the folate molecule can undergo scission in man yielding fragmentation; this view was supported by presence of isoxanthopterin (Figure 1-23), a possible metabolite of a pterin fragment,in the urine (Blair, 1958).

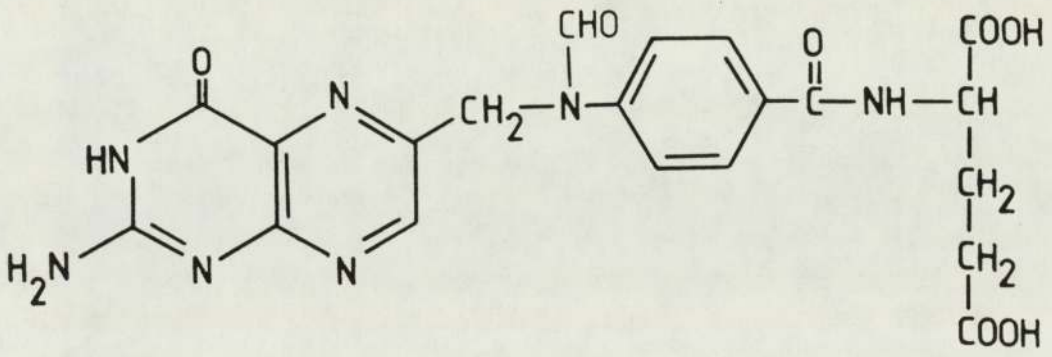
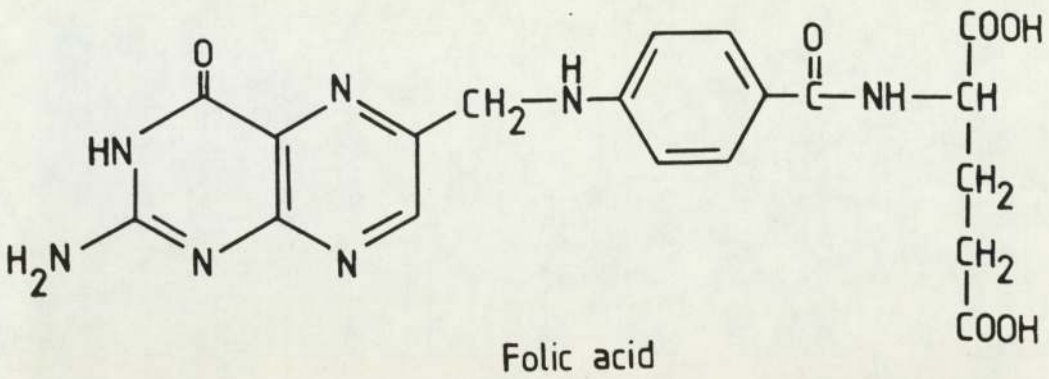
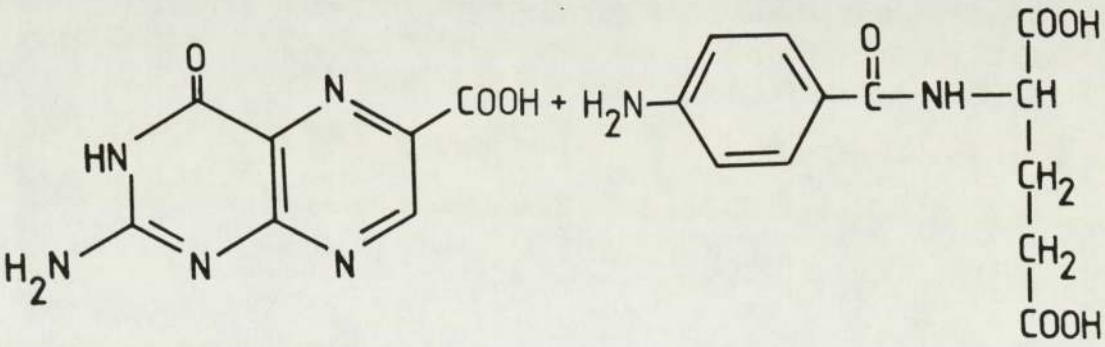


Figure 1-18 : 10-Formylfolate



Folic acid

9-10 Scission



Pterin-6-Carboxylic acid

P-aminobenzoyl-L-glutamic acid

Figure 1-19 : The suggested cleavage of folic acid molecule

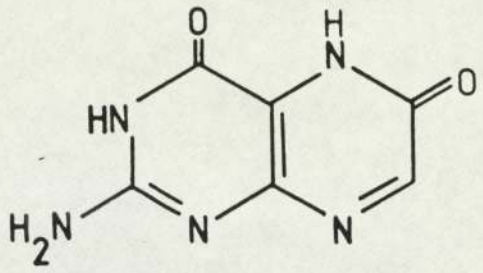


Figure 1-20 : Xanthopterin

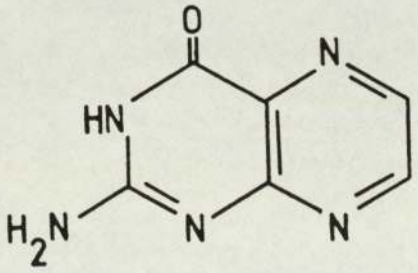


Figure 1-21 : Pterin

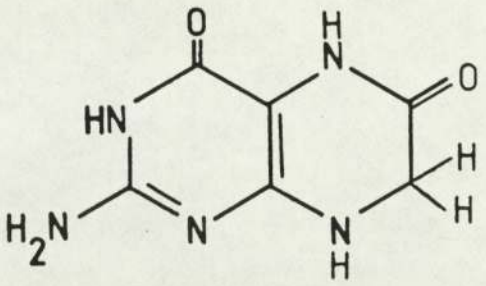


Figure 1-22 : Dihydroxanthopterin

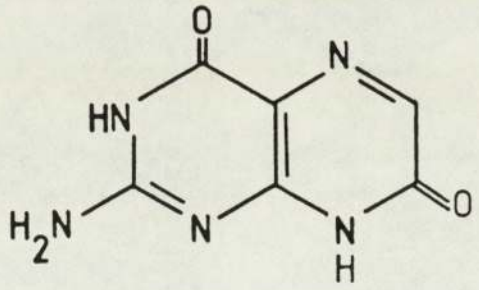


Figure 1-23 : Isoxanthopterin

Considerable catabolism has been shown to occur during folate metabolism in man and rat (Blakley, 1969; Rowe, 1978; Pheasant et al., 1981). Blair, (1958) and more recently, Murphy et al., (1978), suggested that a possible metabolic route would be via cleavage of the C_9-N_{10} bond in the tissue to give p-aminobenzoyl-L-glutamate and a pterin which would undergo further metabolism. An increase of catabolism of folate has been suggested as the cause of folate deficiency of many clinical conditions, such as, pregnancy, malignancy, haemolytic anaemia and inflammatory disease (Hoffbrand 1971) and to be associated with the anticonvulsant drug phenytoin (Hawkins and Meynell, 1958; Klipstein, 1964; Reynolds, 1975) and alcoholism (Kelly, et al., 1981). Until recently, the mechanism of folate catabolism was not known. Studies in vivo on rats showed that the catabolism of ^{3}H-folic acid proceeds via cleavage of the C_9-N_{10} bond of the molecule to yield pteridines which are retained by the liver and released slowly (Reed et al., 1978) and p-aminobenzoylglutamate most of which is acetylated to produce p-acetamidobenzoyl-L-glutamate (p-AcABG) (Figure 1-24) which is rapidly excreted in the urine (Murphy et al., 1976; Reed et al., 1978).

The identification of ^{3}H-acetylated derivatives of p-aminobenzoate and p-aminobenzoyl-L-glutamate confirms that scission of the folate molecule occurs in vivo since acetylation must occur before excretion (Pheasant et al., 1981). However Johns et al., (1961) have found the ^{3}H-aminobenzoyl-L-glutamate in human urine after the intraperitoneal (i.p.) administration of ^{3}H-folic acid. Another catabolite was identified as p-acetamidobenzoate (Figure 1-25) after the administration of radiolabelled 10-CHOFA, folic acid and 10-CHOTHF to rat (Connor, et al., 1979). Previously Dinning,

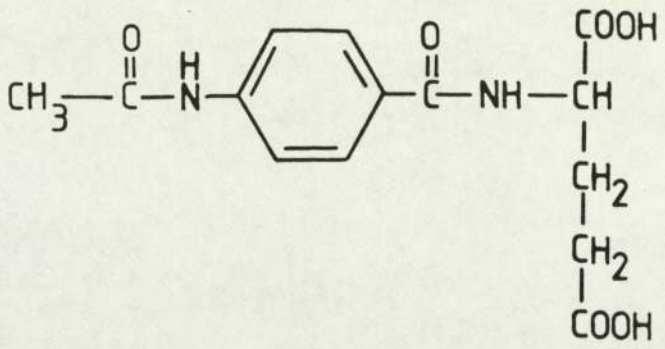


Figure 1-24 : P-acetamidobenzoyl-L-glutamic acid

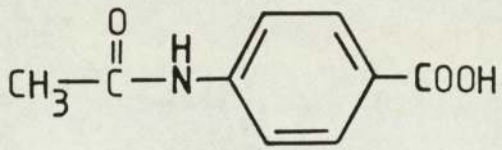


Figure 1-25 : P-acetamidobenzoic acid

et al., (1957) reported an increase in free and acetylated urine diazotizable amines after the administration of large amount of folic acid or 5-formylTHF (Figure 1-26) to rats. ^{3}H-p-acetamidobenzoate (p-AcABA) and p-AcABG have been identified as folate catabolites present in guinea pig urine (Choolun et al., 1980) and in human urine (Saleh, et al., 1980) after an oral dose of ^{3}H-folic acid, 10-formylfolic acid or (^{3}H, ^{14}C)-folic acid. Pheasant et al., (1981) also have found both ^{3}H-p-AcABG and p-AcABA in rat urine after the oral dose of (^{3}H, ^{14}C)-folic acid, mixed labelled of 10-formylpteroyl-L-glutamate and 10-formyl-pteroylpentaglutamate.

The temporal distribution of these two catabolites, i.e., the appearance of large amounts of p-AcABA in the first 24 hr and the persistence of p-AcABG in later samples, suggests that they arise by metabolically distinct routes (Pheasant et al., 1981). A hypothesis was put forward to explain the appearance of the two tritiated folate catabolites (Pheasant et al., 1981) that the catabolite formation is regionalized within the body into distinct areas.

1) The generation of p-aminobenzoyl-L-glutamate within the tissues by either non-specific chemical oxidation or possibly enzymic degradation of retained labile folate polyglutamate is followed by acetylation and excretion of p-AcABG in later urine samples.
2) Folate monoglutamates are secreted in bile, broken down chemically, enzymically, or by the gut and metabolised to p-AcABA during reabsorption.

1.4 Factors affecting folate metabolism

A) Pathology of folate deficiency, malabsorption and other enzymes deficiencies

Folate is an essential intermediate in the metabolism of nucleic acid and plays an important role in the process of cell

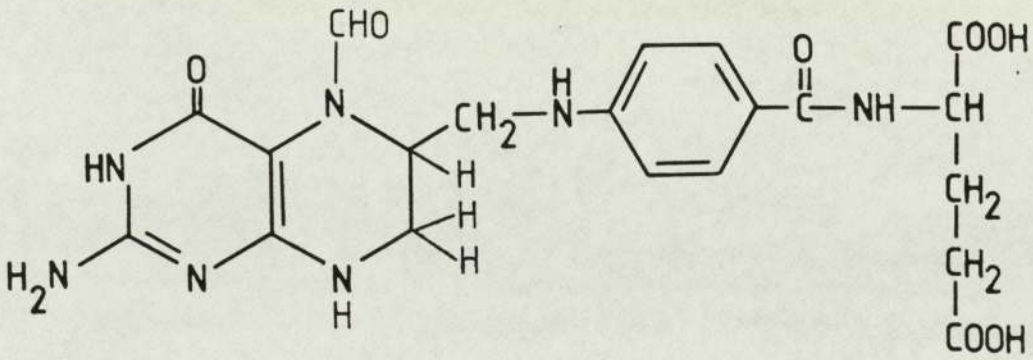


Figure 1-26 : 5-Formyltetrahydrofolic acid (5-CHOTHF)

replication. With such a key position, it is clear that folate deficiency would produce a number of clinical disorders in man. The causes, effects and detection of folate deficiency in both human beings and experimental animals have been extensively reviewed (Blakley, 1969; Stokstad, 1968). The causes of folate deficiency can be summarized in human, in five basic groups.

The first is nutritional folate deficiency which is considered to be the most common hypovitaminosis of man (Blakley, 1969).

The second is malabsorption of folate derivatives such as occurs in tropical sprue and coeliac disease (Anderson et al., 1960; Klipstein, 1963; Freedman et al., 1973; Halsted et al., 1978).

A third group is the lack of vitamin B_{12} to liberate THF from the methyl THF storage form, by either its deficiency in the diet or the absence of the intrinsic factor in pernicious anaemia. In all these conditions, folate deficiency causes anaemia (Rowe, 1978).

In a fourth group, folate deficiency may cause amentia, dementia or depression (Wong, et al., 1977; Mudd, et al., 1972 and J. Blair, personal communication). In the final group a lack of the enzyme 5,10-methyleneTHF reductase causes a much reduced CSF folate and produces severe mental retardation in afflicted children (Erbe, 1979).

B) Methotrexate and other antifolate drugs

The folate metabolic cycle has been the target for a number of drugs, and a most important one is methotrexate (MTX) (4-amino-10-methyl folic acid) (Figure 1-27). It is a folate antagonist which is a powerful inhibitor of DHFR (Bertino et al., 1964; Waxman et al., 1970). Inhibition of DHFR by MTX would lead to a build up of dihydrofolate polyglutamates (Saleh et al., 1982). However the in vivo situation is complex and a number of interacting

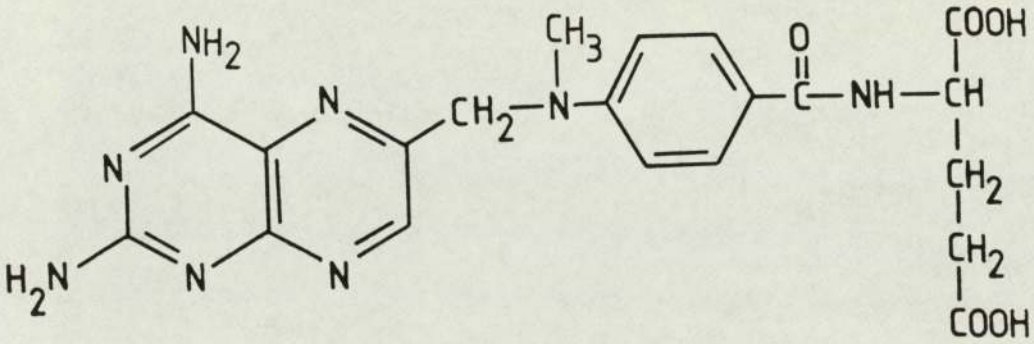


Figure 1-27 : Methotrexate (MTX)

mechanisms may be involved in methotrexate cytotoxicity (Goldman, 1977). When MTX was added to an established human haematoblastoid cell line, total dihydrofolate reductase rose to twice the level in the control; the increase was due to methotrexate-enzyme complex which appeared to have stabilized against normal proteolytic breakdown (Hillcoat et al., 1967). MTX has been used with some success in cancer chemotherapy by inhibition of these enzymes (Johns and Bertino, 1973; Chabner et al., 1975) leading to depletion of the cellular pool of folate coenzymes and thus a lack of purine and pyrimidine and ultimately, cell death (Blakley, 1969). It is also known that MTX inhibits other folate dependent enzymes, such as thymidylate synthetase (Borsa and Whitmore, 1969), dihydropteridine reductase (EC 1.6.99.7) (Craine et al., 1972) and 5,10-CH_2THF reductase (Mangum et al., 1979). Thus the dihydrofolate reductase is not the sole enzyme affected by MTX in vivo (Saleh et al., 1981). Moreover, MTX increases the catabolism of folate by increasing the scission product (Barford et al., 1980). This possibly occurs by inhibiting reduction of DHF to THF by dihydrofolate reductase or by inhibition of dihydropteridine reductase, an enzyme which may maintain tetrahydrofolates in the fully reduced state (Pollock and Kaufman, 1978), therefore leading to increased breakdown via dihydrofolate derivatives. However, a corresponding fall in the amount of folate polyglutamate retained in rat liver was also found which may be due to decreasing the uptake of radiolabel into the liver (Barford et al., 1980).

Apart from anticonvulsants, such as diphenylhydantoin and the barbiturates, a number of other drugs, such as oral contraceptives, have been associated with impaired absorption and utilization of folates, but the mechanism of their action is obscure (Rowe, 1978,

1983). The antiepileptic drugs, such as phenobarbitone, diphenylhydantoin and primidone have no inhibitory action on DHFR, methyleneTHF dehydrogenase or formylTHF synthetase (Hamfelt and Wilmanns, 1965). Folate deficiency is frequently found in a high proportion of cases, who received either phenobarbitone or diphenylhydantoin or both with associated low serum concentration of folate derivatives. However there are some evidences that chronic anticonvulsant therapy causes megaloblastic anaemia which is due to induction of the enzymes responsible for folate breakdown in vivo (Maxwell et al., 1972; Guest, et al., 1983). Various reports suggested the use of antiepileptic drugs in pregnant women provide a considerable risk to the foetus (South, 1972; Smith, 1980).

C) "Methyl trap" hypothesis, vitamin B_{12} and nitrous oxide, N_2O

According to the consideration of interaction of vitamin B_{12} (Cobalamin) and folates (Figure 1-28), the methyl trap hypothesis was put forward to account for the anaemia and high serum folate found in the B_{12} deficiency disorder pernicious anaemia (Herbert and Zalusky, 1962; Noronha and Silverman, 1962a; Nixon and Bertino, 1970; Nixon, 1974). In this hypothesis, the cobalamin dependent methyl transferase reaction is much reduced; the utilization of 5-CH_3THF for methionine biosynthesis is decreased secondary to this loss of activity and as a result, the total body pool of folate becomes predominantly 5-CH_3THF. The decreased availability of other reduced folate derivatives slows the rates of reactions for which they are essential cofactors, i.e. the rates of purine, pyrimidine and nucleic acid synthesis.

In recent years, the nitrous oxide-treated animal has been used extensively as a model for human vitamin B_{12} deficiency.

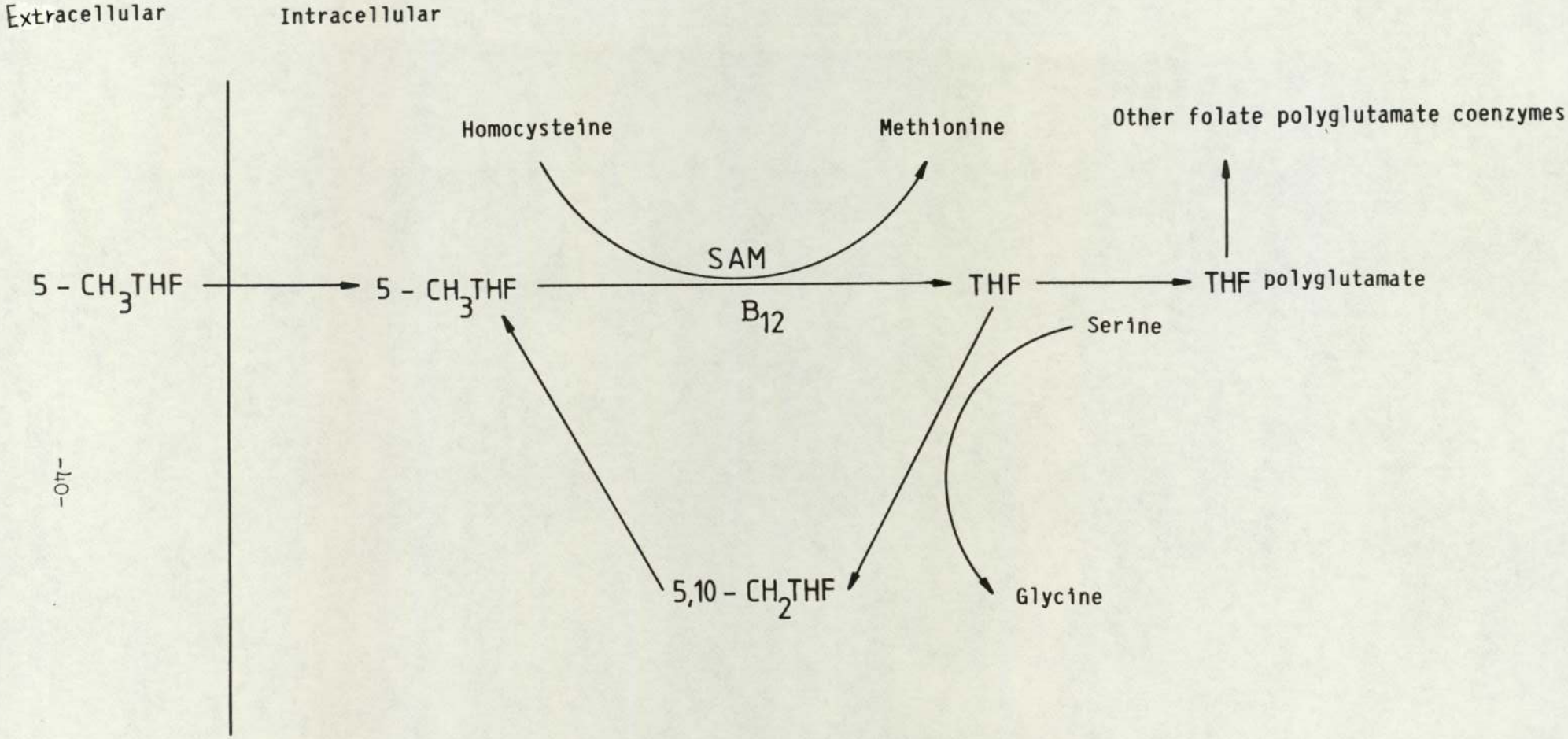


Figure 1-28 : The interaction of B_{12} and folates

N_2O inactivates the B_{12} coenzyme involved in methionine synthesis and interrupts formation of the folate coenzyme (folate polyglutamate) (Chanarin, et al., 1980) by oxidation of B_{12} from the active reduced form cob(I)alamin to inactive oxidized form cob(III)alamin (Banks et al., 1968). N_2O may be regarded as a specific inactivator of the enzyme methionine synthetase which requires cob(I) alamin (Deacon, et al., 1980; Chanarin, 1979; Deacon et al., 1978; Lumb et al., 1981). N_2O also impaired the uptake of folate analogues by rat liver (Perry et al.,1979) and causes cessation of folate polyglutamate synthesis from unsubstituted folates (McGing et al., 1978) and from methyltetrahydrofolate (Perry, et al., 1979; McGing et al., 1978). Scott et al., (1979) and Deacon et al., (1980) have observed the impairment of the conversion of deoxyuridine to thymidine in the presence of N_2O.

1.5 Methods for studying folate metabolism

Human beings are entirely dependent upon dietary sources for their supply of folate. The minimum daily requirement for folate is approximately 50-100µg/day in the normal healthy adult (Herbert, 1977; Sullivan and Herbert, 1964). A normal infant probably needs 20-50µg/day (Sullivan et al., 1966).

However, pregnancy or other conditions associated with high levels of cell replication can increase this requirement several-fold (Hillman and Steinberg, 1982; Rowe, 1983). Therefore a minimal daily requirement of 300-400µg/day in pregnancy is suggested (Willoughby,1967). The predominant form of dietary folate is methyltetrahydrofolate polyglutamate (Hillman and Steinberg, 1982).

Because of the very considerable importance of the folate metabolic cycle, great efforts have been made to study it in detail, but these studies have faced difficulties (Blakley, 1969).

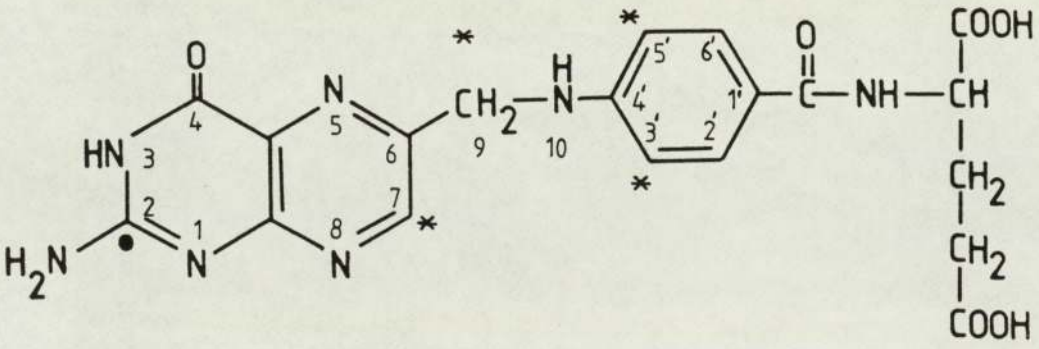
Firstly, in all tissues of man or rat, folates are present in very low concentration (10^{-6}-10^{-8}M) which makes it very difficult to isolate and handle. Secondly, these folate studies must distinguish between endogenous and exogenous folate. Thirdly, all these compounds are very unstable to oxidation and other chemical changes, therefore it is difficult to avoid changing them during the process of isolation. Fourthly, they all have intractable chemical properties, therefore no rigorous characterization is possible. Thus many studies up until the mid 1960s failed to provide adequate protection against photodecomposition and oxidation of folate derivatives during their excretion from tissue (Rowe, 1978).

Although microbiological assay was a major method and a satisfactory assay of total concentration of naturally occurring folates, using three organisms frequently, Lactobacillus casei, Streptococcus faecalis and Pediococcus cervisiae, it does not permit identification of all the various derivatives of folate which are present in given biological samples(Blakley, 1969; Beavon and Blair, 1975). New techniques were evolved to avoid these difficulties, by using radiolabelled compound such as folic acid derivatives, in which the label has been introduced by ^{14}C or by ^{3}H (Anderson et al., 1960; Burgen, 1961). Antioxidants of which the common one is ascorbate were used to solve the instability to oxidation (Wittenberg et al., 1962). Sequential chromatography, including ion exchange, gel permeation, proved to be very useful in the purification and identification of folate coenzymes (Bird et al., 1965; Noronha and Silverman, 1962b;Schertel et al., 1965; Thenen and Stokstad, 1973; Shin et al., 1972), paper chromatography (Winsten and Eigen, 1950; Wieland, et al., 1952; Connor and Blair,

1980), thin layer chromatography (Scott, 1980; Brown et al., 1973a) and finally high performance chromatography which has the advantage of better separation and yet using a smaller sample (Allen and Newman, 1980).

Therefore by using these chromatographic techniques either singly or together it is possible obtain characterization of a very high degree of probability. Mixed labelled folate is also used to improve the identification in which both ^{3}H and ^{14}C label as [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (Figure 1-29) is introduced to measure the ratio of ^{3}H:^{14}C, and observe whether or not it matches with anticipated ratio (Beavon and Blair, 1971; Nixon and Bertino, 1971; Pheasant et al., 1979).

Recently it was found that parenteraly and orally administered radiolabelled folates are rapidly cleared from the serum to the tissues, nevertheless, the mechanisms are still obscure (Rowe, 1983). In series of experiments with radiolabelled 5-formyl and 5-methyltetrahydrofolate on a human subject, Nixon and Bertino (1970) have found that 5-CH_3THF urinary excretion was directly related to the serum level while 5-formylTHF was excreted even when it could not be detected in the serum. Blair and Dransfield (1971) found 5-CH_3THF with other unidentified metabolites in rat urine after an oral dose of [2-^{14}C]-folic acid. While Vidal and Stokstad (1974) reported the appearance of 5-CH_3THF, 5-CHOTHF and 10-CHOTHF in the urine of rats after administration of ^{3}H-folic acid orally. Barford et al., (1978) reported the scission of the folate molecule shortly after administration of [2-^{14}C]-folic acid and a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid to rats producing a metabolite labelled with ^{3}H only. Pheasant and Blair (1979) also



* = ^{3}H (Tritium)

• = ^{14}C

Figure 1-29 : Mixed label folic acid with both [2-^{14}C] and [3′, 5′, 7, 9-^{3}H]

identified metabolites, such as 5-CH_3THF, 10-CHOFA and 10-CHOTHF and in addition the scission products p-acetamidobenzoyl-L-glutamate and p-acetamidobenzoate in rat urine after an oral dose of a mixture of [^{3}H, ^{14}C]-folic acid. These studies in general investigated the metabolic fate of folic acid in vivo, indicating that folate derivatives are removed primarily by renal excretion.

1.6 Rationale for present study

Folates are essential to a variety of reactions involved in nucleoprotein synthesis and cell turnover (Halsted, 1980b). The present study has developed Syrian golden hamsters as new experimental animals to observe the differentiation of the rate of folate catabolism in vivo with other animals. Although no detailed studies are available about the rate of folate degradation in vivo in mammals, or the mechanism by which folate catabolism occurs, these experiments were undertaken to study folate catabolism in vivo and the effect of other agents (xanthopterin, allopurinol, dihydro-orotic acid and growth hormone)on the catabolic rate of folate in an attempt to elucidate the identities and origin of all the metabolites formed including any scission product of folate catabolism and its mechanism. However, the purpose of using Syrian hamsters was to investigate the mechanism of kidney hypertrophy caused by xanthopterin. Therefore, this study uses Syrian hamsters pretreated with four agents; allopurinol, xanthopterin, dihydro-orotic acid and growth hormone (somatotopin), followed by an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid. Three of them (allopurinol, xanthopterin and dihydro-orotic acid) generate superoxide anion ($O_2^{\cdot-}$) during their oxidation reaction in vivo (more details in further chapters, 3 and 4). Growth hormone may increase the superoxide anion formation also by the pathway which is

mentioned above, because of its action to increase DNA and RNA synthesis which therefore may increase purine formation.

Detailed studies were carried out of the oxidation of THF some years ago.

Blair and Pearson (1974) suggested that tetrahydrofolate (FH_4) is oxidized to quinonoid dihydrofolate (qFH_2) by molecular oxygen by a free radical reaction. The first line (equation (1)) shows the initiation step which can occur in a variety of ways. This species reacts with molecular oxygen by electron removal to yield qFH_2 plus superoxide anion and a proton. The $O_2^{\cdot-}$, then, reacts with FH_4 to yield the hydroperoxyl anion plus $FH_3^{\cdot}$ as free radical and that is fed back.

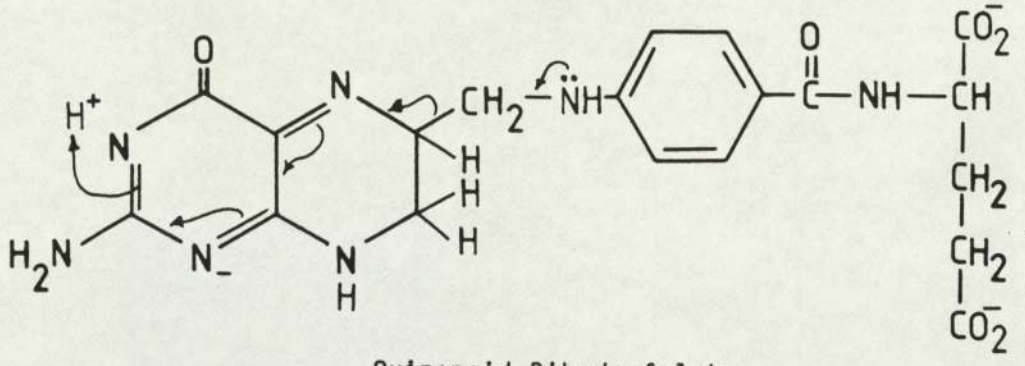
$$FH_4 \longrightarrow FH_3^{\cdot} \qquad (1)\ \text{(initiation step)}$$

$$FH_3^{\cdot} + O_2 \longrightarrow qFH_2 + O_2^{\cdot-} + H^+ \qquad (2)$$

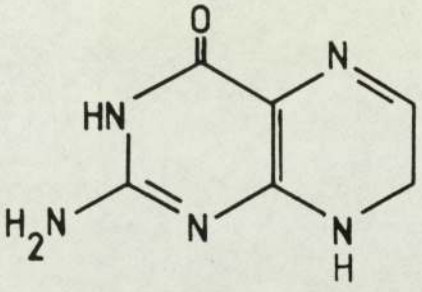
$$FH_4 + O_2^{\cdot-} \longrightarrow FH_3^{\cdot} + HO_2^- \qquad (3)$$

Any increase in $O_2^{\cdot-}$ by other processes will accelerate this breakdown. Thus, it is suggested that tetrahydrofolate in the tissues will be oxidized to the qFH_2 species by $O_2^{\cdot-}$ in the cell itself formed from molecular oxygen, in the animal from the xanthine oxidase stimulated oxidation of various substrates (J. Blair, personal communication). The dihydrofolate species (FH_2) which occurred from this oxidation of FH_4 by $O_2^{\cdot-}$ is known as quinonoid DHF. It is extremely unstable and rapidly undergoes scission reactions (Blair and Pearson, 1974) to yield 7,8-dihydropterin which can then undergo a variety of further reactions (Mager and Berends, 1965) and p-aminobenzoyl-L-glutamate.

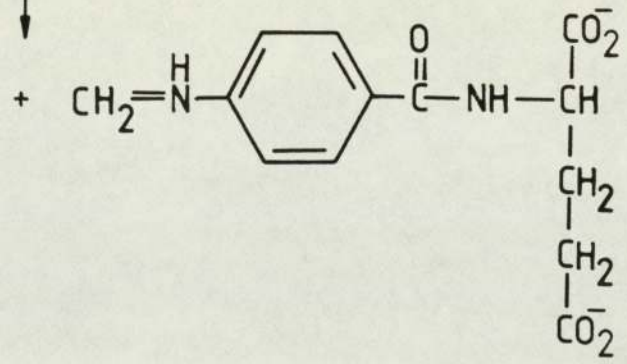
The possible rearrangements of the quinonoid intermediate is shown in Scheme (2). It is, therefore, interpreted that the oxidation of THF via quinonoid dihydrofolate gives ultimately pterin



Quinonoid Dihydrofolate

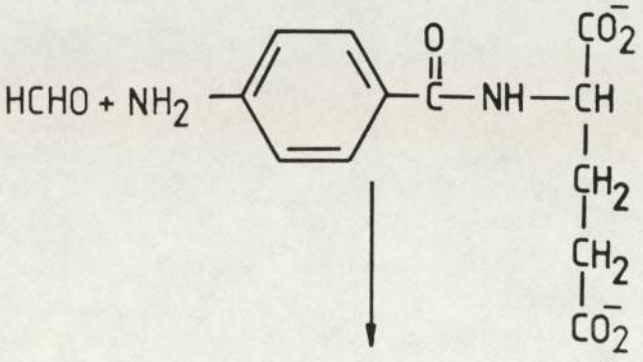


7,8-Dihydropterin



Derivative of P-aminobenzoyl-L-glutamic acid

Further steps



Further steps

Scheme 2 : The quinonoid intermediate DHF reaction

and xanthopterin as the final products (Blair and Pearson, 1974). This, then, suggests that tetrahydrofolate derivatives may be involved in the catabolic process (Saleh et al., 1982). It was established that dihydropteridine reductase can use quinonoid dihydrofolate as substrate (Lind, 1972). This enzyme may act in the maintenance of the reduced folates (Pollock and Kaufman, 1978). Recently, 5,10-CH_2THF reductase has also been shown to have dihydropteridine reductase activity and to reduce quinonoid DHF to THF (Matthews and Kaufman, 1980).

CHAPTER 2

Materials and Methods

CHEMICALS AND REAGENTS

Commercial materials used in this study were:

Folic acid, xanthopterin and pterin [Koch-Light Laboratories Limited (Colnbrook, Bucks, U.K.)]; 5-methyltetrahydrofolate [Eprova Research Laboratories, (Basle, Switzerland)]; p-aminobenzoic acid and p-acetamidobenzoic acid [Aldrich Chemical Co. Limited (Wembley, Middlesex, U.K.)]; p-aminobenzoyl-L-glutamate [Sigma Chemical Co. Limited (London, U.K.)]; [2-^{14}C]-folic acid (specific activity 58.2μCi/mmol) (96% radiochemically pure) and [3',5',7,9-^{3}H]-folic acid (specific activity 500μCi/mmol) (95% radiochemically pure) [Radiochemical Centre Amersham, Bucks, U.K.].

Pterin-6-carboxylic acid (Zakrewski et al., 1970) was prepared by Dr. M. Connor, in this laboratory; p-acetamidobenzoyl-L-glutamate was prepared by the method of Baker et al., (1964) and 10-formylfolate by the method of Blakley (1959).

CHROMATOGRAPHY

1. Ion - Exchange Chromatography

Ion exchange columns were prepared as follows:-

Diethylaminoethyl cellulose (DE-52 Whatman Limited, Maidstone, Kent, U.K.) (50-80gm) was washed with distilled water and equilibrated in 0.05 M-phosphate, pH 7.0 containing 5mg of dithiothreitol/100ml until the washings were of constant ionic strength and pH 7.0.

After degassing, the prepared DE-52 was packed into a column (2cm x 40cm) plugged with glasswool. Samples of urine (10-20ml) were diluted to the conductivity of starting buffer with distilled water before loading on the columns. Appropriate standards were applied while loading. The columns were eluted with standard linear

gradients of 0-1.2 M NaCl 0.05 M-sodium phosphate buffer, pH 7.0 containing 5mg of dithiothreitol/100ml as starting buffer automatically using an LKB Ultra Grad attached to a peristaltic pump (LKB instruments, Croydon, Surrey, U.K.)

The eluant was passed through a U.V. Monitor (LKB Uvicord II, LKB instruments) which measured the U.V. absorbance of compounds eluting from the column. The column effluent was collected in 5ml fractions using an LKB Ultrarac Fraction Collector (LKB instruments). When large volumes of samples were used, fractions (10ml) were also collected during sample loading. The radioactivity in column effluents was determined and the salt gradient, usually eluted over eight hours, was measured by determining the conductivity of every tenth fraction with a Mullard conductivity cell.

2. Gel Filtration

Sephadex-G15 was obtained from Pharmacia (Uppsala, Sweden). The Sephadex gel columns were prepared by soaking the Sephadex-G15 gel in 0.05 M phosphate buffer, pH 7.0 containing 5mg of dithiothreitol/100ml for at least 4 hours, then packing into a (2cm x 60cm) Perspex column (Wright Scientific Limited, Surrey, U.K.) under high pressure. After loading the urine sample (10-25ml) and appropriate standards, using 0.05 M phosphate buffer, pH 7.00 with 5mg%(w/v) dithiothreitol, the column was eluted with the same buffer and the 5ml fractions collected and counted for radioactivity as above.

The elution pattern of relevant folates, pteridines and p-aminobenzoic acid derivatives on DE-52 and Sephadex-G15 is summarized in Table 2.1.

3. Paper Chromatography

Descending paper chromatography was used with propanol/aq. NH_3 (sp.gr.0.88)/water (200:1:99 V/V) as mobile phase and Whatman 3MM chromatography paper as stationary support. The samples (^3H - scission products) and standards (p-aminobenzoyl-L-glutamate, p-aminobenzoic acid and their acetyl derivatives, 10-formylfolic acid) were spotted on the paper and then developed overnight in the dark. The positions of the standards were then observed as dark absorbing or fluorescing spots by viewing under U.V. light at 254 nm or 356 nm.

The sample positions were determined by cutting out strips of paper, immersion in scintillation cocktail and the radioactivity determined in a Beckman LS 7500 liquid scintillation counter. Table 2.2 summarizes the chromatographic behaviour of standard compounds on Whatman 3MM paper with solvent propanol: NH_3: water (200:1:99 V/V). Table 2.3 shows the behaviour of p-aminobenzoic acid derivatives on Whatman 3MM paper with different solvents (Saleh, 1981).

ANIMALS

Normal male Syrian golden hamsters weighing between 80-100gm and aged 8-10 wks (Bantin and Kingman Limited [Aldbrough]) received an oral dose of a mixture of 1.0μCi [2-^{14}C] and 2.0μCi [3',5',7,9-^3H]-folic acid (100μg/kg body wt.). After the administration of labelled folates, the animals were housed in metabolism cages (Jencons Metabowls, Jencons (Scientific) Limited, Hemel Hempstead, Herts, U.K.) with a dark/light cycle of 12 hours and at 21°C. The cages allowed the separate collection of urine and faeces and gave free access to food (Breeding Diet, Heygates Limited) and water. Urine was collected into 5ml of 0.05

M-phosphate buffer, pH 7.00 containing 2% (w/v) sodium ascorbate and dithiothreitol (5mg/100ml) and protected from light by surrounding the urine flask with aluminium foil to prevent the oxidation and degradation of folates. The urine was stored at - 20°C until the time required.

For a few groups in the whole experiments CO_2 was collected by pumping air through sealed Metabowls (connected in series) and passed through 5 M KOH (20ml). Test compounds were given orally, by intra-peritoneal injection (i.p.) or by intra-muscular injection. Most of those compounds were dissolved in peritoneal saline (0.15 M NaCl) and the rest in distilled water or in 0.05 M-phosphate buffer, pH 7.00 containing (2% w/v) sodium ascorbate, except allopurinol which was suspended in Mazola cooking oil because of its insolubility in any of the above solvents (more details in chapter 3). At the end of the experiment the animals were then killed by cervical dislocation. The organs were removed for determination of the distribution of radioactivity and the identification of the retained folates.

PATIENTS

The metabolism of folic acid in man was investigated at the General Hospital in Birmingham, U.K. using patients suffering from malignant disease, chronic alcoholism and control groups. Further details of those patients are given in the relevant chapter. All patients received an oral dose of labelled folic acid (5μCi [2-^{14}C] and 20μCi [3',5',7,9-^{3}H]-folic acid) and various doses of unlabelled folic acid. The urine was collected on to 10gm of sodium ascorbate in a plastic container to prevent the oxidation and degradation of the folates. The urine samples were kept frozen

at -20°C until the time of analysis. All patients were allowed a normal diet throughout the experiment.

MEASUREMENT OF TOTAL RADIOACTIVITY

Urine radioactivity (early samples) was measured in a Nuclear Enterprises Liquid Scintillation Counter Type 8310 (Nuclear Enterprises Limited, Edinburgh) with 10ml of Fisons Dioxan D "Cocktail" after calibrating the channels for ^{14}C and ^{3}H; corrections were made for quenching, background and ^{14}C spillover into the ^{3}H channel. Later samples were measured in Beckman LS 7500 liquid scintillation counter (Beckman Instruments Inc. Scientific Instruments, Division, Irvine, California 92713), with automatic quench compensation, using the same technique of preparation as in the previous counter. Tissue radioactivity was measured initially by total oxidation and latterly by solubilisation.

1. Oxidation

The liver, kidney and faeces were freeze-dried and ground to give a homogeneous powder. The samples (100mg) of powder were oxidized in Beckman Biological Materials Oxidizer, and the oxidised gases passed through traps. Tritium was trapped as tritiated water in a dry ice/methanol cold trap and counted in 10ml of Fisons tritium absorber. ^{14}C was trapped as $^{14}CO_2$ in 15ml of Fisons absorber P (Fisons, Loughbrough, Leics., U.K.), a scintillation cocktail designed for the collection of $^{14}CO_2$. Duplicate determinations of each sample were made together with appropriate controls. Samples were counted for radioactivity in Beckman LS 7500 liquid scintillation counter.

2. Solubilisation

This was done by the method of Wunderly and Mooney (1981). The radioactive tissue samples such as minced or homogenised liver and

kidney (100mg) were placed in a glass scintillation vial, and 1.0ml of Beckman tissue solubiliser (BTS-450, P/N 580691) added. The sample was incubated in a 55-60°C waterbath until it was thoroughly digested. The completely digested sample is a viscous homogeneous mixture with some colouration. Several drops of a decolourising solution of 30% H_2O_2 in water was added and mixed gently by leaving the sample to stand for several hours. Since this may increase chemiluminescence, this step may be deleted if samples show little colouration. The addition of glacial acetic acid in Beckman scintillation cocktail is suggested to degrade the chemiluminescence which might be generated by tissue solubilisers.

Finally the sample was kept overnight in the dark before being counted in the Beckman LS 7500 liquid scintillation counter.

The faeces were freeze-dried and ground to homogeneous powder, then 20mg of the powder was put in the glass scintillation vial, with 0.1ml of distilled water added for 1/2 hour. One ml of BTS-450 solubiliser was added and incubated for 1-2 hours at 40°C (waterbath); 0.5ml of a mixure of isopropanol and 0.5ml of H_2O_2 were added and the sample left to stand for 10 minutes. It was then incubated for two hours at 40°C, 5 ml of distilled water was added to dilute the sample. Finally 10ml of Beckman scintillation cocktail containing 7% of glacial acetic acid was added. The addition of glacial acetic acid was for the same reason as above. The sample was left in the dark overnight before being counted in the Beckman LS 7500 liquid scintillation counter.

3. Extraction of Folates from Livers

The liver was removed after opening the abdominal cavity of the animal and quickly chilled and washed in ice-cold 0.05 M-phosphate buffer pH 7.0 to prevent the breakdown of folate polyglutamate.

Then the liver was dropped into a solution of boiling 0.05 M-phosphate buffer pH 7.0 containing 2% w/v sodium ascorbate and 5mg of dithiothreitol/100ml and maintained at 100°C for 10 mins (Stokstad and Thenen 1973). After heating the extract was cooled, homogenised and centrifuged to remove the precipitated protein and the supernatant was stored at -15°C until the time of chromatography on Sephadex-G15 and counted with Beckman LS 7500 liquid scintillation counter.

4. CO_2 Collection

The exhaled CO_2 was collected for radioassay by pumping the expired air from the sealed Metabowl in which the hamsters were kept into a trap containing 20ml of 5M KOH. The collection was for every 24 hr. The radioactivity was measured as $^{14}CO_2$ using the scintillation cocktail.

The samples were counted in Beckman LS 7500 liquid scintillation counter.

STATISTICAL ANALYSIS

Throughout the thesis all tests of statistical significance were performed using Student's "t" test.

Table 2.1

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

Compound	Elution Position	
	Sephadex-G15 (Fraction No.)	DEAE-Cellulose (Molarity Na Cl)
Folic acid	37	0.96
* Pteroylheptaglutamate	11	0.60
10-CHOFA	21	0.53
10-CHOTHF	18	0.45
5-CHOTHF	28	0.59
5-CH_3THF	37	0.67
* 5,10-CH_2-THF	25	0.64
* Methotrexate	60	-
* Triazene	16	0.40
Folate (X)	-	0.7
Folate (Z)	-	0.6
Pterin	35	0.30
* Pterin - 6 - CHO	43	0.70
Pterin - 6 - COOH	30	0.60
* Pterin - 6 - CH_3	44	-
* Pterin - 6 - Sulphonicacid	34	-
* D-neopterin	28	-
* Xanthopterin	57	0.57
* Isoxanthopterin	45	0.49
* Dihydroxanthopterin	76	-
* 6 - Hydroxymethylpterin	32	-
* 6 - Methyltetrahydropterin	36	-
* 6 - COOH - Lumazine	27	-
* 6 - OXO - Lumazine	34	-
* Lumazine	30	-
* 2 - acetamido - 4 - hydroxypteridine	27	-
* 2 - acetamido - 4,6 - hydroxypteridine	28	-
* 2 - acetamido - 4 - hydroxy 6 - formylpteridine	30	-
Metabolite (A)	45	0.40
Metabolite (B)	-	0.35
Metabolite (C)	32	0.38
p-aminobenzoic acid	35	0.40
p-acetamidobenzoic acid	36	0.43
p-aminobenzoyl-L-glutamate	18	0.40
p-acetamidobenzoyl-L-glutamate	19	0.43
* p-aminohippuric acid	33	-
* p-acetamidohippuric acid	27	0.35
^3H_2O	21	0.0
* Urea	21	0.0

Elution conditions are described in the text.
* Elution position taken from Saleh (1981)

Table 2.2

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

Compound	R_f Values in Solvent (Δ)
p-aminobenzoic acid	0.5
p-acetamidobenzoic acid	0.7
p-aminobenzoyl-L-glutamate	0.21
p-acetamidobenzoyl-L-glutamate	0.52
10-formylfolic acid	0.19

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

* Table 2.3

The chromatographic behaviour of p-aminobenzoic acid derivatives on Whatman 3MM paper, with different solvents.

Compound	R_f Values in Solvent		
	A	B	C
p-aminobenzoic acid	0.65	0.21	0.81
p-acetamidobenzoic acid	0.56	0.36	0.84
p-aminobenzoyl-L-glutamate	0.85	0.04	0.43
p-acetamidobenzoyl-L-glutamate	0.85	0.09	0.58

A = 1% (V/V) acetic acid in water
B = Butanol/ethanol/aq NH_3 (sq. gr. 0.88)/Water (10:10:1:4 by vol.)
C = n-Butanol/pyridine/water/glacial acetic acid (6:4:3:1 by vol.)

* = The data of this Table taken from Saleh (1981).

CHAPTER 3

The metabolism of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid over 0-48 hours in normal Syrian golden hamsters and hamsters treated with xanthopterin orally.

3.1 Folic acid metabolism in the normal hamsters

Folate metabolism has been studied using microbiological assay (Blakley, 1969) and radioactive labelling (Anderson et al., 1960; Burgen, 1961) as previously described.

In this set of experiments, the Syrian golden hamster was examined:

1) to compare with folate metabolism in man, the rat and guinea pig.
2) to examine a possible mechanism for xanthopterin stimulated kidney hypertrophy.
3) to obtain further analyses of possible routes of folate catabolism in vivo.

Materials and Methods

14 male Syrian hamsters (80-100gm body wt), each received an oral dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (100μg/kg body wt). Two animals were housed per Metabowl. Urine was collected every 24hr, either of each pair separately or pooled together. Faeces were collected at the end of the experiment at 48hr. Livers and kidneys were removed after 48hr by the technique described in chapter 2 .

The determination of radioactivity in the urine, faeces, livers and kidneys, the column chromatography and paper chromatography were as previously discussed (chapter 2). The liver extract was chromatographed on Sephadex-G15 (see chapter 2). The faeces as a homogeneous powder from all animals were mixed, suspended in 1% of aqueous ammonia and centrifuged at room temperature. The supernatant was filtered and stored at -20°C as faecal extract until required for chromatography on DE-52 chromatography, Sephadex-G15

column and paper chromatography (descending method). The $^{14}CO_2$ radioactivity was measured as described in chapter 2.

Results

a) Radioactivity in urine, faeces and tissues

Table (3.1.1) shows the distribution of radioactivity in urine, faeces, livers and kidneys in 0-48hr. Quantitative analysis of 0-24hr and 24-48hr urine samples showed, significantly, more ^{3}H than ^{14}C was excreted ($p<0.001$). The greater urinary excretion of radioactivity occurred in the first 24hr and declined in the second 24hr.

The hepatic level of radioactivity showed no significant difference in the recovery of the ^{3}H and ^{14}C dose. Similar recoveries of the ^{3}H and ^{14}C dose appeared in the kidney. The percentage of recovery of ^{3}H and ^{14}C dose in the faeces was similar (23.97% ^{3}H, 23.53% ^{14}C).

b) Urinary metabolites

Qualitative and quantitative analysis of urine samples was carried out by sequential chromatography on DE-52 and Sephadex-G15 columns with, where appropriate paper chromatography. Species were identified by cochromatography with known compounds. In the 0-24hr samples (Table 3.1.2), 5-CH_3THF (II) was present as the major folate together with two other mixed-label species, (I)folate (X) and (III)folate (Z) (Figure 3.1.1). On account of the similarity of their ^{3}H:^{14}C ratio to that of the starting material, these are thought to be folate species. Folic acid was absent. p-AcABA and p-AcABG were also present but no free amines (Figure 3.1.2). Tritiated water ($^{3}H_2O$), urea and an unidentified ^{14}C-labelled only species, were also found Pk (VI, VII).
The 48hr urine sample had a similar pattern of metabolites (Table

3.1.2) that of the 24hr samples, although the levels of both unidentified folates and 5-CH_3THF declined. There was a small amount of p-AcABA and some increase in the p-AcABG level (Figure 3.1.3).

c) Faeces extracts

The 0-48hr faecal extracts (Figure 3.1.4) showed folic acid and folate X (I.II) accompanied by a small amount of ^{14}C species only (IV) eluting at the void volume of both column systems. Tritiated scission products (III) were found with p-AcABG, a major peak, and p-AcABA (Figure 3.1.5). Paper chromatography contributed a contrasting pattern with a high level of p-AcABA instead (Figure 3.1.6).

d) Liver extracts

Sephadex-G15 column chromatography of 0-48hr liver extracts (Figure 3.1.7) showed folate polyglutamates only. No folate monoglutamates were seen.

3.2 Folic acid metabolism in normal hamsters following oral xanthopterin administration

Previous work with hamsters has shown that xanthopterin induces renal hypertrophy (Haddow et al., 1972). Xanthopterin and other pterins inducing kidney hypertrophy may operate through inhibition of xanthine oxidase, so leading to the accumulation of abnormal amounts of xanthine and hypoxanthine for the synthesis of nucleic acid (Haddow, et al., 1972). Kidney hypertrophy from xanthopterin dosing is probably not due to the kidney damage arising from the deposition of leucopterin in the kidney tubules, but is due to some biochemical effects (Brokaw, 1953; Agnew, 1955; Lotspeich 1965 and Reiter, 1968). Folic acid has a similar capacity to induce kidney proliferation in the rat (Threlfall, et al., 1964; Threlfall, 1968). It seemed, therefore, that xanthopterin might reduce the

folate breakdown in the kidney thereby raising folate concentration and this could give rise to kidney hypertrophy. The effect of xanthopterin on the metabolism of labelled folic acid in vivo has not been previously studied. The administration of xanthopterin along with a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid in the Syrian hamster was used in order to examine the breakdown of folate.

Materials and Methods

Male Syrian hamsters (80-100g body wt) each received oral doses of xanthopterin (100mg/0.5 ml distilled water) every 24hr for two days. On the third day, they were dosed with a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (100µg/kg body wt) and housed in Metabowl cages as described before (see chapter 2). After 48hr, the animals were killed. The collection of urine, faeces, livers and kidneys was carried out as described in chapter 2. The preparation of the liver extract, the determination of radioactivity and column chromatography were carried out as previously discussed (chapter 2). The carbon dioxide radioactivity ($^{14}CO_2$) was collected and measured as was described earlier in chapter 2.

Results

Table 3.2.1 summarizes the 0-48hr recovery of ^{3}H and ^{14}C in urine, faeces and tissues of hamsters pre-treated with xanthopterin orally. The recovery of radioactivity of 0-24hr urine sample declined significantly (12.32 ±3.92% ^{3}H, 5.56 ±1.76% ^{14}C of the dose) compared with the control hamsters (19.06 ±3.17% ^{3}H, 9.22 ±2.97% ^{14}C of the dose) [$p<0.05$].

There were no significant differences in the recovery of radioactivity over the 24-48hr urine sample between those two groups. Excess ^{3}H over ^{14}C was detected in the urine.

The faecal recovery of radioactivity over the 0-48hr time period was lower in those experimental hamsters (15.96 ±4.38% ^{3}H, 15.01 ±4.83% ^{14}C of the dose) than of the control group (23.97 ±7.84% ^{3}H, 23.53 ±6.18% ^{14}C of the dose) and no excess of ^{14}C over ^{3}H appeared. The hepatic and kidney recovery of radioactivity were similar in both experimental and control groups.

Urinary metabolites

The pattern was similar to that of controls (Table 3.2.2). There was a slight decrease of intact folates in the experimental group and also a slight fall in scission products particularly p-AcABA. DE-52 chromatography and Sephadex-G15 column chromatography of 24hr and 48hr urine samples were shown in Figures (3.2.1, 3.2.2, 3.2.3) with the disappearance of two mixed labelled species [folate (X) and folate (Z)] in the later urine sample (48hr). 5-CH_3THF was the only intact metabolite seen.

Liver extracts

Sephadex-G15 column of 0-48hr liver extracts had a similar pattern to that of control hamsters with folate polyglutamate only.

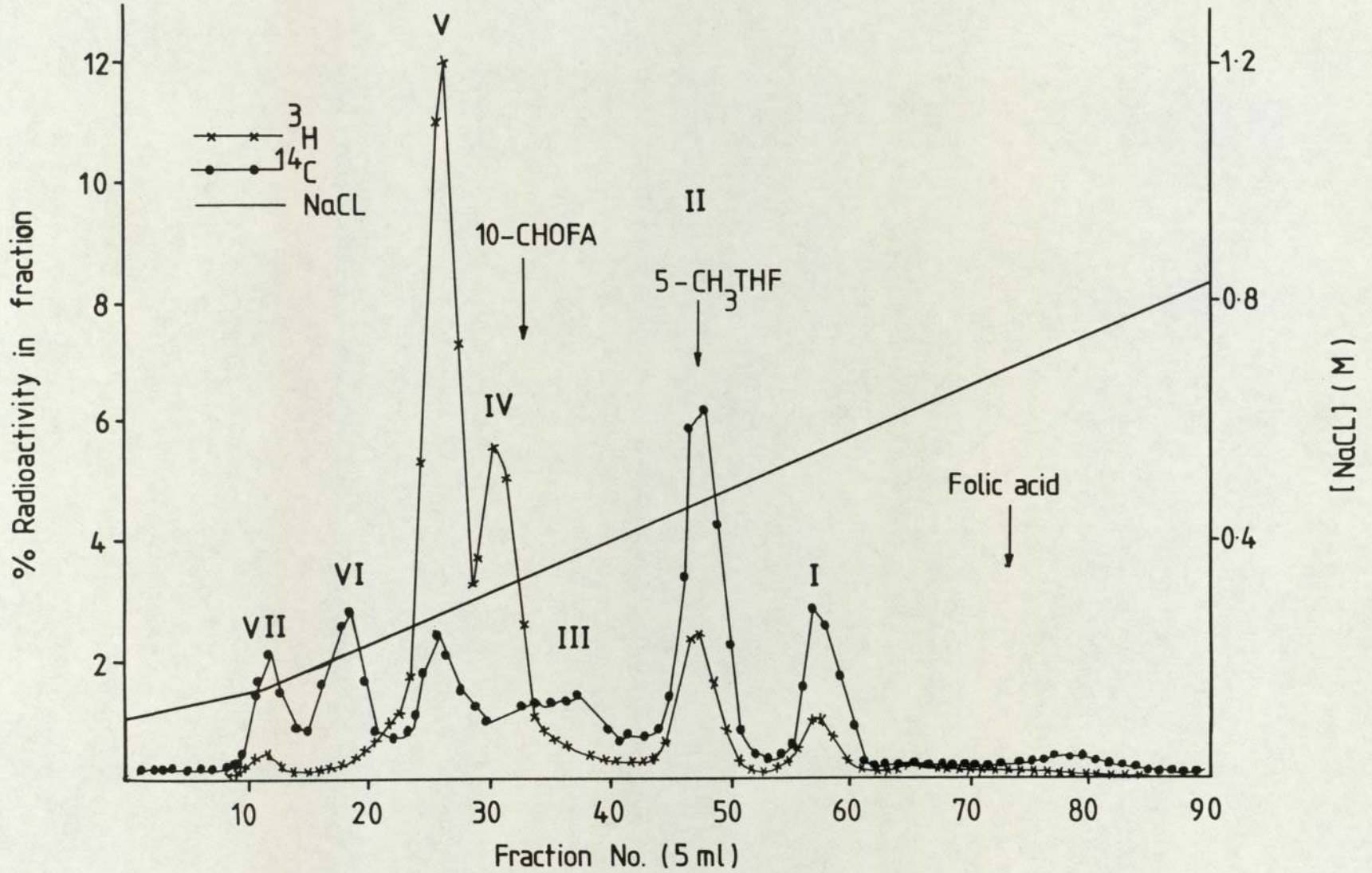


Fig. 3.1.1 DE-52 chromatography of the (0-24hr) urine sample of normal Syrian hamster receiving a mixture of $^{14}C + ^{3}H$ folic acid (100µg/kg body wt).

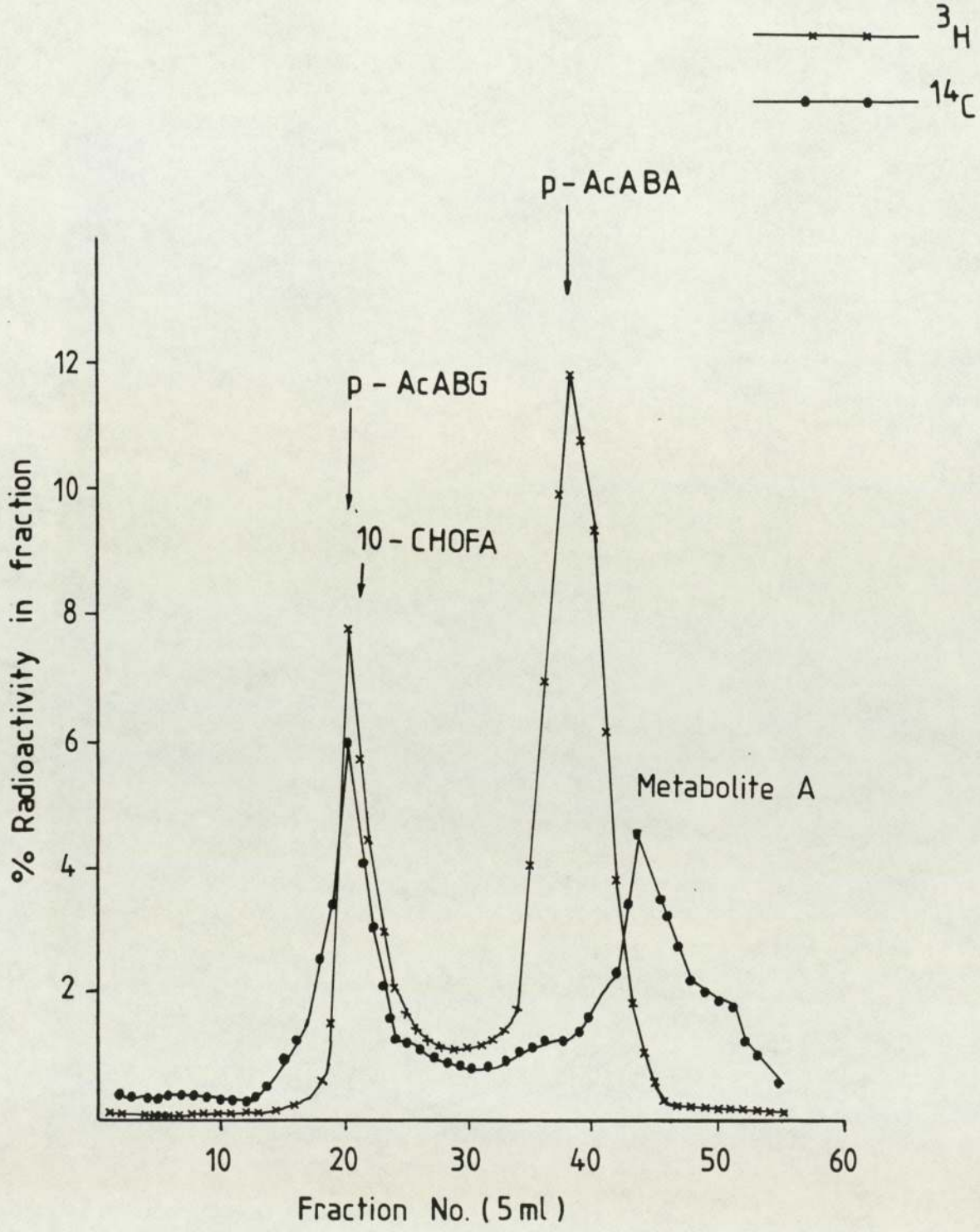


Fig. 3.1.2. Sephadex-G15 chromatography of the peaks IV and V from DE-52 chromatography of 0-24hr urine sample of normal hamster receiving labelled folic acid (^{3}H + ^{14}C).

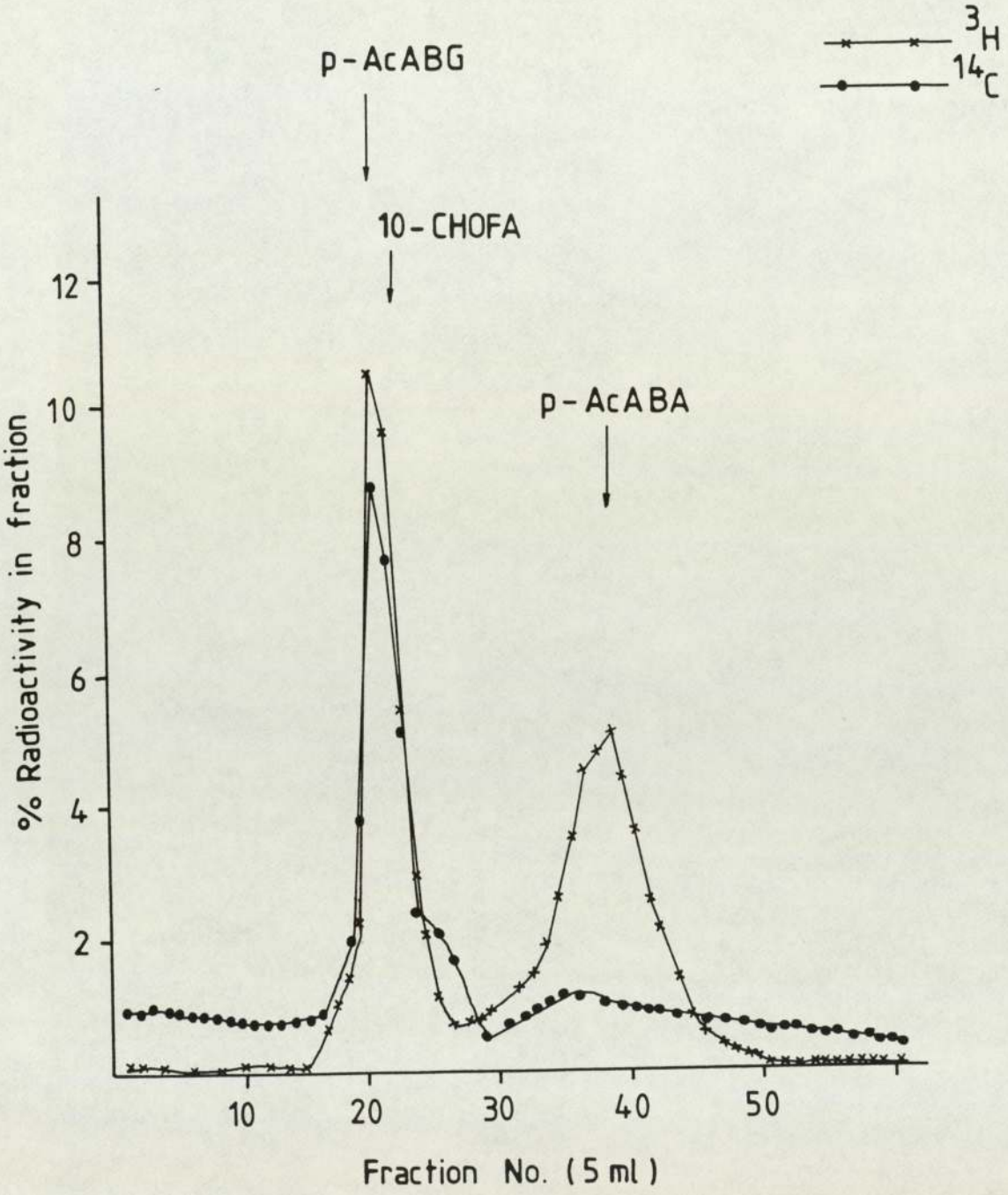


Fig. 3.1.3. Sephadex-G15 chromatography of the tritiated peak from DE-52 chromatography of 24-48hr urine sample of normal hamster receiving labelled folic acid (^3H + ^{14}C).

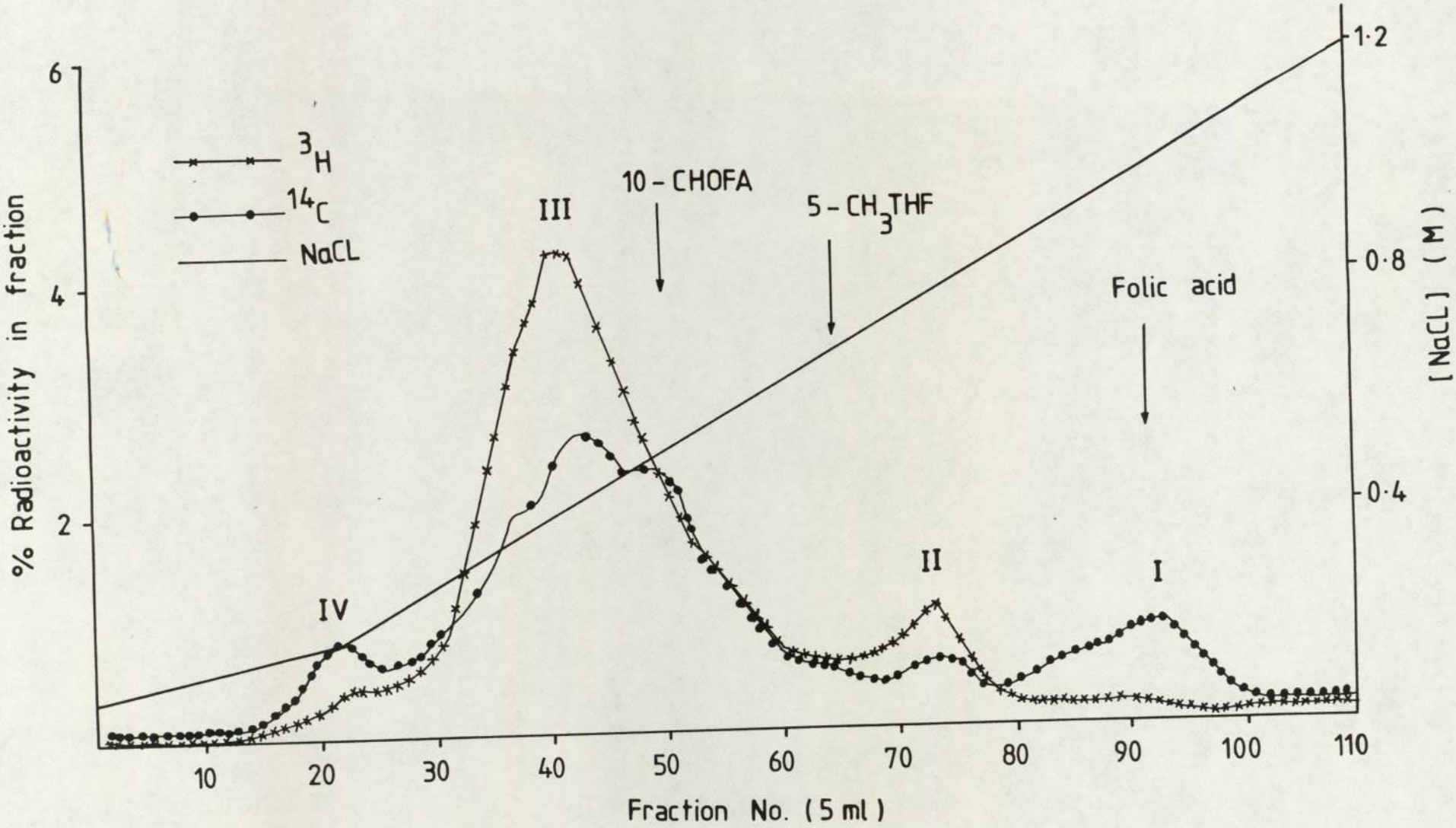


Fig. 3.1.4. DE-52 chromatography of collected faeces extract (0–48hr) of normal hamster after the administration of labelled folic acid (^3H + ^{14}C) (100 µg/kg body wt).

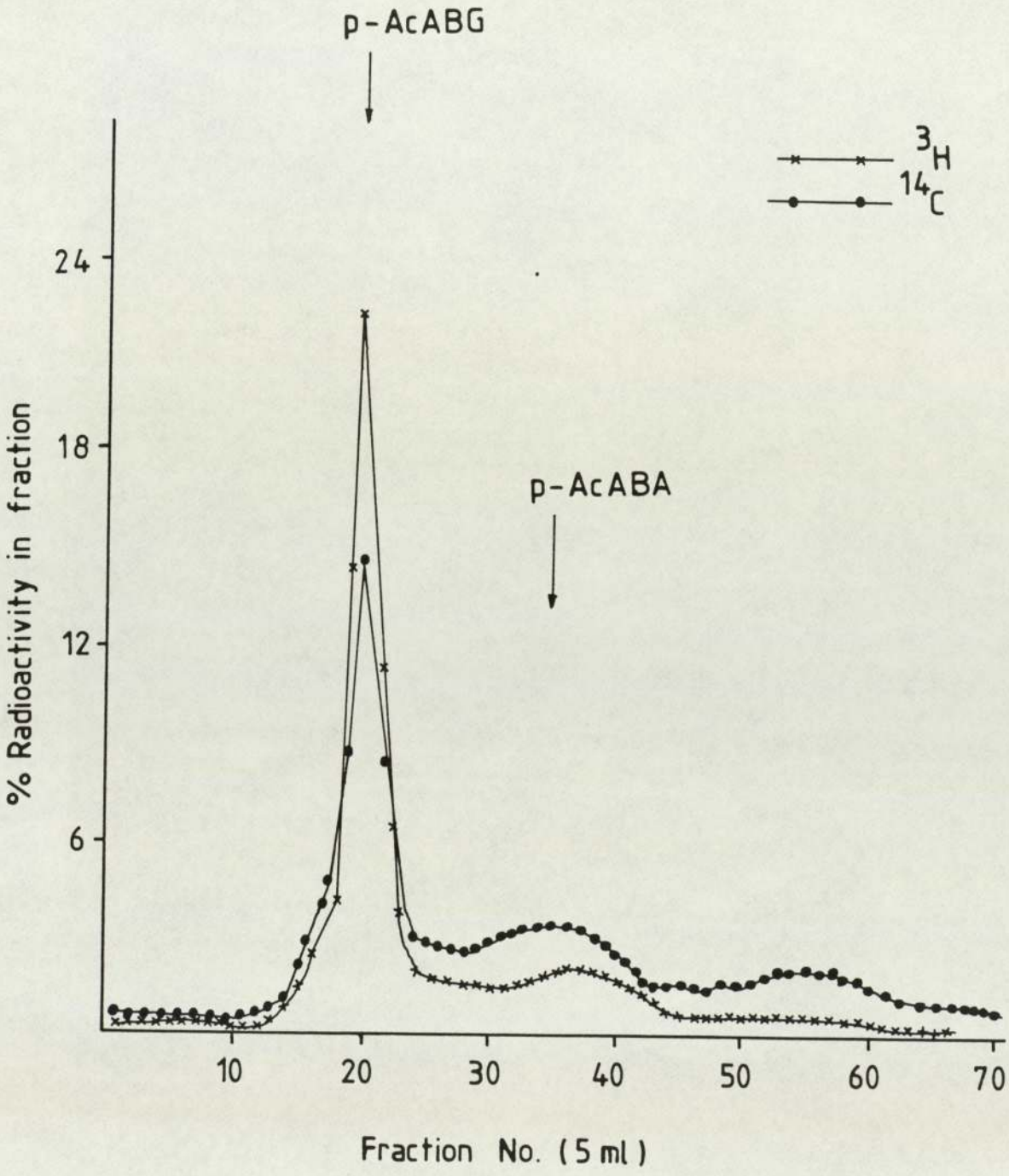


Fig. 3.1.5. Sephadex-G15 chromatography of the peak III from DE-52 chromatography of 0-48hr faeces of normal hamster after the administration of labelled folic acid (^{3}H + ^{14}C).

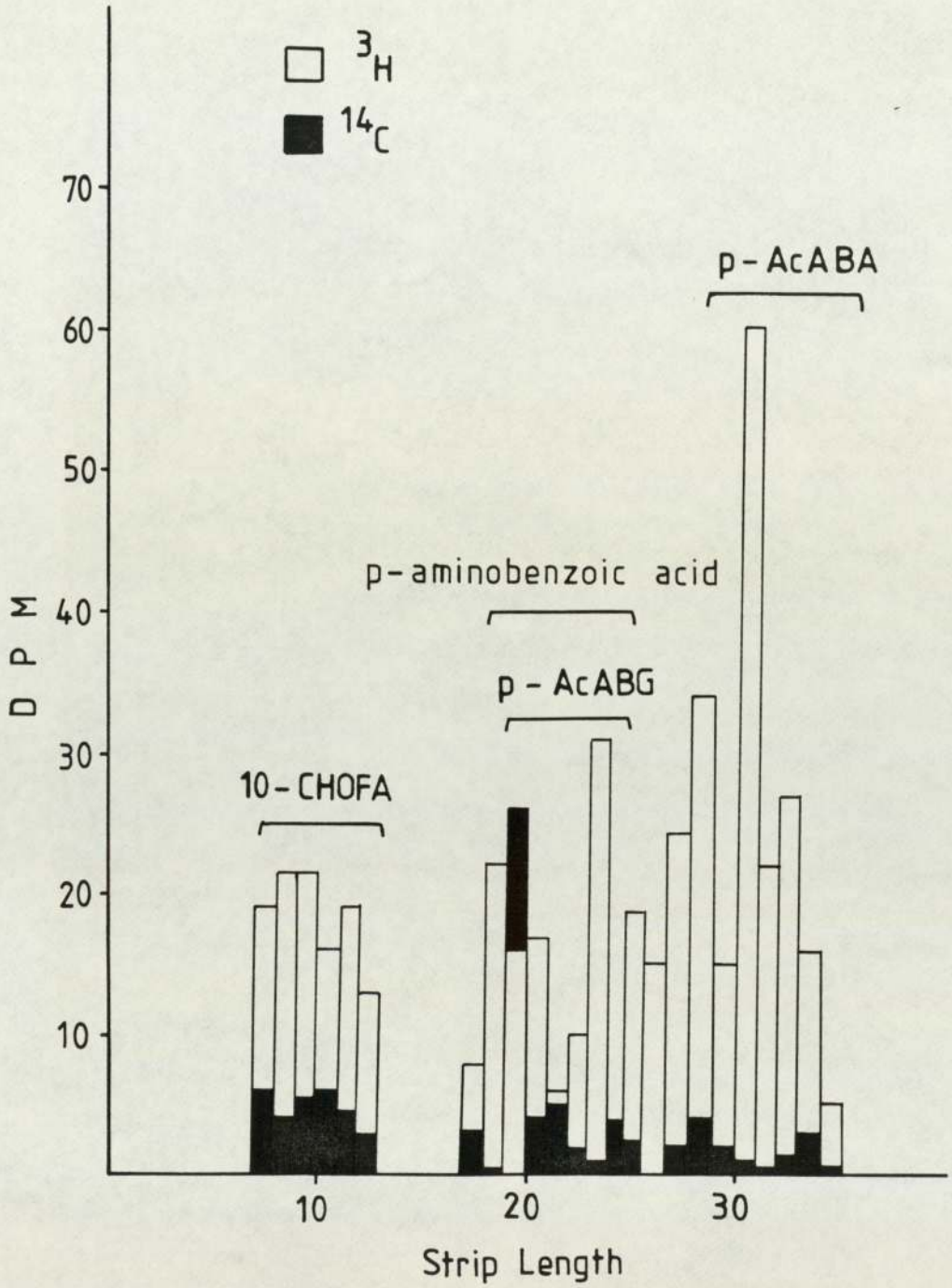


Fig. 3.1.6 Paper Chromatography (Whatman 3MM paper) of the tritiated peak (III) from DE-52 Chromatography of 0-48hr faeces of normal hamsters receiving labelled folic acid (^{3}H + ^{14}C) orally in solvent (propanol/eq. NH_3/water).

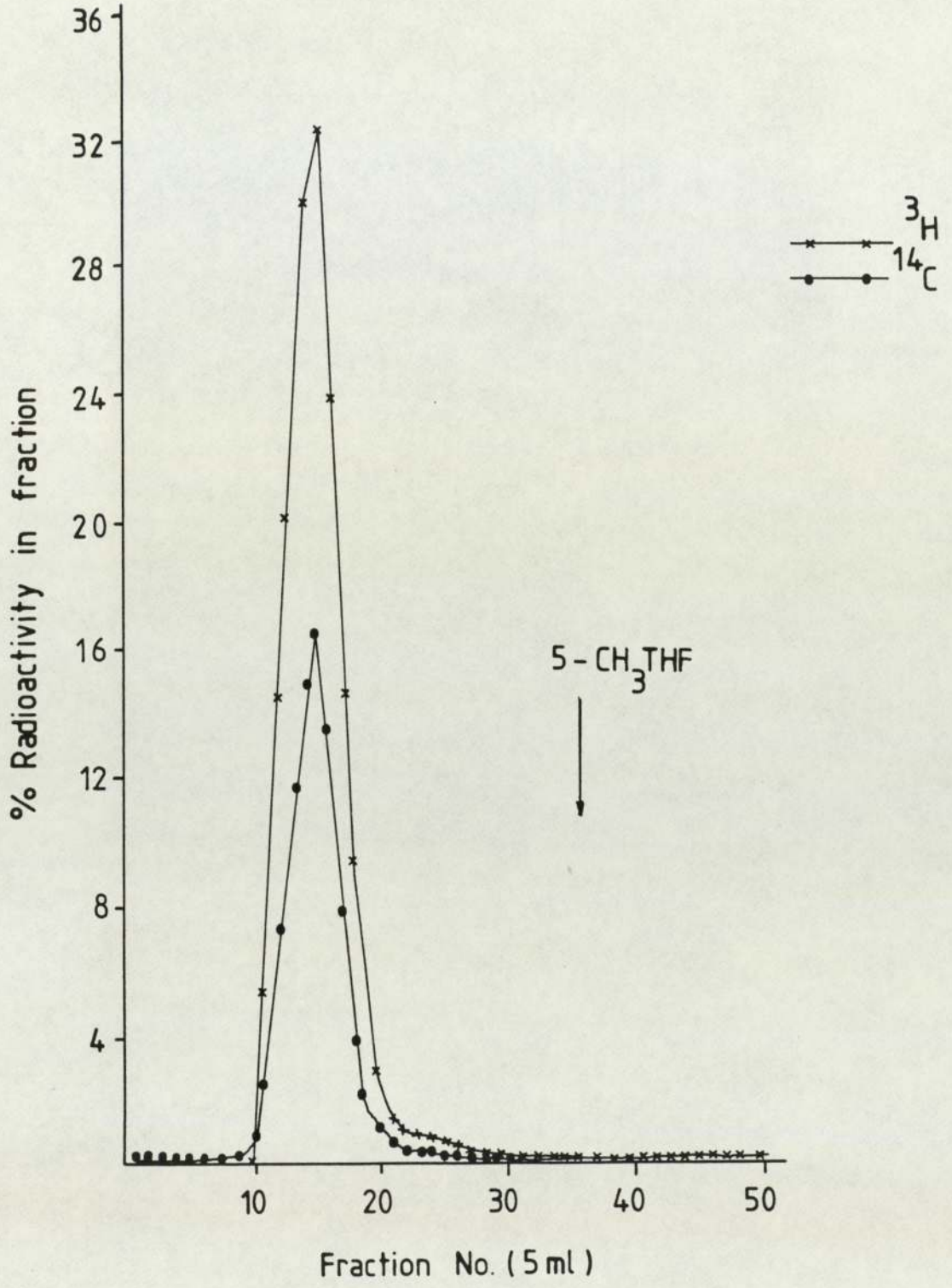


Fig. 3.1.7. Sephadex-G15 chromatography of 0-48hr liver extract of normal hamsters receiving labelled folic acid (^{3}H + ^{14}C).

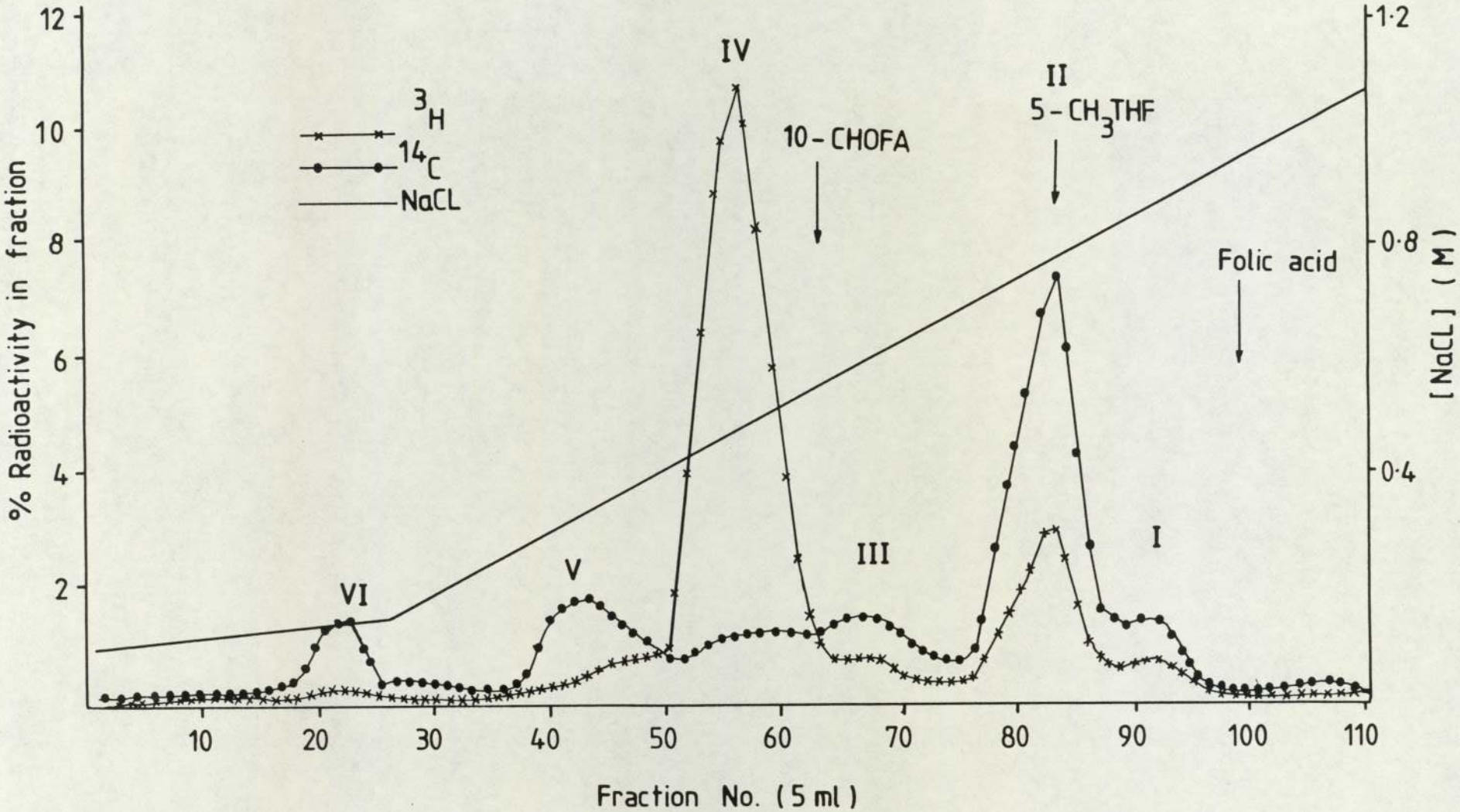


Fig. 3.2.1 DE-52 chromatography of 0-24hr urine sample of normal hamsters treated with xanthopterin plus a mixture of ^3H + ^{14}C folic acid orally.

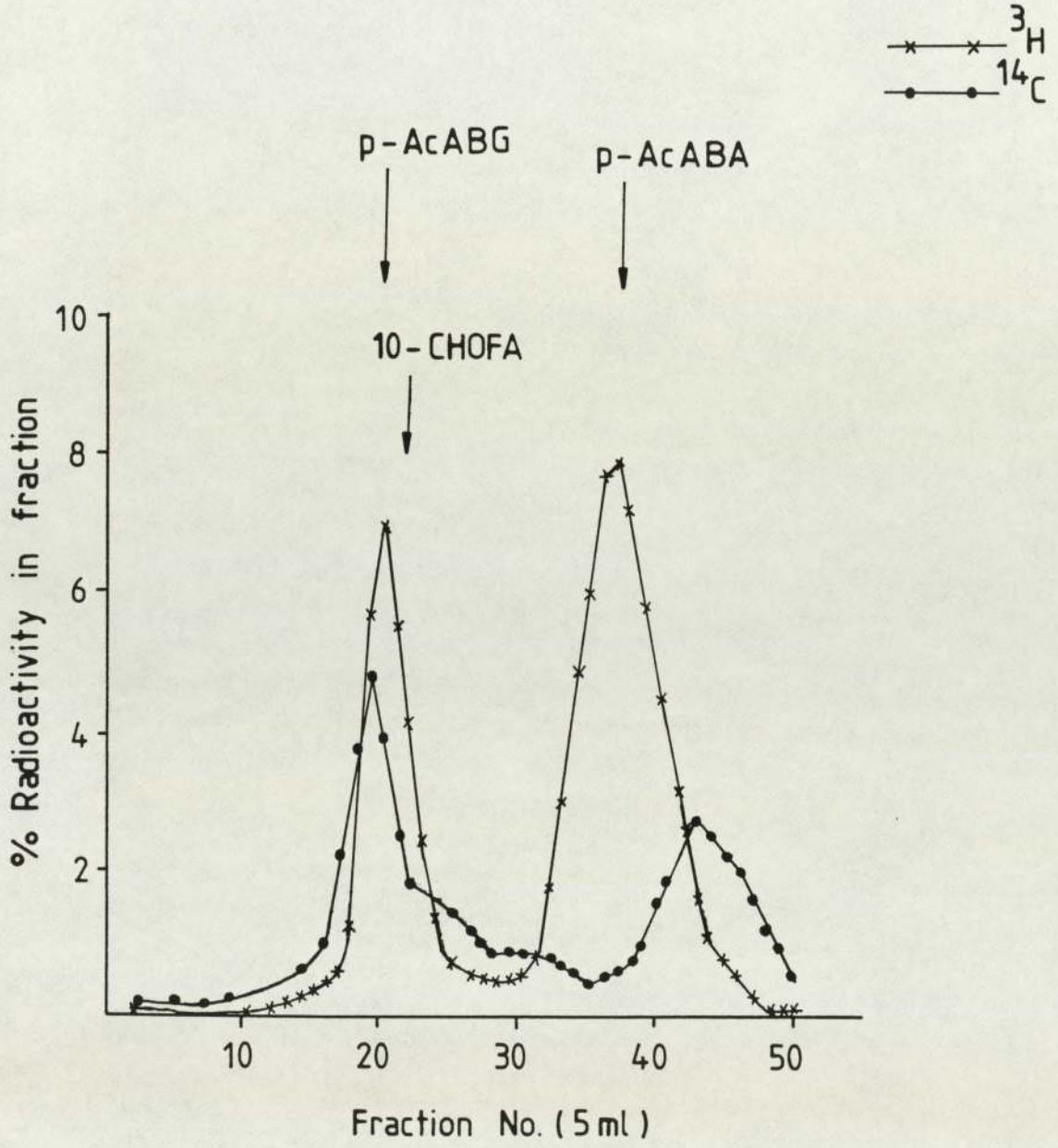


Fig. 3.2.2. Sephadex-G15 chromatography of the tritiated peak (IV) from DE-52 chromatography of 0-24hr urine sample of the normal hamsters treated with xanthopterin plus labelled folic acid (^{3}H + ^{14}C) orally.

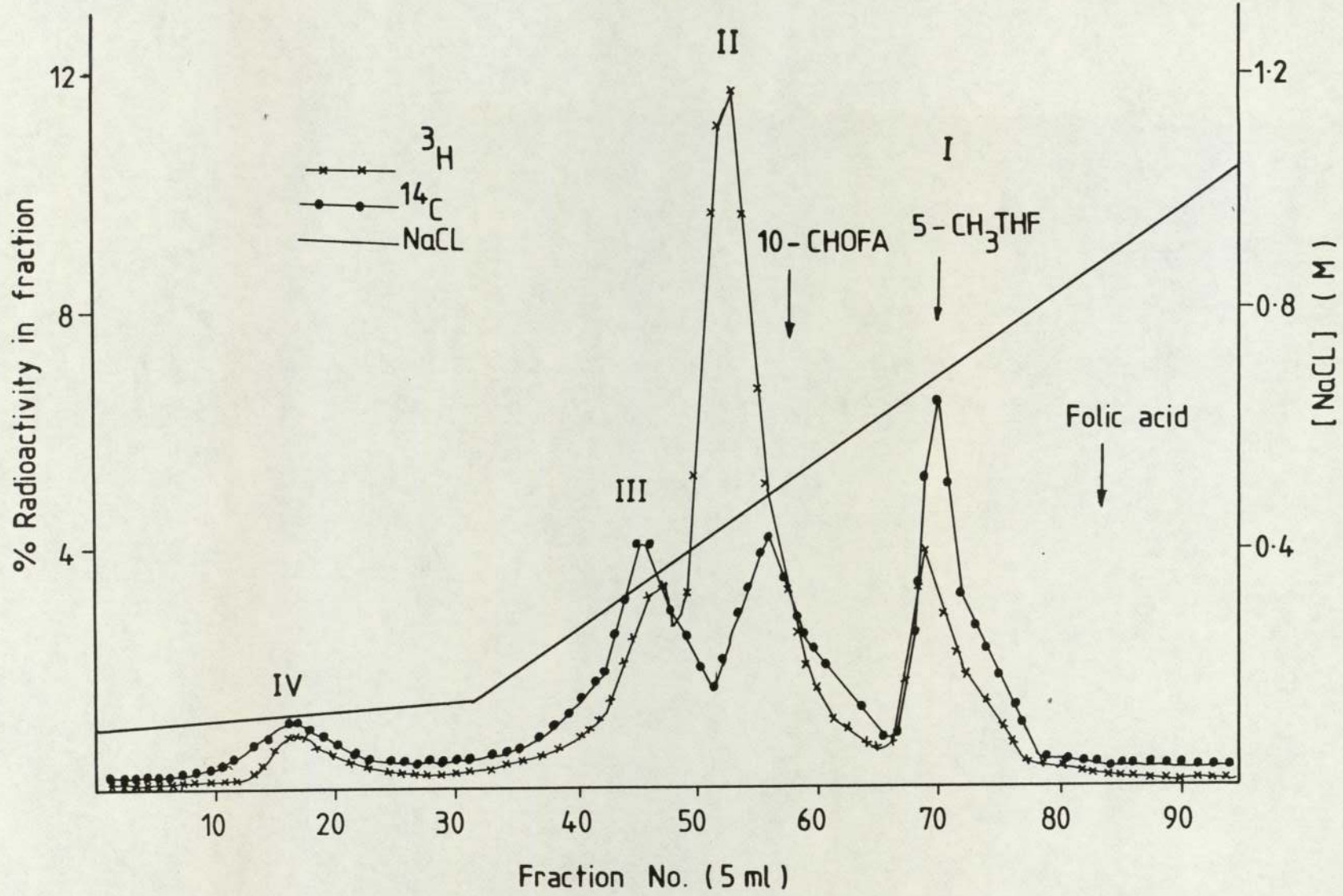


Fig. 3.2.3. DE-52 chromatography of the (24-48hr) urine sample of control hamsters receiving labelled folic acid (^{3}H + ^{14}C) and xanthopterin only.

Table 3.1.1

Animals (No)	Urine				Faeces	
	0-24hr		24-48hr		0-48hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters (7)	±17.59 ± 5.86	± 6.79 ± 4.08	± 6.03 ± 4.78	± 2.16 ± 1.44	±22.12 ± 8.98	±20.35 ± 7.79
Hamsters (7)	±20.53 ± 0.48	±11.65 ± 1.86	± 3.42 ± 1.64	± 1.87 ± 0.87	±25.81 ± 6.7	±26.70 ± 4.57
Average	±19.06 ± 3.17	± 9.22 ± 2.97	± 4.73 ± 3.21	± 2.02 ± 1.12	±23.97 ± 7.84	±23.53 ± 6.18

Animals (No)	Liver		Kidney		Total	
	0-48hr		0-48hr		0-48hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters (7)	±16.82 ± 5.99	±15.41 ± 5.28	± 1.84 ± 0.70	± 1.72 ± 0.72	64.4	39.64
Hamsters (7)	±11.69 ± 5.99	±10.24 ± 5.32	± 0.92 ± 0.22	± 0.74 ± 0.15	62.37	51.2
Average	±14.26 ± 5.99	±12.83 ± 5.3	± 1.38 ± 0.46	± 1.23 ± 0.44	63.39	45.42

Recovery of radioactivity in the urine, faeces and tissues for 0-48hr following the administration of (100µg/kg body wt) of a mixture of labelled folic acid (^{14}C + ^{3}H) to each normal hamster. The results are expressed as the percentage of the dose (mean ± standard deviation).

Table 3.1.2

Animals (No)	Time Period	Folic acid		5-CH_3THF		10-CHOFA	Folate X		Folate Z	
		^3H	^{14}C	^3H	^{14}C	^{14}C	^3H	^{14}C	^3H	^{14}C
Hamsters (7)	0-24hr	N.d	N.d	2.15	2.02	0.46	0.82	0.87	0.72	0.58
	24-48hr	N.d	N.d	0.42	0.35	0.39	0.2	0.17	0.15	0.14
Hamsters (7)	0-24hr	N.d	N.d	5.06	5.02	0.46	0.91	0.96	1.38	1.24
	24-48hr	N.d	N.d	0.26	0.24	0.26	N.d	N.d	N.d	N.d

Animals (No.)	Time Period	p-AcABG	p-AcABA	Metabolite(A)		Metabolite(B)	
		^3H	^3H	^3H	^{14}C	^3H	^{14}C
Hamsters (7)	0-24hr	2.63	6.36	-	0.89	0.8	0.91
	24-48hr	0.65	1.34	-	0.14	0.3	0.34
Hamsters (7)	0-24hr	3.94	5.6	-	0.64	0.36	1.27
	24-48hr	0.66	1.13	-	0.37	0.54	0.49

Metabolites present in urine of the normal hamsters (0-48hr) following the administration of (100µg/kg body wt.) of labelled folic acid (^3H + ^{14}C). The results are expressed as the percentage of the dose.

Table 3.1.2 (Cont'd)

Animals (No.)	Time Period	Urea	$^{3}H_2O$	$^{14}CO_2$	Total	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters (7)	0-24hr	0.57	0.28	0.41	13.76	6.71
	24-48hr	0.28	0.30	0.25	2.02	2.06
Hamsters (7)	0-24hr	0.74	0.19	N.d	17.44	10.33
	24-48hr	0.28	0.14	N.d	2.73	1.64

N.d = not detected in the normal hamster (control assay)

Table 3.2.1

Animals (No)	Urine				Faeces	
	0-24hr		24-48hr		0-48hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters (3)	± 16.09 1.4	± 5.88 0.14	± 3.70 0.36	± 1.67 0.6	± 20.13 0.36	± 14.65 1.05
Hamsters (7)	± 10.16 3.12	± 6.37 2.44	± 4.95 1.90	± 3.68 2.0	± 17.7 11.60	± 20.3 11.46
Hamsters (5)	± 10.71 7.24	± 4.44 2.7	± 1.68 0.35	± 1.00 0.3	± 10.04 1.17	± 10.09 1.99
Average	± 12.32 3.92	± 5.56 1.76	± 3.44 0.87	± 2.12 0.97	± 15.96 4.38	± 15.01 4.83

Animals (No)	Liver		Kidney		Total	
	0-48hr		0-48hr		0-48hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters (3)	± 15.06 6.51	± 13.44 0.61	± 2.17 1.32	± 1.17 0.67	57.15	36.81
Hamsters (7)	± 14.52 4.60	± 12.55 3.69	± 2.01 0.36	± 0.99 0.10	49.34	43.89
Hamsters (5)	± 11.27 3.87	± 8.58 3.75	± 1.51 0.56	± 0.8 0.34	35.21	24.91
Average	± 13.62 4.99	± 11.52 2.68	± 1.90 0.75	± 0.99 0.37	47.23	35.20

Recovery of radioactivity in the urine, faeces and tissues of the normal hamsters for 0-48hr following the administration of 100mg/Kg body wt. of xanthopterin and 100µg/kg body wt of a mixture of labelled folic acid (^{14}C + ^{3}H). The results are expressed as the percentage of the dose.

Table 3.2.2

Animals (No)	Time Period	Folic acid		5-CH_3THF		10-CHOFA	Folate X		Folate Z	
		^3H	^{14}C	^3H	^{14}C	^{14}C	^3H	^{14}C	^3H	^{14}C
Hamsters (3)	0-24hr	N.d	N.d	1.38	1.33	0.49	1.02	0.9	N.d	N.d
	24-48hr	N.d	N.d	0.46	0.36	0.2	0.05	0.05	N.d	N.d
Hamsters (7)	0-24hr	N.d	N.d	3.33	2.97	0.57	N.d	N.d	N.d	N.d
	24-48hr	N.d.	N.d	0.75	0.65	0.08	0.22	0.22	N.d	N.d
Hamsters (5)	0-24hr	N.d	N.d	1.86	1.92	0.32	0.37	0.32	0.43	1.70
	24-48hr	N.d	N.d	0.29	0.25	0.19	N.d	N.d	N.d	N.d

Animals (No.)	Time Period	p-AcABG	p-AcABA	Metabolite(A)		Metabolite(B)	
		^3H	^3H	^3H	^{14}C	^3H	^{14}C
Hamsters (3)	0-24hr	2.65	5.33	-	0.34	0.78	1.13
	24-48hr	0.70	1.26	-	0.1	0.54	0.43
Hamsters (7)	0-24hr	2.12	2.32	-	0.85	0.65	1.29
	24-48hr	1.04	0.57	-	0.04	0.42	0.96
Hamsters (5)	0-24hr	1.43	3.18	-	0.23	0.69	0.66
	24-48hr	0.3	0.38	-	0.02	0.32	0.25

Metabolites present in urine of the normal hamsters (0-48hr) and expired air after the administration (100mg) of xanthopterin orally and the dose (100µg/kg body wt) of labelled folic acid (^3H + ^{14}C). The results are expressed as the percentage of the dose.

Table 3.2.2 cont'd

Animals (No.)	Time Period	Urea	$^{3}H_2O$	$^{14}CO_2$	Total	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters	0-24hr	0.64	0.38	0.85	11.54	5.68
(3)	24-48hr	0.17	0.18	0.71	3.19	1.85
Hamsters	0-24hr	N.d	N.d	0.5	8.42	6.18
(7)	24-48hr	0.17	0.01	0.23	3.01	2.35
Hamsters	0-24hr	0.3	0.13	N.d	8.09	5.45
(5)	24-48hr	0.06	0.06	N.d	1.35	0.77

N.d = not detected in the normal hamster treated with xanthopterin

CHAPTER 4

The metabolism of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid over 0-72hr in normal Syrian hamsters and in normal Syrian hamsters treated with specific agents

4.1 The metabolism of folic acid in normal hamsters over 0 - 72 hours

Recent studies (Blair, 1976; Krumdieck, et al., 1978; Pheasant, et al., 1981) have suggested that there are two metabolically distinct pools of folate: a short term pool corresponding to newly absorbed folate and folate in the enterohepatic circulation and a long term pool representing the retained tissue folates and that folate is catabolised in both pools. This chapter describes a longer time period of investigation (0-72hr) than that discussed earlier (chapter 3) and in addition the effect of administering compounds such as xanthopterin, allopurinol, growth hormone (somatotropin) and dihydro-orotic acid on folate metabolism.

Materials and Methods

15 Syrian hamsters (80-100gm body wt.) each received an oral dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (1.00μCi, 2.0μCi) as (100μg/kg body wt.). Animals were housed in Metabowl cages. Urine, faeces, livers and kidneys were collected at three times, 0-24hr, 24-48hr and 48-72hr as was described previously in chapter 2. The liver extract preparation, the determination of radioactivity and the column chromatography were carried out as in chapter 2.

Results

a) Urine, faeces and tissue radioactivity

The recovery of radioactivity in the urine, faeces, and tissues is shown in Table 4.1.1. Over the first 24 hours the recovery of ^{3}H over ^{14}C was significantly higher (13.98% ^{3}H, 9.04% ^{14}C of the dose) ($p<0.001$) and this significance was found in the 24-48hr and 48-72hr urine samples. An excess of ^{3}H over ^{14}C was present in the faeces also during all time periods (24.82%^{3}H,18.19% ^{14}C

of the dose) which was statistically significant ($p<0.001$).

The recovery of radioactivity in the liver shows significantly less ^{3}H than ^{14}C at 24hr (10.89% ^{3}H, 17.15% ^{14}C) ($0.005<p<0.001$). The percentage increased in the 48 hours (13.11% ^{3}H, 20.26% ^{14}C of the dose) ($0.005<p<0.001$). A smaller percentage of the dose of ^{3}H than ^{14}C was found in the kidneys.

b) Urinary metabolites

Qualitative analysis of the 0-24hr urine sample (Table 4.1.2) showed that 5-CH_3THF (II) was the major intact folate in the urine, in addition to another unidentified folate X (I). The ratio of ^{3}H:^{14}C was similar to that of starting materials. Scission products appeared with greater amount of p-AcABA than that of p-AcABG. However, $^{3}H_2O$, urea and other ^{14}C labelled only species appeared (IV)(B) as well as unidentified metabolite A in pk III (Table 4.1.2). No free amine was found. Folic acid was absent. The qualitative analysis of 24-48hr and 48-72hr urine sample gave the same pattern as 0-24hr (Table 4.1.2). The excretion of 5-CH_3THF fell in the later urine sample, and other intact folates appeared as folate Z (III) and 10-CHOTHF (IV). DE-52 chromatography and Sephadex-G15 column chromatography of the two times of urine sample are illustrated in figures (4.1.1, 4.1.2, 4.1.3, 4.1.4).

c) Liver extracts

Figures (4.1.5, 4.1.6) illustrate the chromatography of the liver extracts prepared as described in chapter 2 on Sephadex-G15. At all time periods a single radioactive peak labelled with both ^{3}H and ^{14}C was found in the void volume of the column and chromatographed in the same position as folate polyglutamate (Connor et al., 1977).

4.2 Folic acid metabolism in normal hamsters following i.p. xanthopterin administration

The investigation was carried out to study folate metabolism after the intraperitoneal injection of xanthopterin. It was hoped that this inquiry would clarify the mechanism of folate catabolism *in vivo* and assist our understanding of the role of xanthopterin in the kidney hypertrophy.

Materials and Methods

15 male Syrian golden hamsters (80-100gm body wt.) were anaesthetized with ether and each received an intraperitoneal injection (i.p) of xanthopterin (Sigma, London) (2.5mg dissolved in 0.5ml of 0.15 M NaCl). Twenty four hours later each animal received a second dose of xanthopterin (2.5mg/kg body wt.). At the same time as the last dose of xanthopterin, animals were given an oral dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid [1.0μCi, 2.0μCi) as (100μg/kg body wt). Thereafter every five animals were housed separately in wire bottomed Metabowl cages fitted with a urine-faeces separator, food and tap water were given ad libitum (see chapter 2). 5 animals were killed at each 24 hours, the collection of urine, faeces, the removal of livers and kidneys at three time periods of 0-24hr, 24-48hr, and 48-72hr, the preparation of liver extracts, the determination of radioactivity and column chromatography are similar to that were described in chapter 2.

Results

The recovery of radioactivity in urine, faeces, livers and kidneys is shown in Table 4.2.1. Quantitative analysis of the urine radioactivity showed that at all times, the percentage of the ^{3}H was significantly higher ($P<0.001$) than ^{14}C. There was no significant difference in the total radioactivity recovery in the

urine of the control and test animals. In the faecal recovery, the percentages of the dose of ^{3}H and ^{14}C are similar. In the liver, it was observed that there was an excess of ^{14}C over ^{3}H (7.43% ^{3}H, 8.24% ^{14}C of the dose). A similar excess of ^{14}C over ^{3}H was found in the kidneys.

Urinary metabolites

Table 4.2.2 shows the relative distribution of each metabolite in the 0-24hr urine sample of normal hamsters treated with xanthopterin (i.p). The pattern was similar to that of the control, with a slight difference in the excretion of 5-CH_3THF (II) and in addition, the appearance of 10-CHOFA (III). The qualitative analysis of urine sample on both column systems was illustrated in figures (4.2.1), (4.2.2). The 48 hours urine sample had a similar pattern to the 24hr urine sample (Table 4.2.2).

The third urine sample (48-72hr) has similar metabolites to the other urine samples,(Table 4.2.2)with decrease in the excretion of 5-CH_3THF (figure 4.2.3, 4.2.4).

Liver extracts

Chromatography on Sephadex-G15 column of liver extracts for all times 24hr, 48hr and 72hr gave the same pattern as was shown in the control hamsters. The major radioactive peak eluted in the position of folate polyglutamates close to the void volume. No other labelled compound was found.

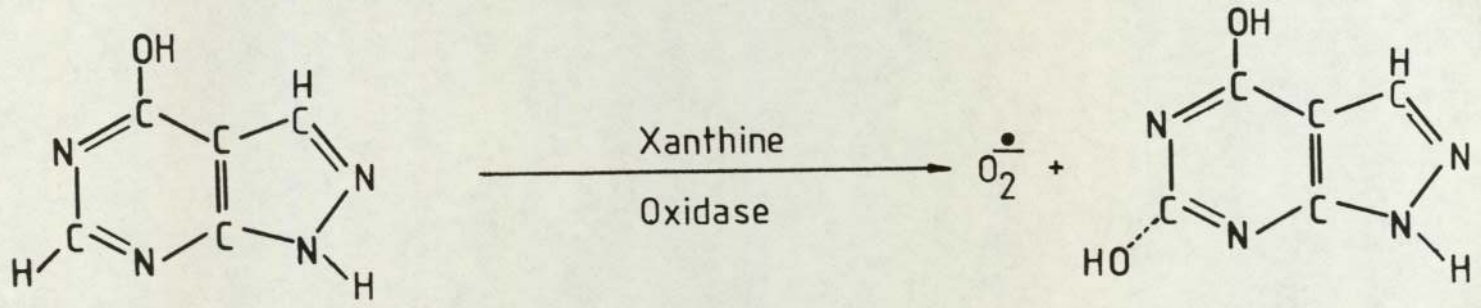
4.3 The metabolism of folic acid in normal hamsters after allopurinol administration

Allopurinol (4-hydroxypyrazolo (3,4-d) pyrimidine) (Webster, 1981) is a hypoxanthine analogue and a xanthine oxidase inhibitor which is widely used to control uric acid production in gout, other hyperuricemic states and the formation of various renal stones (Rundles et al., 1963; Klinenberg et al., 1965; Webster, 1981,).

Allopurinol is also a substrate for xanthine oxidase and is converted to oxipurinol [2,4-dihydroxypyrazolo (3,4-d) pyrimidine,] an analogue of xanthine (Elion, 1978) (Figure 4.3.A.). The biological half-life of allopurinol in man is 1-2 hours (Elion et al., 1966; Hande et al., 1978). Thus its disappearance in the body is extremely rapid. Administration of allopurinol blocks the conversion of hypoxanthine and xanthine to uric acid (Figure 4.3.B) resulting in a fall in serum uric acid concentration (Hande and Chabner, 1980). Allopurinol as a substrate for xanthine oxidase would be expected to form superoxide anion and this has been confirmed (Surdhar, personal communication). Xanthine oxidase is found in the supernatant of liver homogenates (Aebi, et al., 1962; Villela et al., 1955) a fraction which is frequently not separated from the microsomal oxygenase system (Fouts, 1970). The interaction of allopurinol with folate metabolism in Syrian golden hamster was studied to observe if inhibition of xanthine oxidase affected folate catabolism, as it has been previously suggested that it was mediated by xanthine oxidase (Elion, 1966).

Materials and Methods

15 male Syrian golden hamsters (80-100gm body wt) each received an oral dose of allopurinol (2mg/0.5ml Mazola oil) (Aldrich Chemical Co. Limited, England) per day for three days. On the fourth day, all the animals received another dose of allopurinol (2mg/0.5ml oil), in addition to the administration of an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (100 µg/kg body wt). Five hamsters were housed in the Metabowl cages separately and after twenty four hours they were killed with ether, and urine, faeces, livers and kidneys were collected (see chapter 2). The remaining hamsters were given each with further oral dose (2mg) of allopurinol



Allopurinol

[4-hydroxypyrazolo (3,4 - d) pyrimidine]

Oxipurinol

[2-4-dihydroxypyrazolo (3,4 - d) pyrimidine]

Figure 4.3.A : Reaction of oxidation of allopurinol

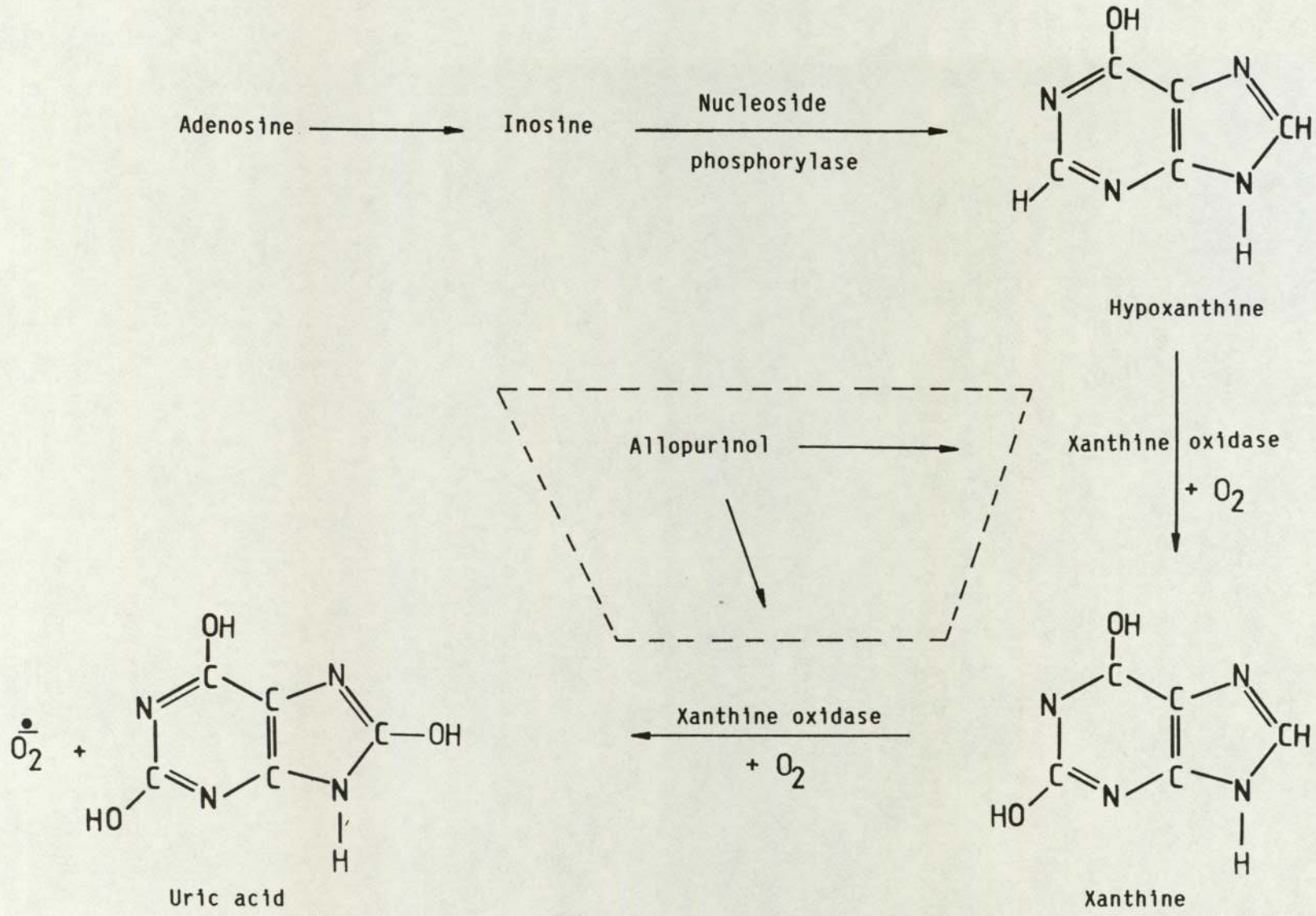


Figure 4.3.B : The metabolism of purine nucleoside to uric acid; Reaction promoted by xanthine oxidase inhibited by allopurinol

then and the second group of five hamsters were housed, and their urine, faeces and tissues were collected as above. The last group of five living hamsters received the last dose of allopurinol (2mg/0.5ml oil) each, and also housed for another twenty four hours, then killed. The collection of urine, faeces and tissues was done as in previous description (chapter 2). The surgical technique, liver extract preparation, the determination of the radioactivity and the column chromatography were as previously described in chapter 2.

Results

Table 4.3.1 records the distribution of radioactivity in urine, faeces and tissues after the oral dose of allopurinol to Syrian hamsters. Quantitative analysis of urine samples at all three time periods showed that the recovery of ^{3}H is higher than ^{14}C (29.38% ^{3}H, 10.67% ^{14}C of the dose) ($p=0.001$). However, the urinary recovery at 24hr (18.86 $\pm$6.6% ^{3}H of the dose) was higher than of the control hamsters (13.98 $\pm$3.21% ^{3}H of the dose) ($p<0.005$). Similarly, the recovery of the 24-48hr urine sample was higher (7.48 $\pm$1.6% ^{3}H, 2.54 $\pm$0.41% ^{14}C of the dose) than that of the control hamsters (2.79 $\pm$1.29% ^{3}H, 1.76 $\pm$0.77% ^{14}C of the dose) ($p=0.001$).

There was no significant difference in the recovery of 48-72hr urine sample (3.04 $\pm$1.04% ^{3}H, 1.65 $\pm$0.81% ^{14}C of the dose) compared with control assay (2.63 $\pm$1.19% ^{3}H, 1.48 $\pm$0.62% ^{14}C of the dose). An excess of ^{14}C dose over ^{3}H was excreted in the faeces particularly during 24hr (16.81 $\pm$2.53% ^{14}C, 9.77 $\pm$3.83% ^{3}H) ($p<0.001$). The hepatic radioactivity in 24hr time

period appeared to be slightly different (14.10 ±6.17% ^{3}H, 18.59 ±6.89% ^{14}C of the dose) to the control hamsters (10.89 ±2.8% ^{3}H, 17.15 ±6.2% ^{14}C of the dose) and the ^{14}C species is higher than the ^{3}H species, while it was similar to that of controls in both 48hr and 72hr time periods. Similar radioactivity was found in kidneys of hamsters pre-treated with allopurinol and the control hamsters and rather more ^{14}C over ^{3}H was recovered.

Urinary metabolites

Qualitative analysis of all urine samples was carried out on both DE-52 chromatography and Sephadex-G15 column chromatography (Figure 4.3.1, 4.3.2, 4.3.3, 4.3.4). In 0-24hr urine sample, the pattern of metabolites was shown in Table 4.3.2 with the presence of 5-CH_3THF (II), 10-CHOFA (III), together with the unidentified folate X (I) as intact folates. The excretion of 5-CH_3THF was very low (1.05%) as the percentage of the dose compared with controls (3.78%). However a higher percentage of p-AcABA (10%) than in the control hamsters (4%) was observed, as well as increased p-AcABG. Urea, $^{3}H_2O$ and unidentified metabolites (A and B) with ^{14}C labelled species were excreted also. No folic acid and amines appeared.
In 48hr and 72hr urine samples, the pattern was similar to that of the 24hr urine sample (Table 4.3.2) except a more rapid fall of intact folate with increasing time was detected. However, the amount of scission products were similar to that of control pattern.

Liver extracts

Sephadex-G15 column chromatography of liver extracts of all time periods showed a similar pattern to the control hamsters

(Figure 4.3.5). A single peak of radioactivity as folate polyglutamate appeared only.

4.4 The effect of growth hormone (somatotropin) on folate metabolism in normal Syrian golden hamsters

Somatotropin (growth hormone) is a pituitary protein hormone which plays an essential role in the biological regulation of growth and exerts influence upon many branches of metabolism (Pankov et al., 1982). It is secreted by the acidophils (eosinophilic cells). As isolated from the pituitary gland of mammalian species, it has a molecular weight of about 21,500 (Harper, 1975) and is a single polypeptide chain of 188 amino acid residues. It has anabolic effects on tissues such as muscle, adipose tissue and liver (Montgomery et al., 1980; Botella-Ilussià, 1973 and Harper, 1975). In muscle, growth hormone can stimulate protein synthesis by increasing the transport of amino acids into the cell (Harper, 1975) as well as by other means. In the intact animal, growth hormone administration results in an increase in DNA and RNA synthesis (Harper, 1975). We have studied somatotropin (growth hormone) in order to see the effect of this hormone stimulating anabolism on the catabolism of folates.

Materials and Methods

15 male Syrian hamsters (80-100gm body wt) each received an intramuscular injection (0.05 i.u./0.1ml of 0.15 M NaCl) of somatotropin dose (4.0, i.u. with 3.2mg of mannitol, balanced phosphate buffer salts each ampoule) (Sigma, London) for two days.

In the second day of somatotropin pre-treatment, they received oral doses of a mixture of [2-^{14}C] and [3', 5',7,9-^{3}H]-folic acid (100µg/kg body wt). The animals were housed in Metabowl cages as described before. The collection of urine, faeces, livers and kidneys for 24hr, 48hr and 72hr has been described before (see chapter 2). The determination of radioactivity, preparation of liver extract and the column chromatography were similar as previously described in chapter 2.

Results

a) Radioactivity in urine, faeces and tissues

Table 4.4.1 summarizes the recovery of ^{3}H and ^{14}C in the urine, faeces and tissues. The urinary radioactivity excretion of 0-24hr was greater in animals pre-treated with somatotropin (22.97 ±6.91% ^{3}H, 10.26 ±3.54% ^{14}C of the dose) than in controls (13.98 ±3.21% ^{3}H, 9.04 ±2.16% ^{14}C of the dose) ($p<0.001$). Moreover, the recovery of 24-48hr urine sample was significantly higher also (5.63 ±1.82% ^{3}H, 2.95 ±0.88% ^{14}C of the dose) than in controls (2.79 ±1.29% ^{3}H, 1.76 ±0.77% ^{14}C of the dose). The recovery of radioactivity of 48-72hr urine sample of both control group and this group was similar. The ratio of ^{3}H:^{14}C dose was rather higher at all times in the urine samples than the starting materials. An excess of ^{14}C over ^{3}H was observed in the faeces and the recovery of radioactivity in 0-24hr was diminished (8.42 ±2.08% ^{3}H, 10.91 ±1.86% ^{14}C of the dose) compared to that in control hamsters (15.28 ±0.62% ^{3}H, 12.76 ±4.62% ^{14}C of the dose) ($p<0.001$). On the other hand there was no significant difference in the faecal recovery of radioactivity in 24-48hr and 48-72hr between this group and the control group.

The hepatic radioactivity at all time periods in this experimental group was similar to controls. Moreover recovery of both isotopes (^{3}H and ^{14}C) in the kidneys was similar and there was a similar recovery of radioactivity at all time periods compared to controls.

b) Urinary metabolites

Urine samples at all times gave similar patterns of labelled metabolites to that of control hamsters (Table 4.4.2) except the disappearance of the unidentified mixed labelled species. An increase of tritiated water ($^{3}H_2O$) during the later time (72hr) urine sample was detected. The percentage of dose as 5-CH_3THF (6.13% ^{3}H, 5.23% ^{14}C) was higher than in controls (3.17% ^{3}H, 3.32% ^{14}C) in the 24hr urine sample and it sharply decreased with increasing time. Also the percentages of the dose as scission products were higher than in controls. p-AcABA was the major catabolite in the presence of somatotropin which gradually decreased during later time periods. DE-52 column chromatography of urine samples is illustrated in figures (4.4.1,4.4.2).

Liver extracts

The Sephadex-G15 column chromatography of liver extracts was again obtained with a single peak of radioactivity labelled with both ^{3}H and ^{14}C folate polyglutamate and similar to the liver extract pattern of control hamsters.

4.5 The effect of dihydro-orotic acid on the folate metabolism in the Syrian golden hamsters

Dihydro-orotic acid is an intermediate in pyrimidine synthesis (Harper, 1975). It is reversibly oxidized to orotic acid by dihydroorotic acid dehydrogenase (DHODase) (EC 1.3.3.1) (Webster,

1981) (Figure 4.5.A), an enzyme found in mammalian cells on the outer surface of the inner mitochondrial membrane (Jones, 1980; Karibian, 1973; Rowe, 1983). DHODase activity is found in all tissues of the rat and it is highest in liver, heart, kidney and brain (Kennedy, 1973). In man, its highest activity is in liver, heart and kidney (Krooth, 1970; Webster, 1981). It is found also in human leukocytes (Smith and Baker, 1959), intestinal mucosa (Rowe, 1983) and cultured fibroblasts (Wuu and Krooth, 1968). Little is known about the natural electron acceptor of this enzyme from a mammalian source (Shoaf and Jones, 1973). It was found that this enzyme from rat liver produces superoxide radicals (Forman and Kennedy, 1975a). The mammalian cytochrome C was catalytically reduced in systems consisting of DHODase, oxygen and dihydro-orotate (Rajagopalan et al., 1962; Handler et al., 1964; Fridovich and Handler, 1962). This suggested that as in the case of xanthine oxidase, and hepatic aldehyde oxidase, dihydroorotic acid dehydrogenase forms superoxide anion which is then responsible for the subsequent reduction of cytochrome C. In this chapter the reaction of dihydro-orotic acid with folate catabolism was studied in vivo using Syrian golden hamsters as experimental animals in order to elucidate the role of superoxide anion in folate catabolism.

Materials and Methods

15 male Syrian golden hamsters (80-100gm body wt) were used. Each hamster received 2mg/0.5ml distilled water of dihydro-orotic acid (Sigma Chemical Co. Limited, London, England) every 24 hours for four days. In the fifth day they received another dose (2mg/0.5ml) of dihydro-orotic acid in addition to the dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (100µg/kg body wt), then every five hamsters were housed for 24hr using the

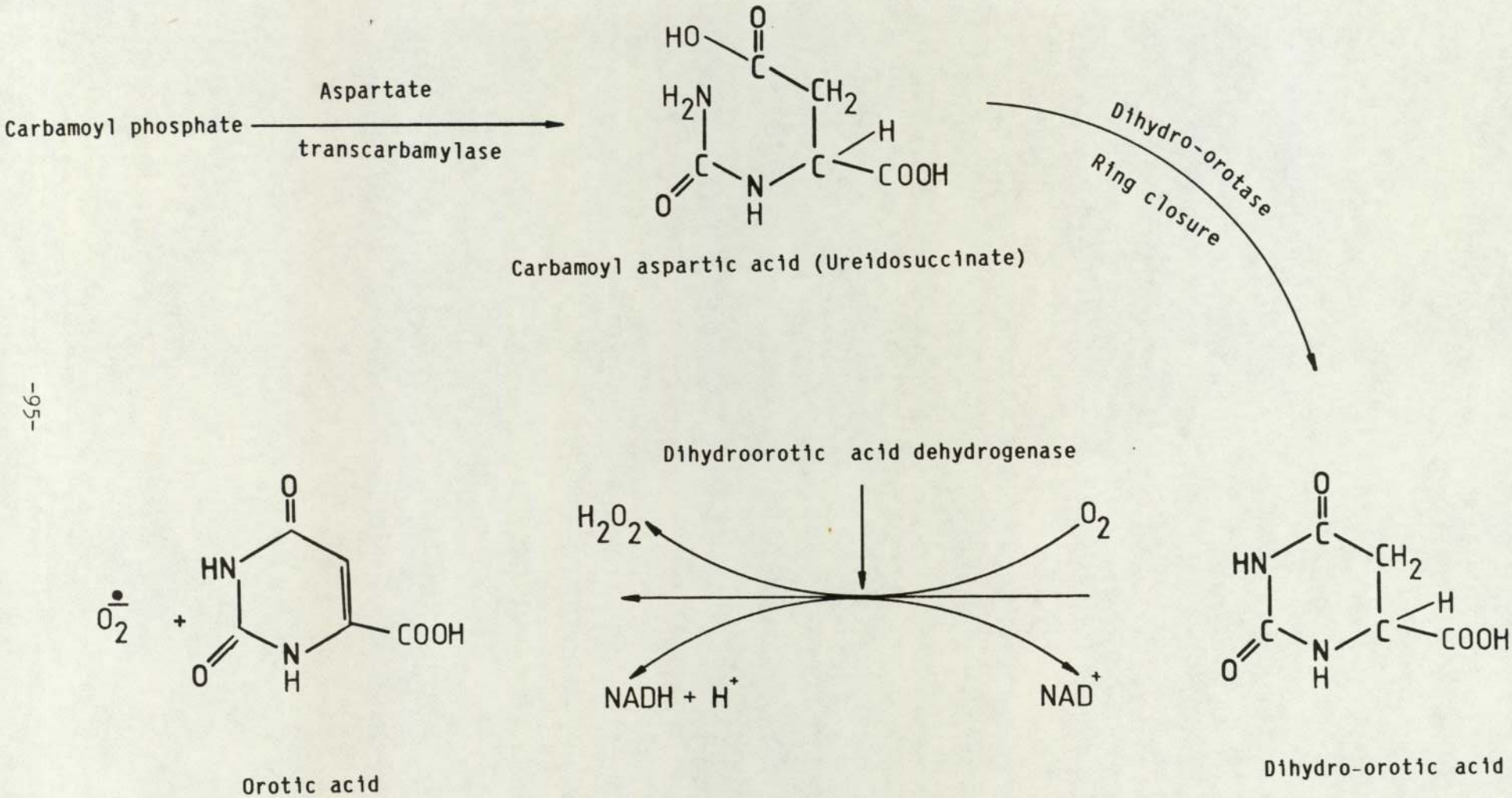


Figure 4.5.A : Part of the biosynthetic pathway for pyrimidine de novo

same techniques as in section 3 of this chapter. The remaining groups of hamsters were dosed each day with 2mg of dihydro-orotic acid until the last day. The collection of urine, faeces and tissues for 0-24hr, 24-48hr and 48-72hr was as described previously in chapter 2. The determination of radioactivity, the liver extract preparation and the column chromatography was similar to that in chapter 2.

Results

Table 4.5.1 summarizes the recovery of radioactivity of ^{3}H and ^{14}C in the urine, faeces, livers and kidneys. The urinary recovery of radioactivity in all three urine samples was generally higher compared with control hamsters. The recovery of radioactivity of 0-24hr urine sample in hamsters pre-treated with dihydro-orotic acid was 24.66 $\pm$14.96% ^{3}H of the dose, while it was 13.98 $\pm$3.21% ^{3}H of the dose in the controls ($p<0.005$). The amount of ^{3}H recovered was doubled that of in control hamsters. The urinary radioactivity in the 48hr urine sample was statistically higher than in control hamsters ($p<0.001$). The urinary radioactivity in 48-72hr (4.5 $\pm$2.1% ^{3}H, 2.04 $\pm$1.02% ^{14}C of the dose) was similar to controls (2.63 $\pm$1.19% ^{3}H, 1.48 $\pm$0.62% ^{14}C of the dose).

Thus in all cases the majority of urinary radioactivity is excreted in the first 24hr and a higher recovery of ^{3}H-isotope than ^{14}C was identified. Quantitative analysis of the faeces showed no differences in the recovery of radioactivity from controls at all time periods and there was a slight excess of recovered ^{14}C over ^{3}H dose. The hepatic recovery of radioactivity in 0-24hr of the experimental group (17.17 $\pm$7.65% ^{3}H, 24.24 $\pm$10.22%

^{14}C of the dose) was significantly higher than in control hamsters (10.89 ±2.82% ^{3}H, 17.15 ±6.2% ^{14}C of the dose) ($p<0.001$). It was similar in the later time periods and an excess of ^{14}C over ^{3}H was found. However the dose recovered in the kidneys was approximately equal to that of the controls.

Urinary metabolites

Table 4.5.2 shows the relative distribution of each metabolite appearing in the urine of hamsters treated with dihydro-orotic acid. In 0-24hr urine sample a similar pattern to that in controls was found, except an increase in the amount of scission products and 5-CH_3THF (II) as a major intact folate which was decreased slightly compared with normal hamsters. The metabolite pattern of both 24-48hr and 48-72hr (Table 4.5.2) was similar to that of the 24hr urine sample pattern with decreases in 5-CH_3THF and scission products. DE-52 column chromatography and Sephadex-G15 column systems of all urine samples were shown in Figures (4.5.1, 4.5.2, 4.5.3).

Liver extracts

Qualitative analysis of liver extracts on Sephadex-G15 (Figure 4.5.4) appeared with similar pattern to that of control group. Folate polyglutamate was the only peak found in all time periods.

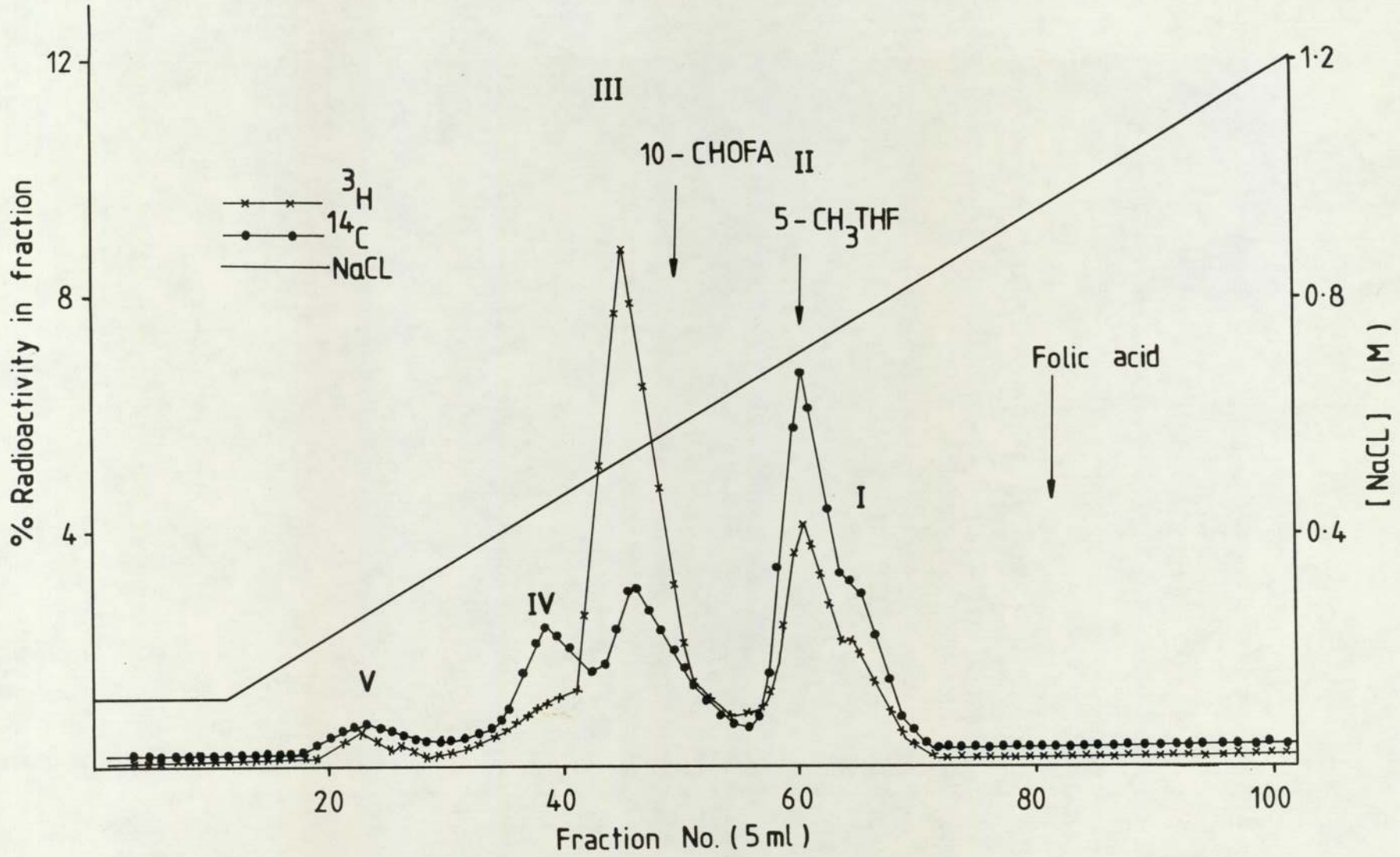


Fig. 4.1.1. DE-52 chromatography of the (0-24hr) urine sample of normal hamsters administered a mixture of the ^{3}H + ^{14}C folic acid (100µg/kg body wt) orally.

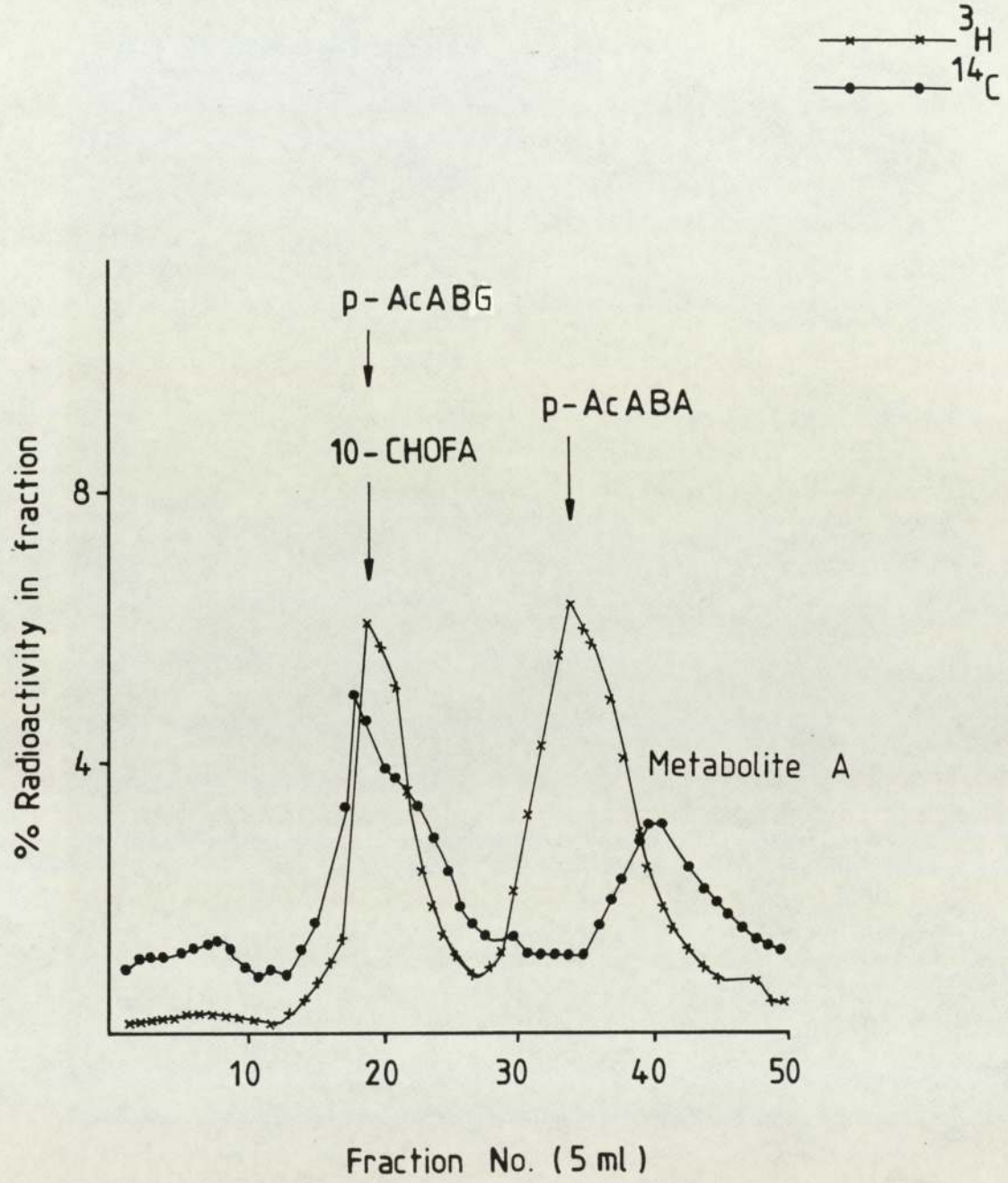


Fig. 4.1.2. Sephadex-G15 chromatography of peak III from DE-52 chromatography of 0-24hr urine sample of normal hamsters after the administration of labelled folic acid (^{3}H + ^{14}C).

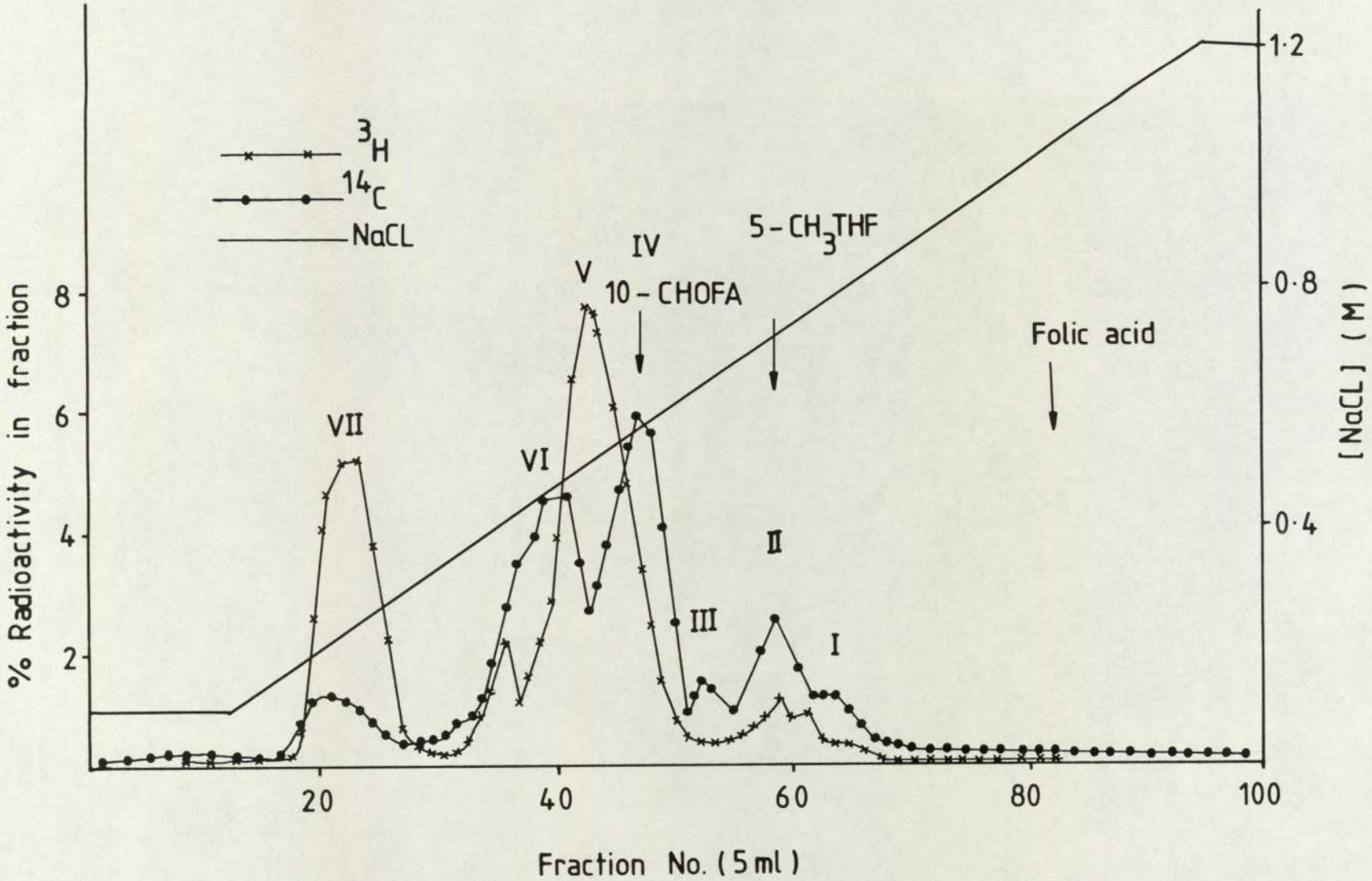


Fig. 4.1.3. DE-52 chromatography of the (48-72hr)urine sample of normal hamsters after the administration of a mixture of ^{3}H + ^{14}C folic acid (100µg/kg body wt.).

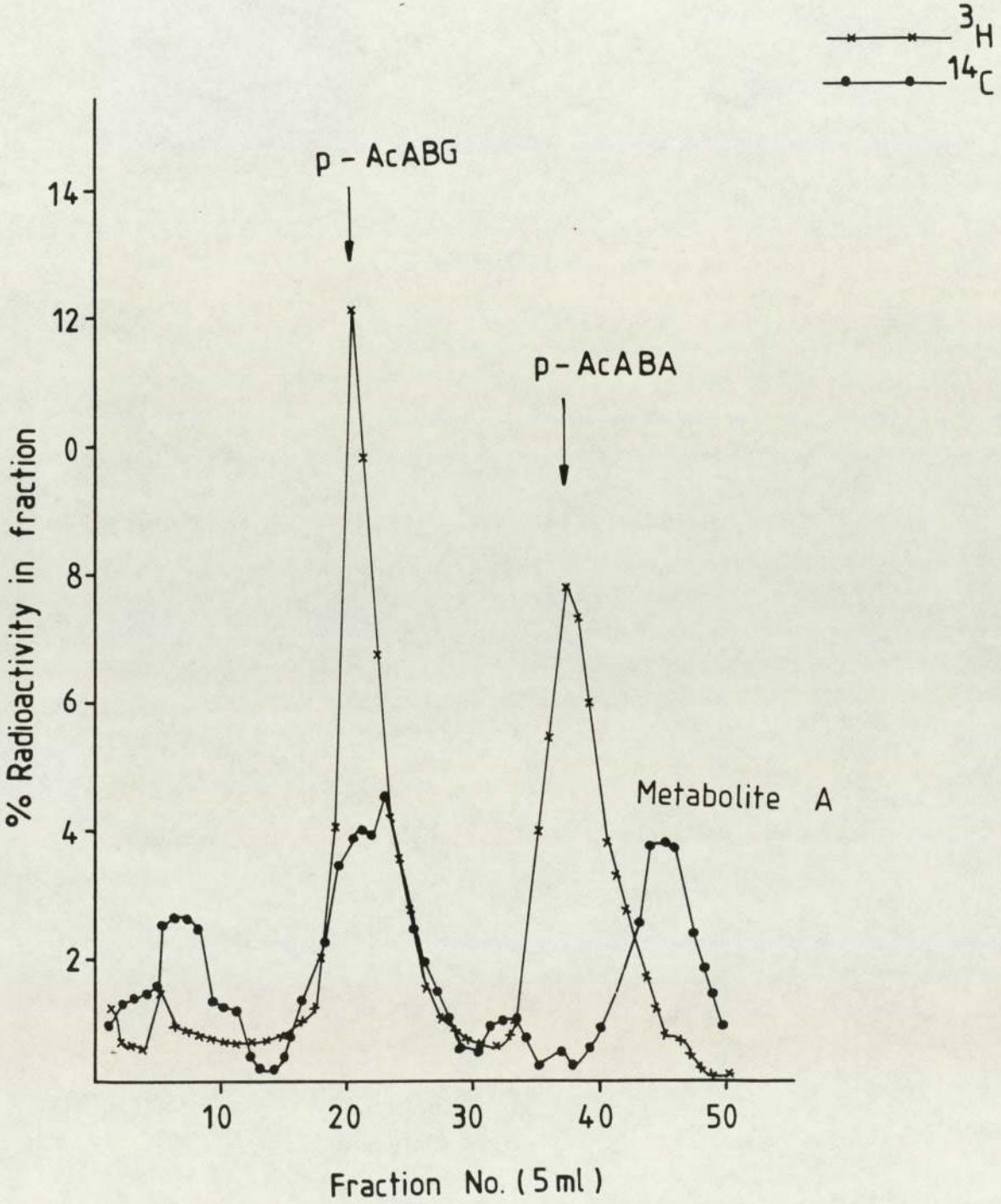


Fig. 4.1.4. Sephadex-G15 chromatography of peak V from DE-52 chromatography of 48-72 hr urine sample of normal hamsters receiving a mixture of ^{3}H + ^{14}C folic acid.

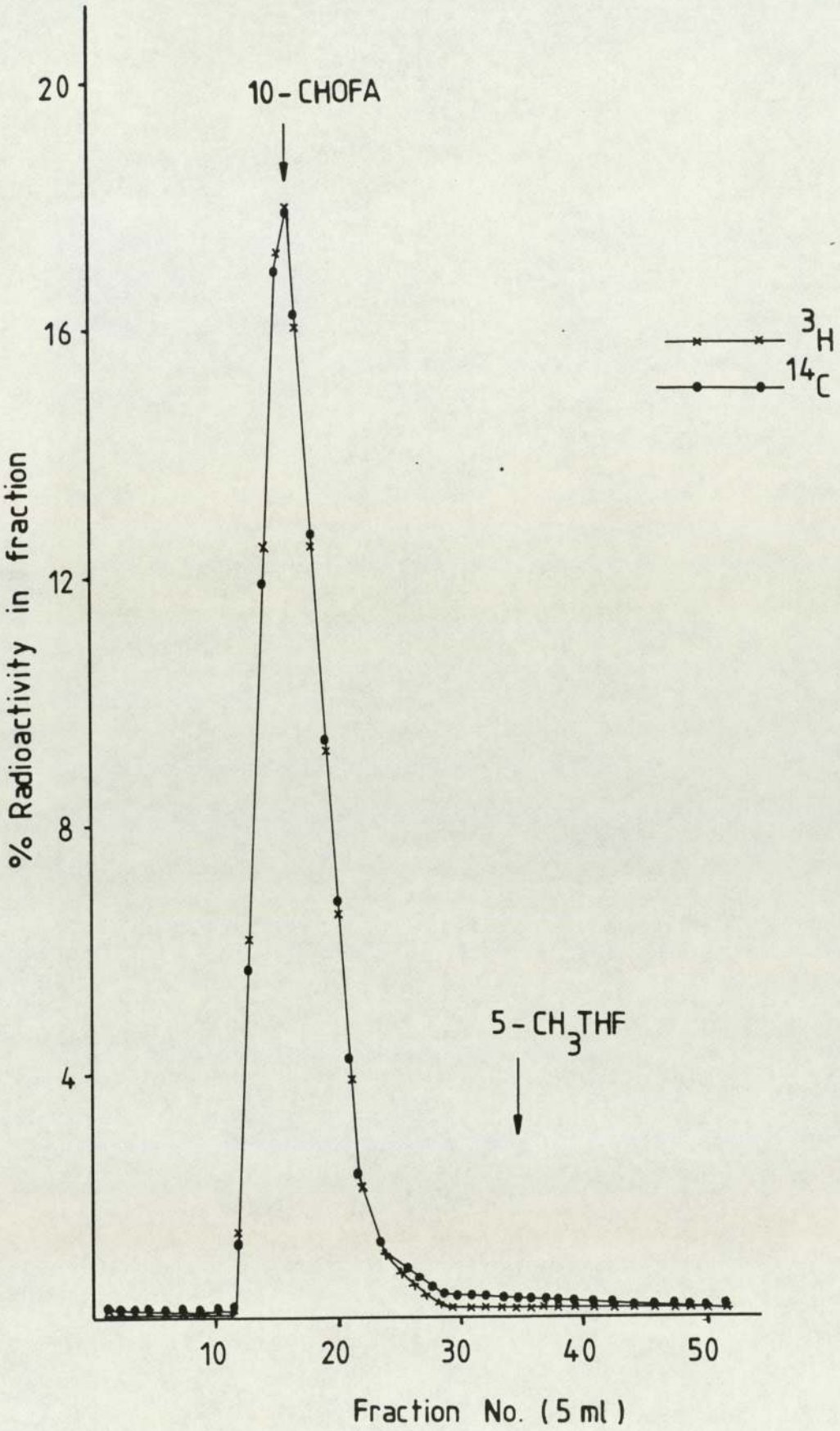


Fig. 4.1.5. Sephadex-G15 chromatography of liver extract at 24hr of normal hamsters after administration of Labelled folic acid (^{3}H + ^{14}C).

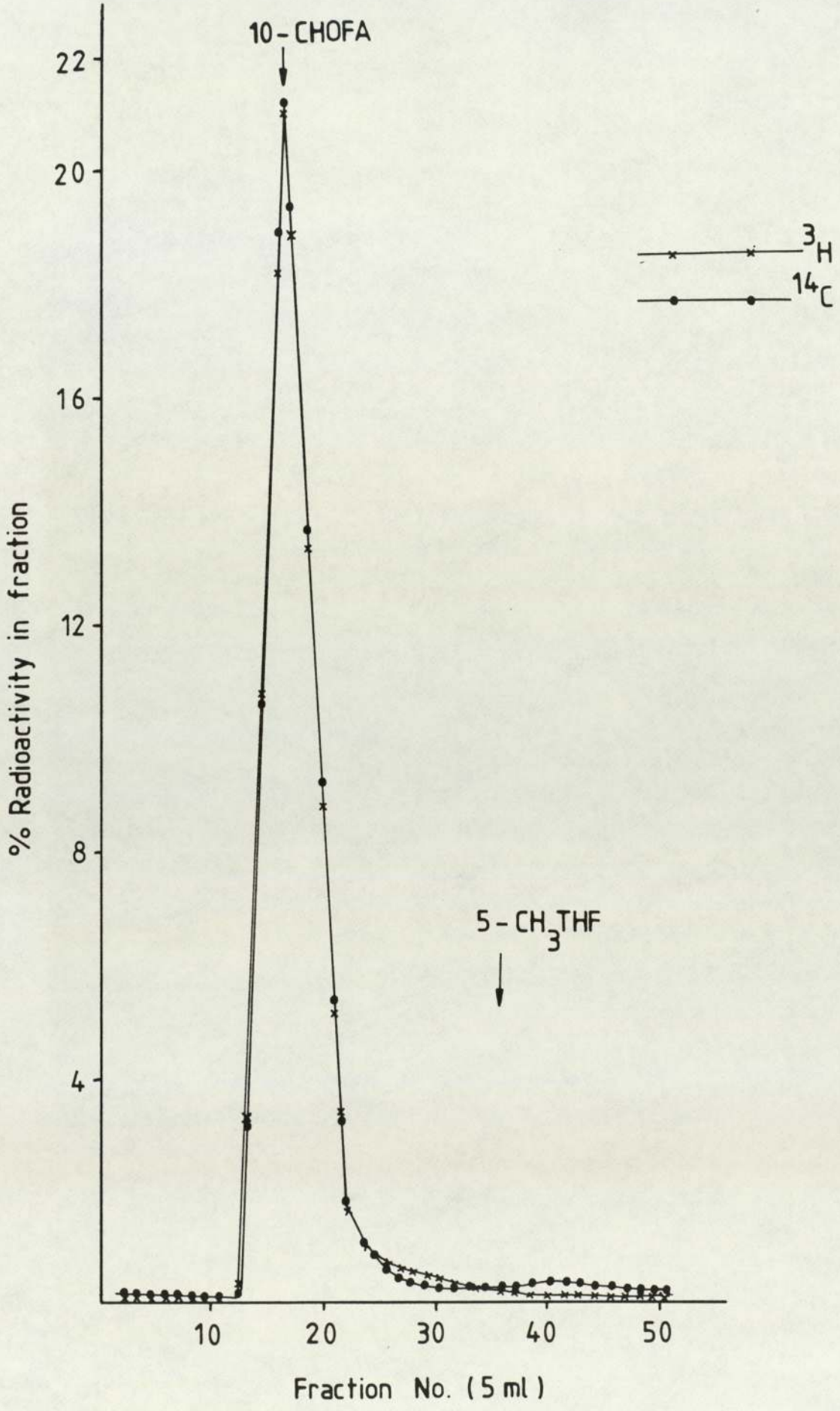


Fig. 4.1.6. Sephadex-G15 chromatography of liver extract at 48-72hr of normal hamsters receiving labelled folic acid (^{3}H + ^{14}C) orally.

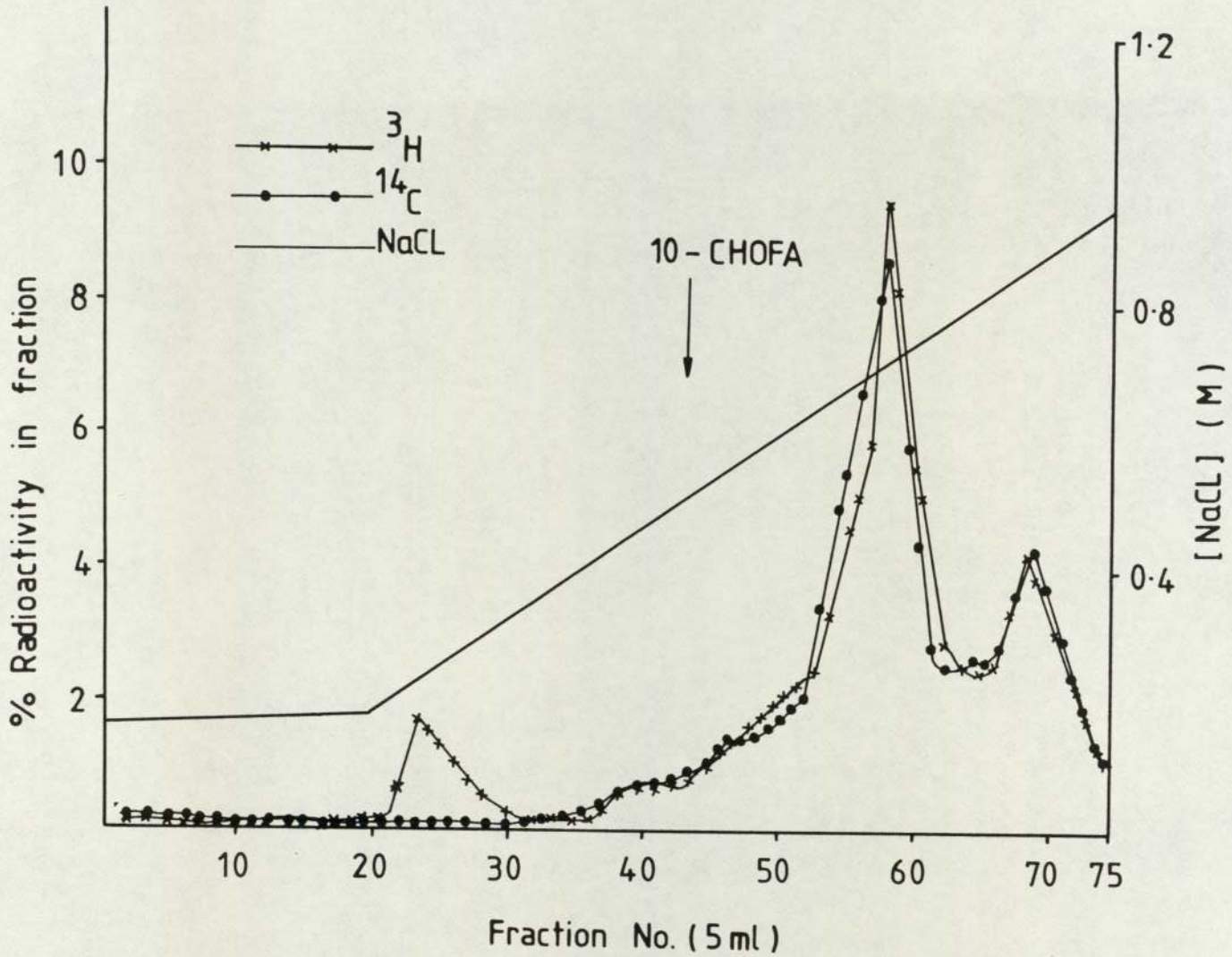


Fig. 4.1.7. DE-52 chromatography of (10-20 tube) fraction from Sephadex-G15 chromatography of 0-24hr liver extract of normal hamsters receiving labelled folic acid (^{3}H + ^{14}C).

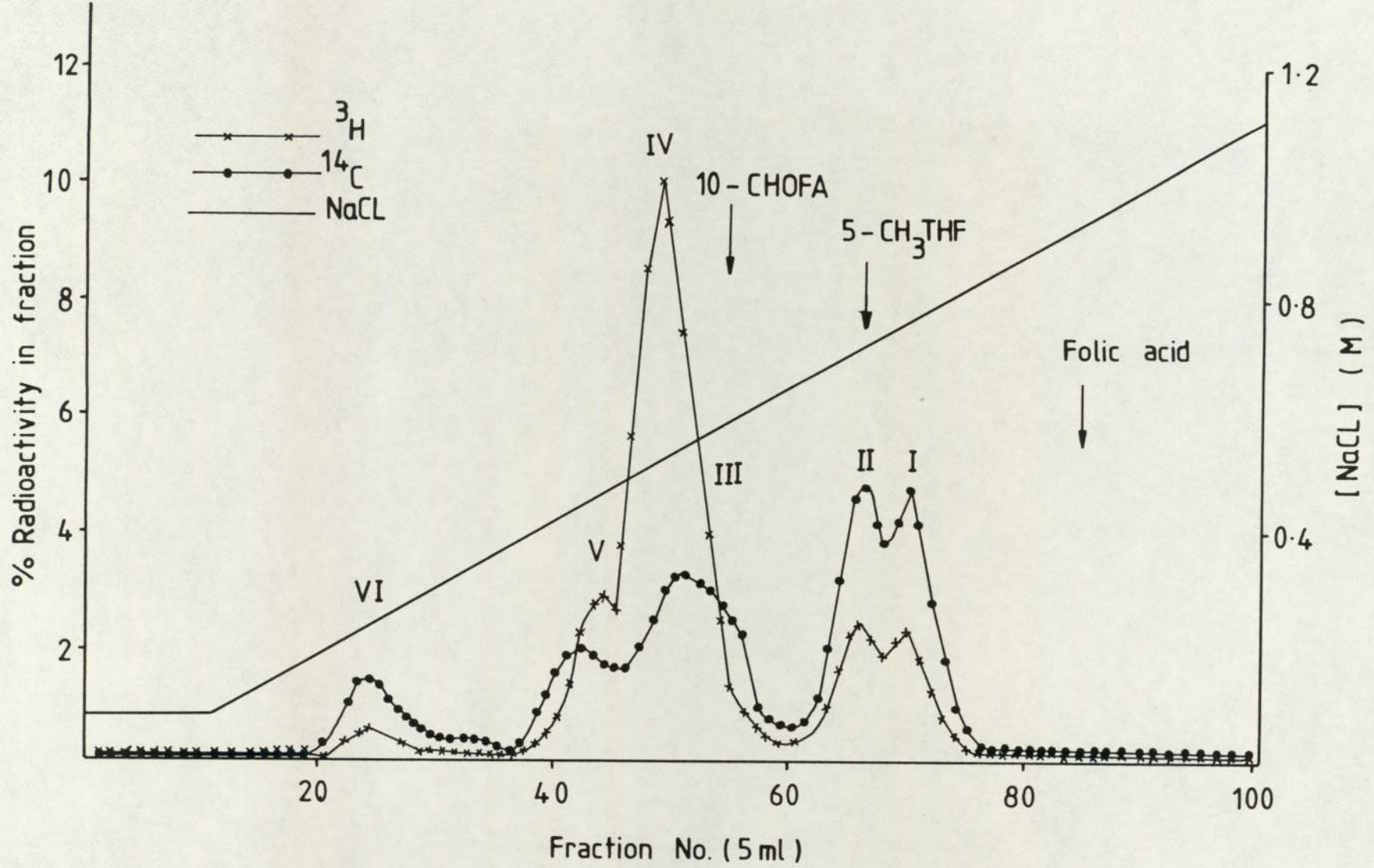


Fig. 4.2.1. DE-52 chromatography of (0-24hr) urine sample of normal hamsters receiving xanthopterin (i.p.) and labelled folic acid (^{3}H + ^{14}C).

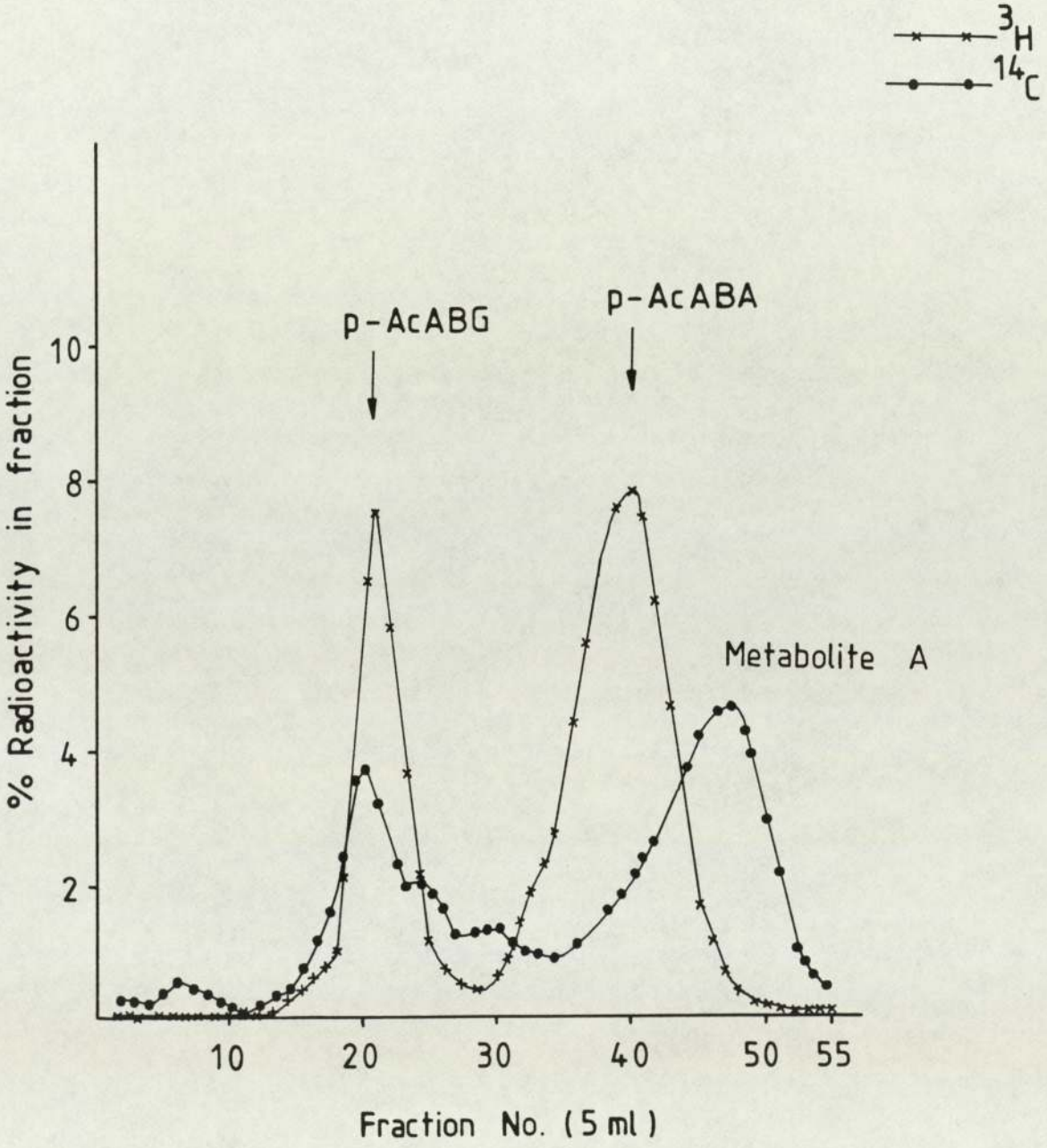


Fig. 4.2.2. Sephadex-G15 chromatography of the tritiated peak (IV) from DE-52 chromatography of urine sample (0-24hr) of normal hamsters pre-treated with xanthopterin (i.p) .

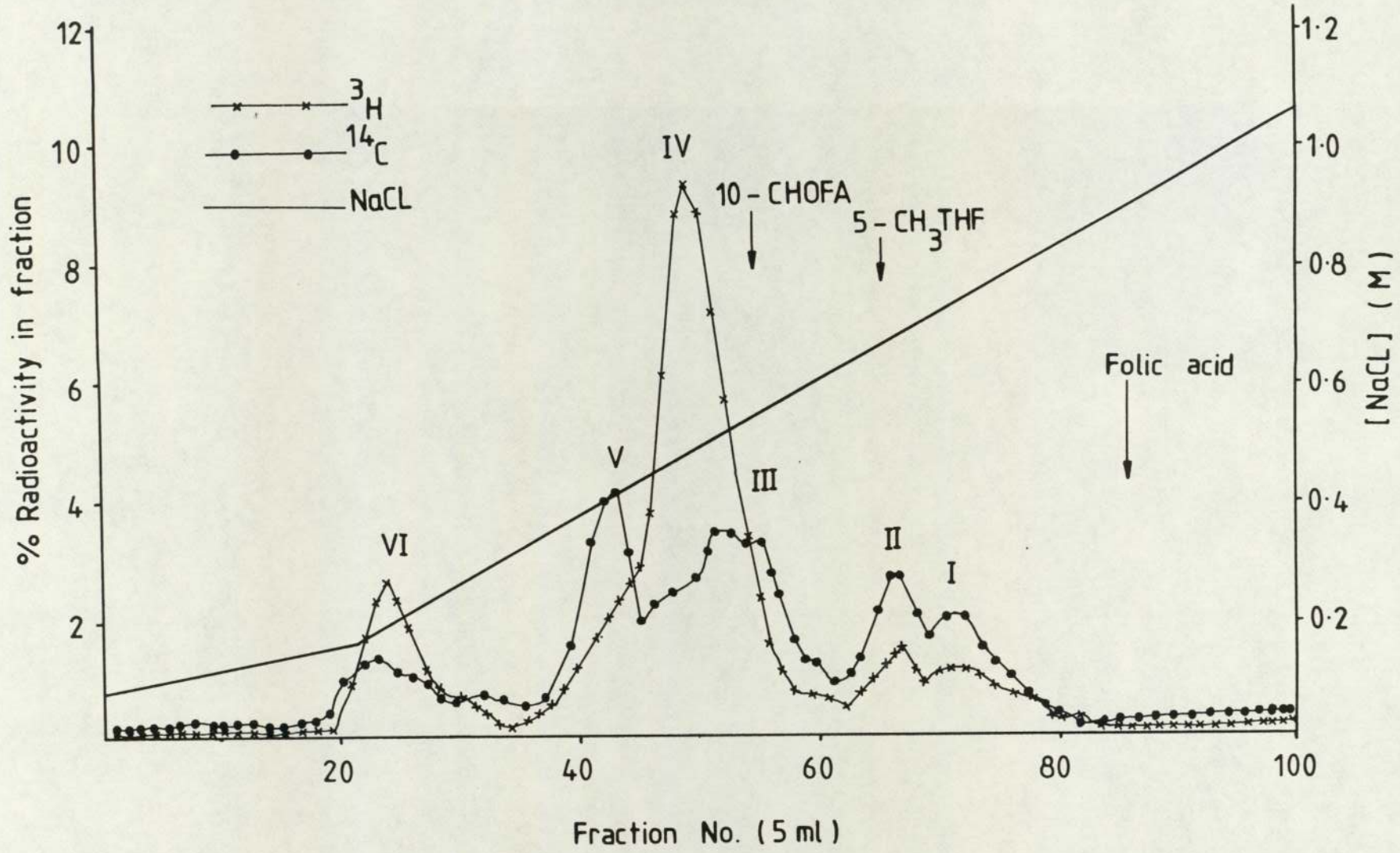


Fig. 4.2.3. DE-52 chromatography of (48-72hr) urine sample of normal hamsters receiving xanthopterin (i.p.) and a mixture of ^{3}H + ^{14}C folic acid.

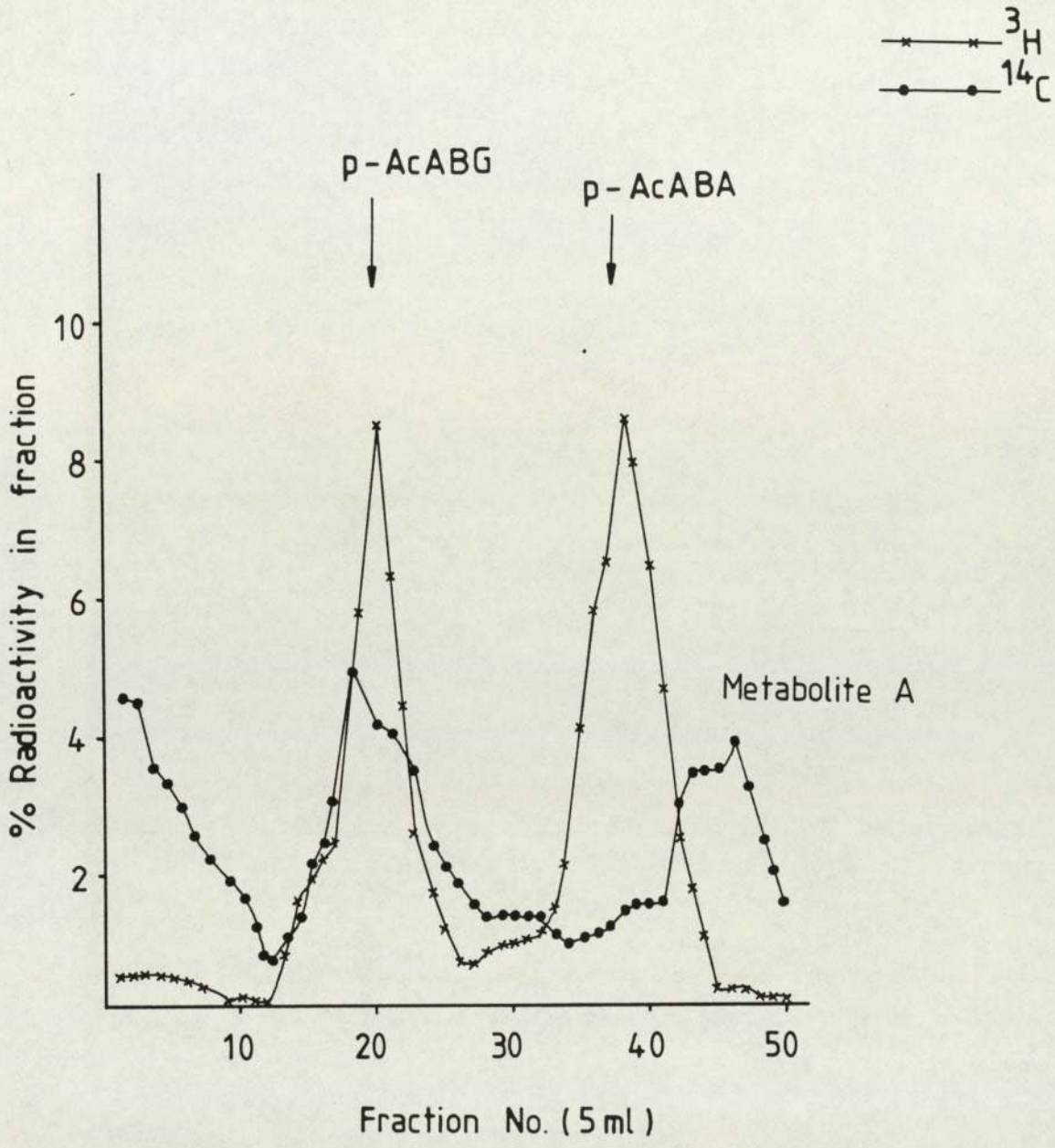


Fig. 4.2.4. Sephadex-G15 chromatography of the tritiated peak (IV) from DE-52 chromatography (48-72hr) of urine sample of normal hamsters given xanthopterin (i.p.) and a mixture of ^{3}H + ^{14}C folic acid.

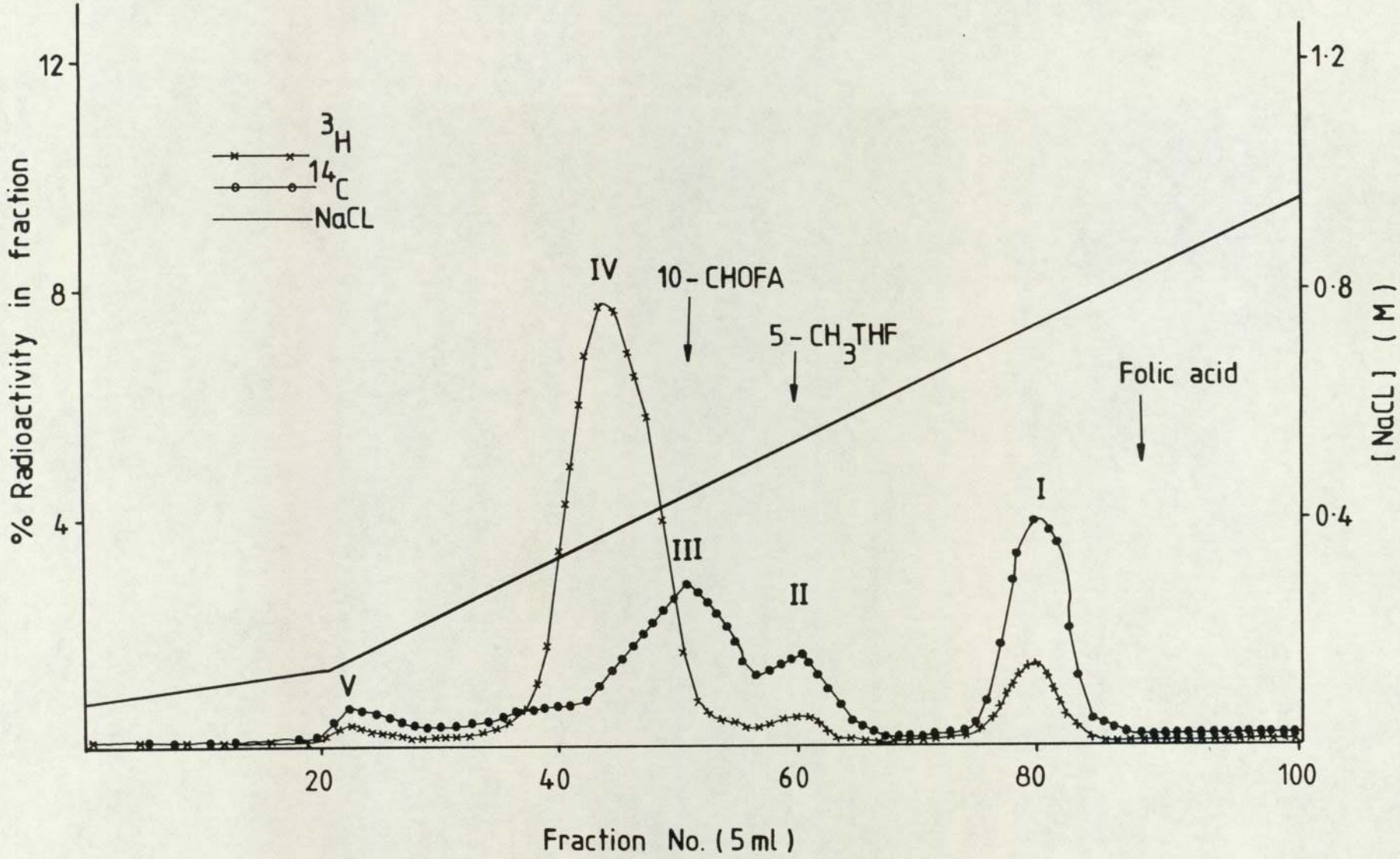


Fig. 4.3.1. DE-52 chromatography of the (0-24hr) urine sample of normal hamsters treated with allopurinol orally.

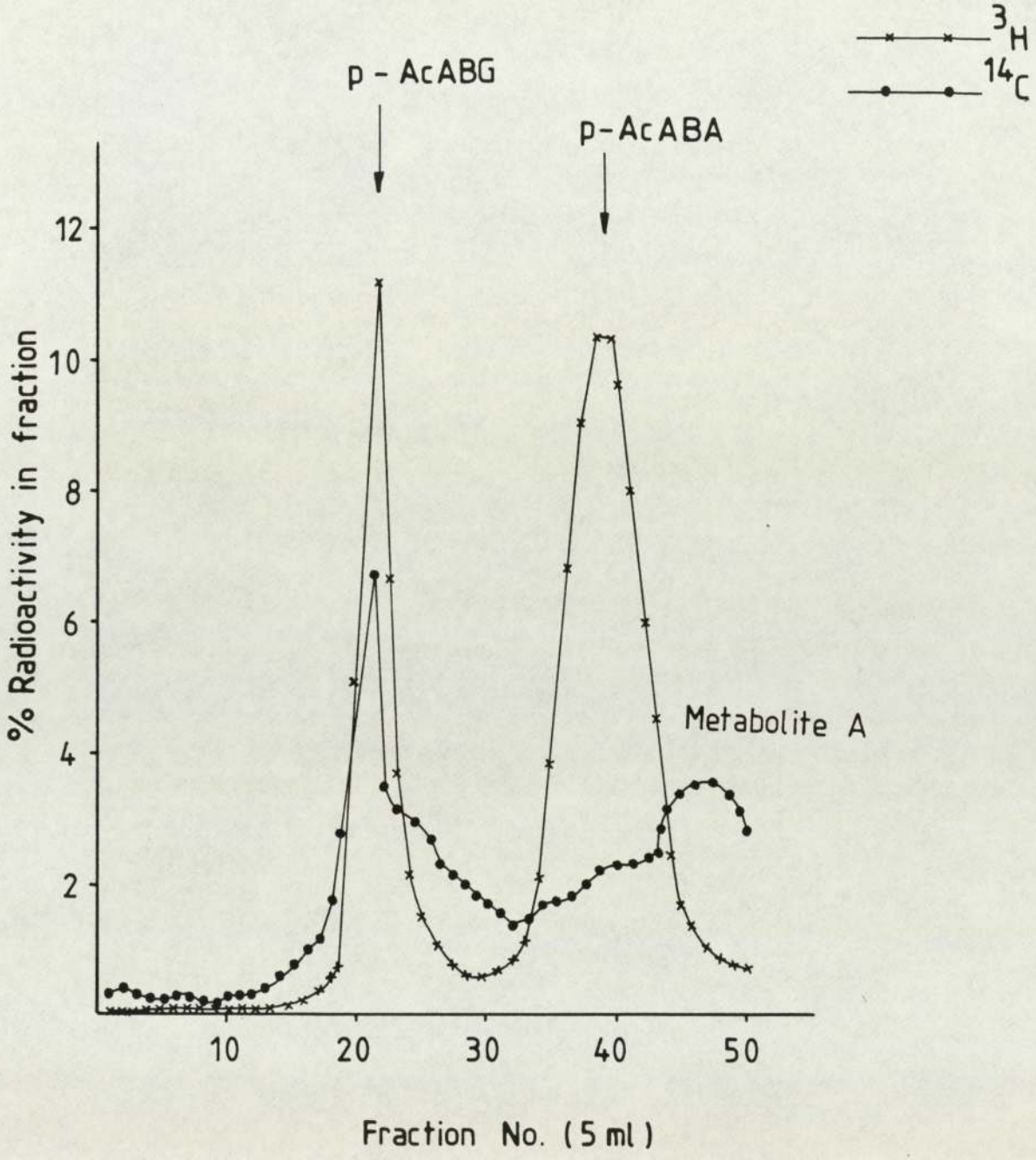


Fig. 4.3.2. Sephadex-G15 chromatography of peak IV from DE-52 chromatography of 0-24hr urine sample of hamsters treated with allopurinol.

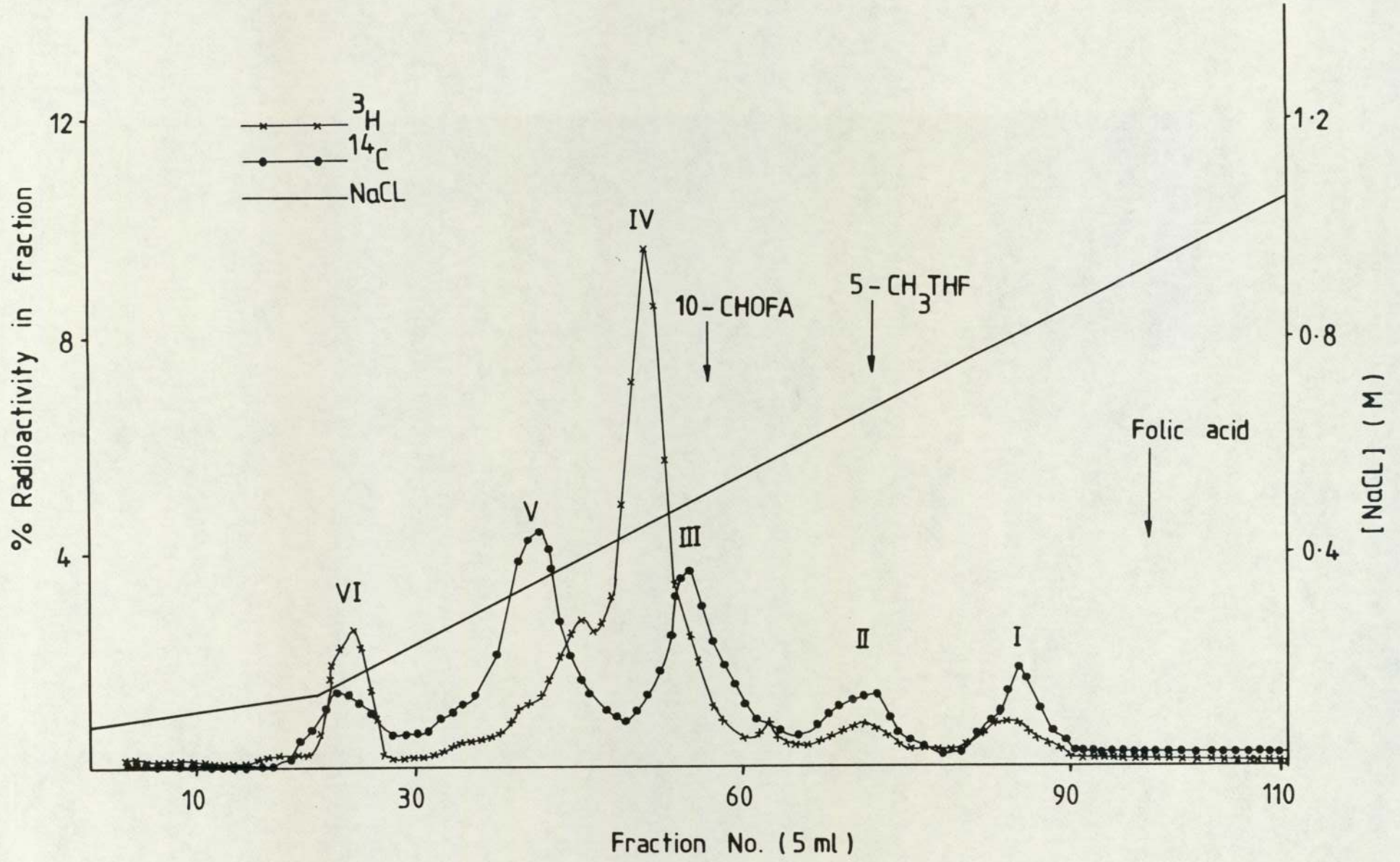


Fig. 4.3.3. DE-52 chromatography of the (48-72hr) urine sample of normal hamsters pre-treated with allopurinol and labelled folic acid (^{3}H + ^{14}C) only orally.

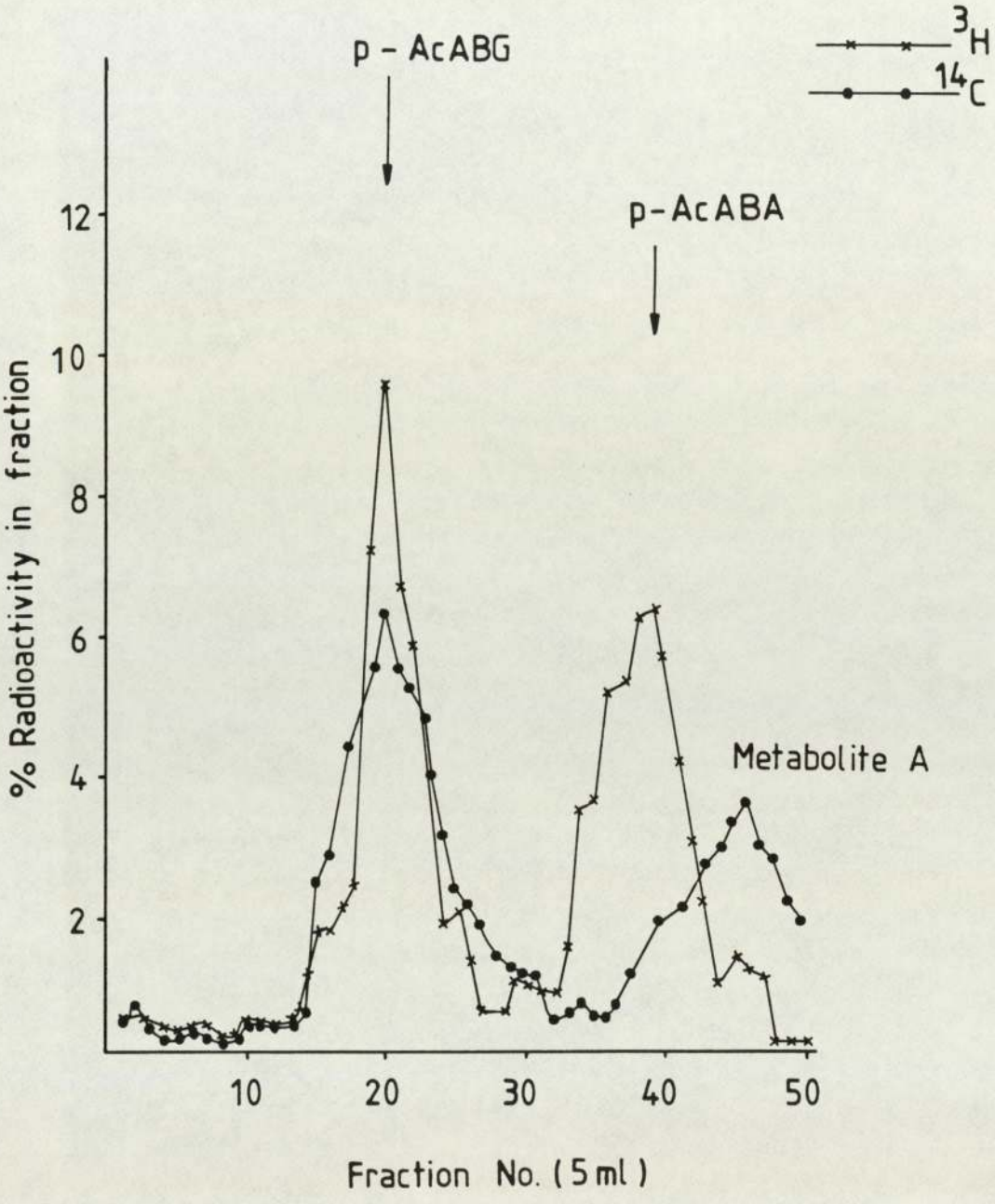


Fig. 4.3.4. Sephadex-G15 chromatography of peak IV from DE-52 chromatography of 48-72 hr urine sample of hamsters pre-treated with allopurinol.

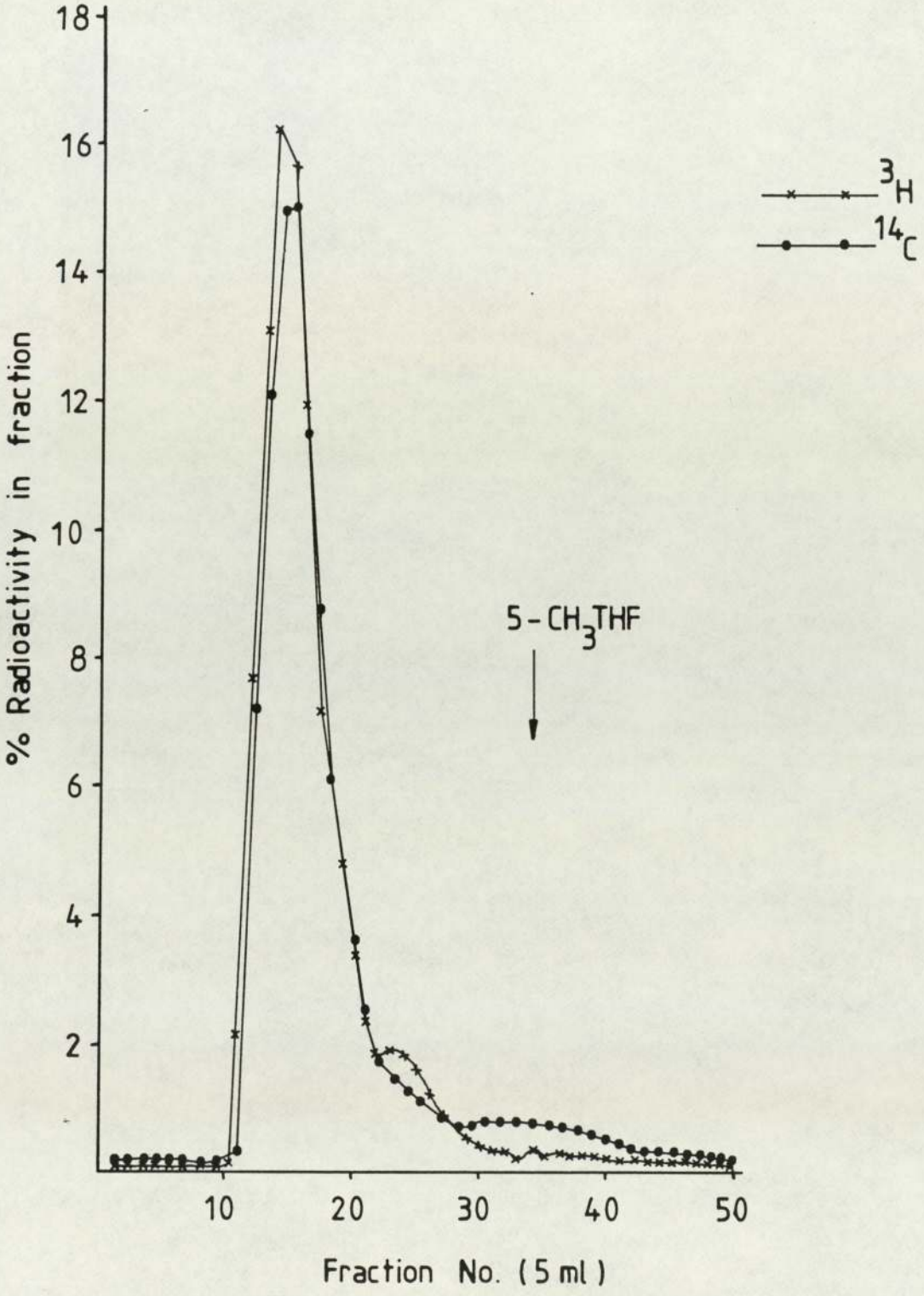


Fig. 4.3.5. Sephadex-G15 chromatography of liver extract at 24hr of normal hamster pre-treated with allopurinol and labelled folic acid (^{3}H + ^{14}C) orally.

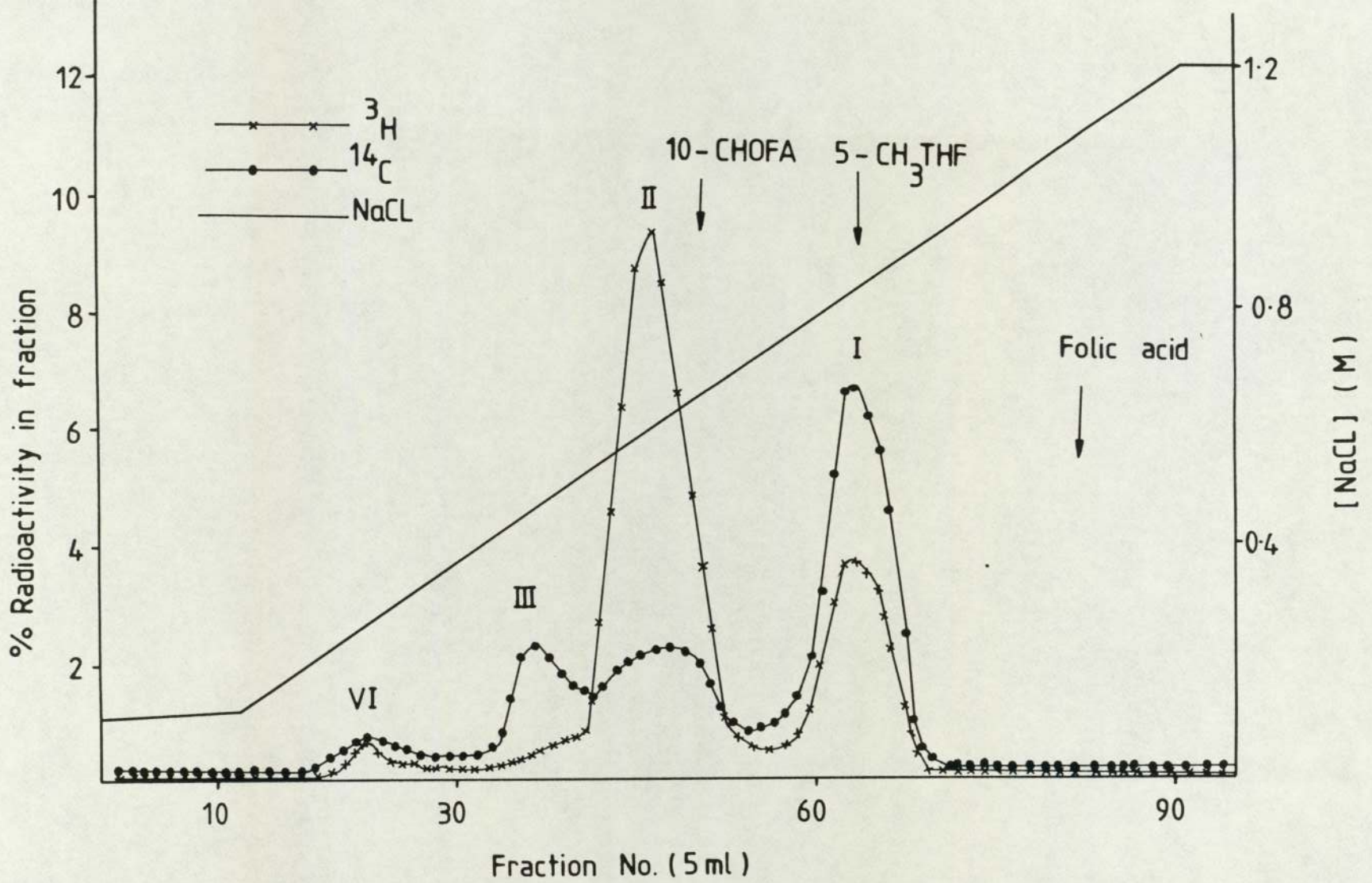


Fig. 4.4.1. DE-52 chromatography of urine sample (0-24hr) of normal hamsters after the administration of somatotropin and labelled folic acid (^{3}H + ^{14}C) orally.

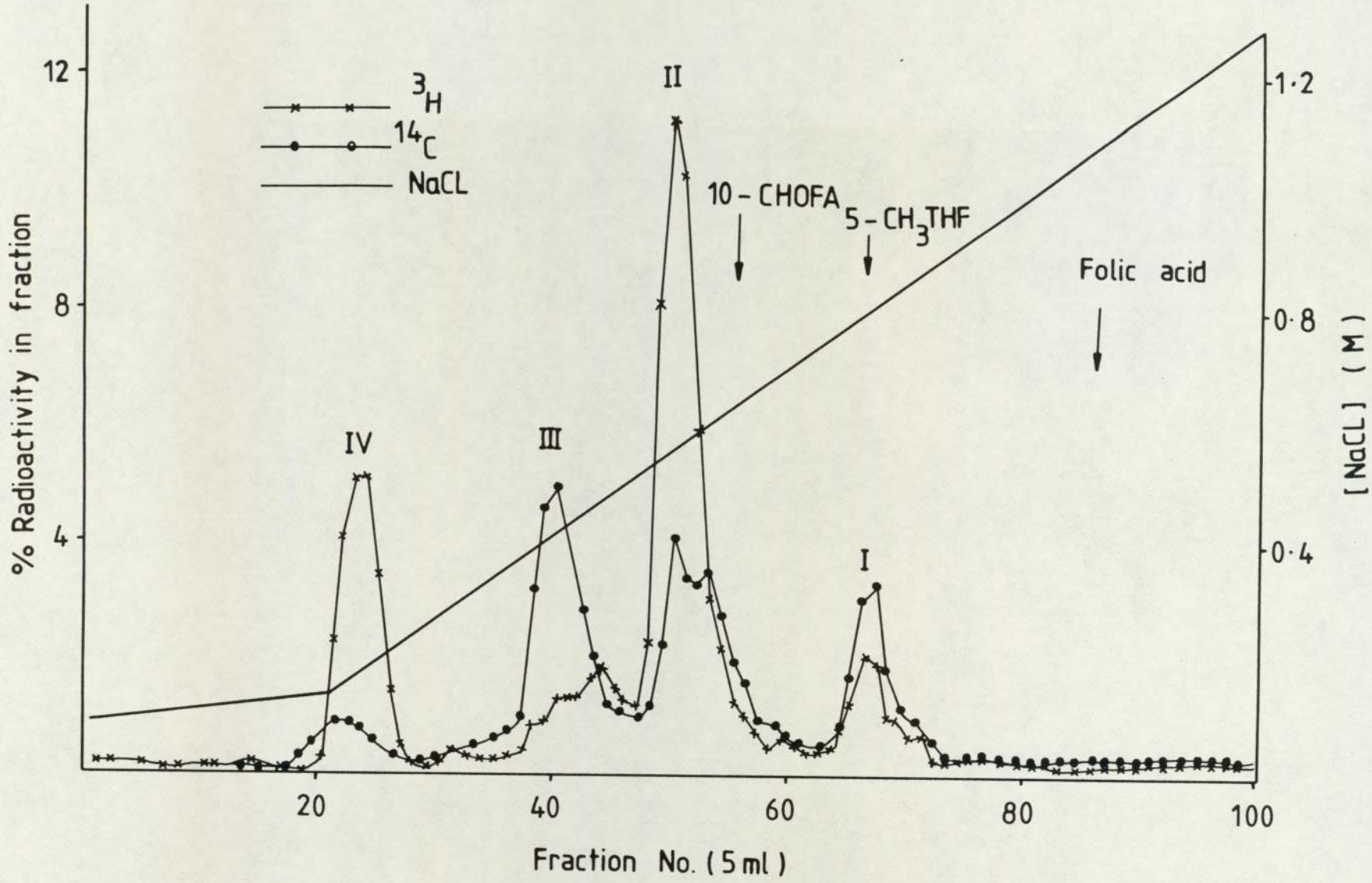


Fig. 4.4.2. DE-52 chromatography of 48-72hr urine sample of normal hamsters pre-treated with somatotropin plus labelled folic acid (^{3}H + ^{14}C) orally.

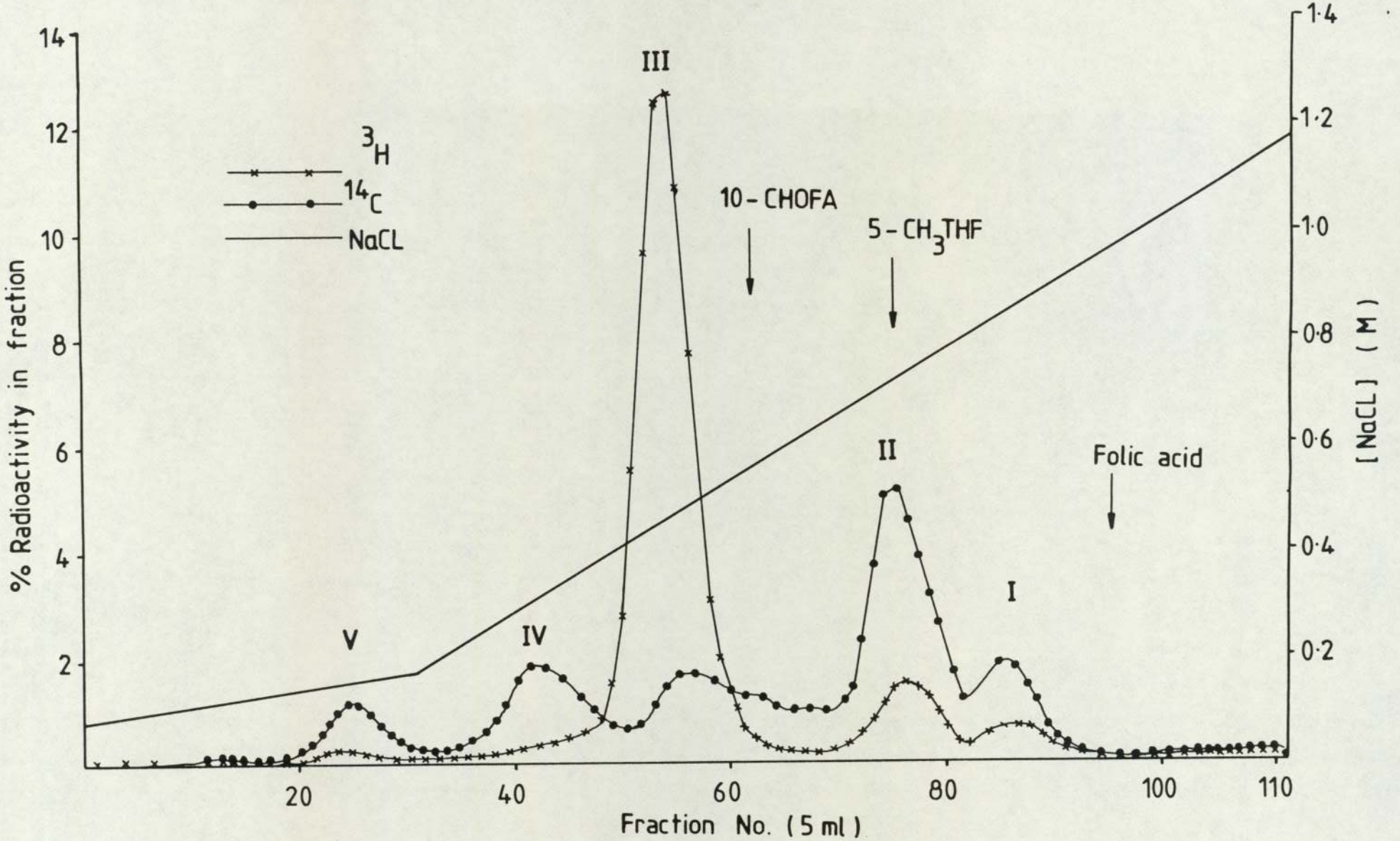


Fig. 4.5.1. DE-52 chromatography of collected urine (0-24hr) of normal hamsters given dihydro-orotic acid and Labelled folic acid (^3H + ^{14}C).

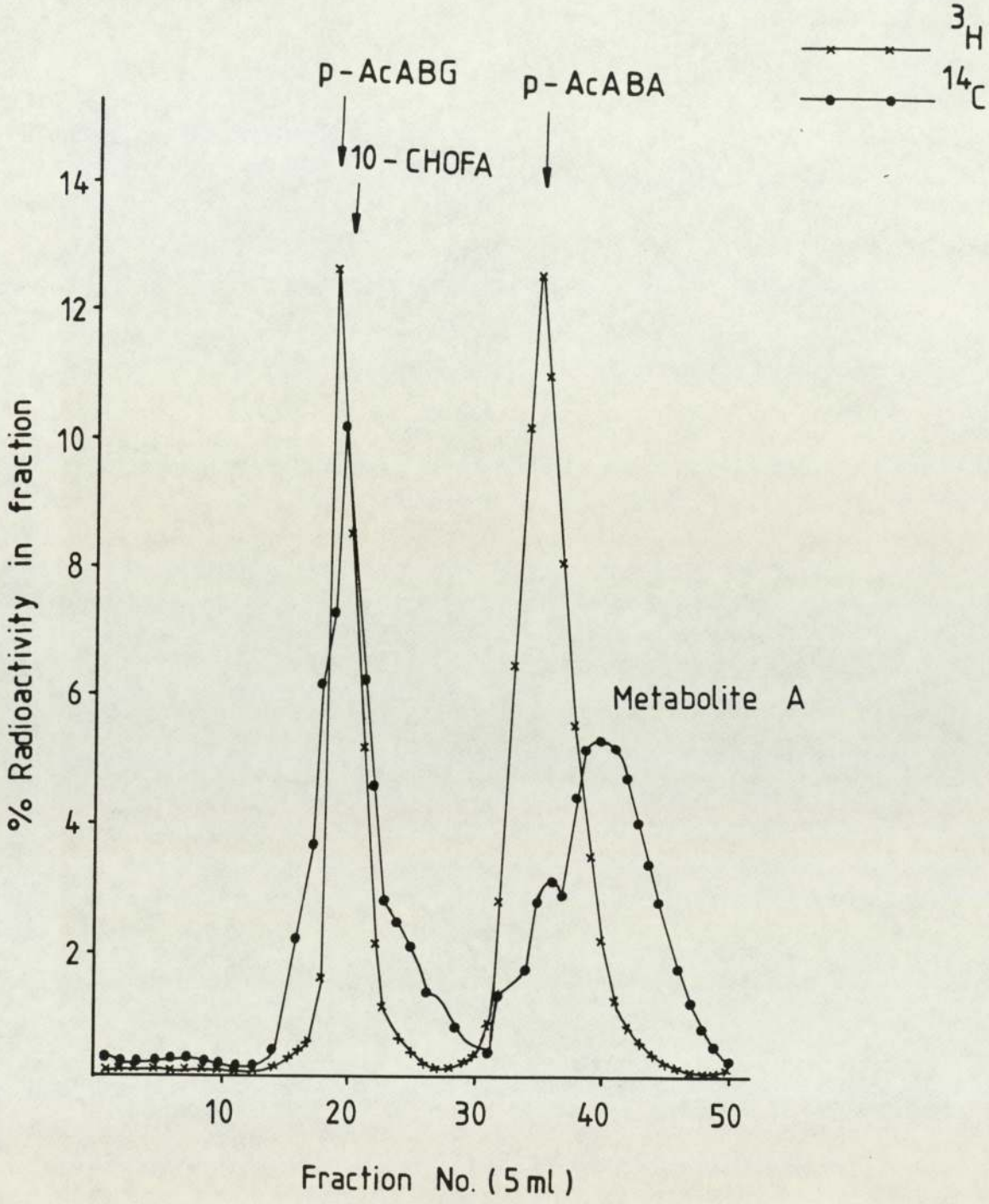


Fig. 4.5.2. Sephadex-G15 chromatography of the tritiated peak (III) from DE-52 chromatography of 0-24hr urine sample of normal hamsters given dihydro-orotic acid and a mixture of ^{3}H + ^{14}C folic acid orally.

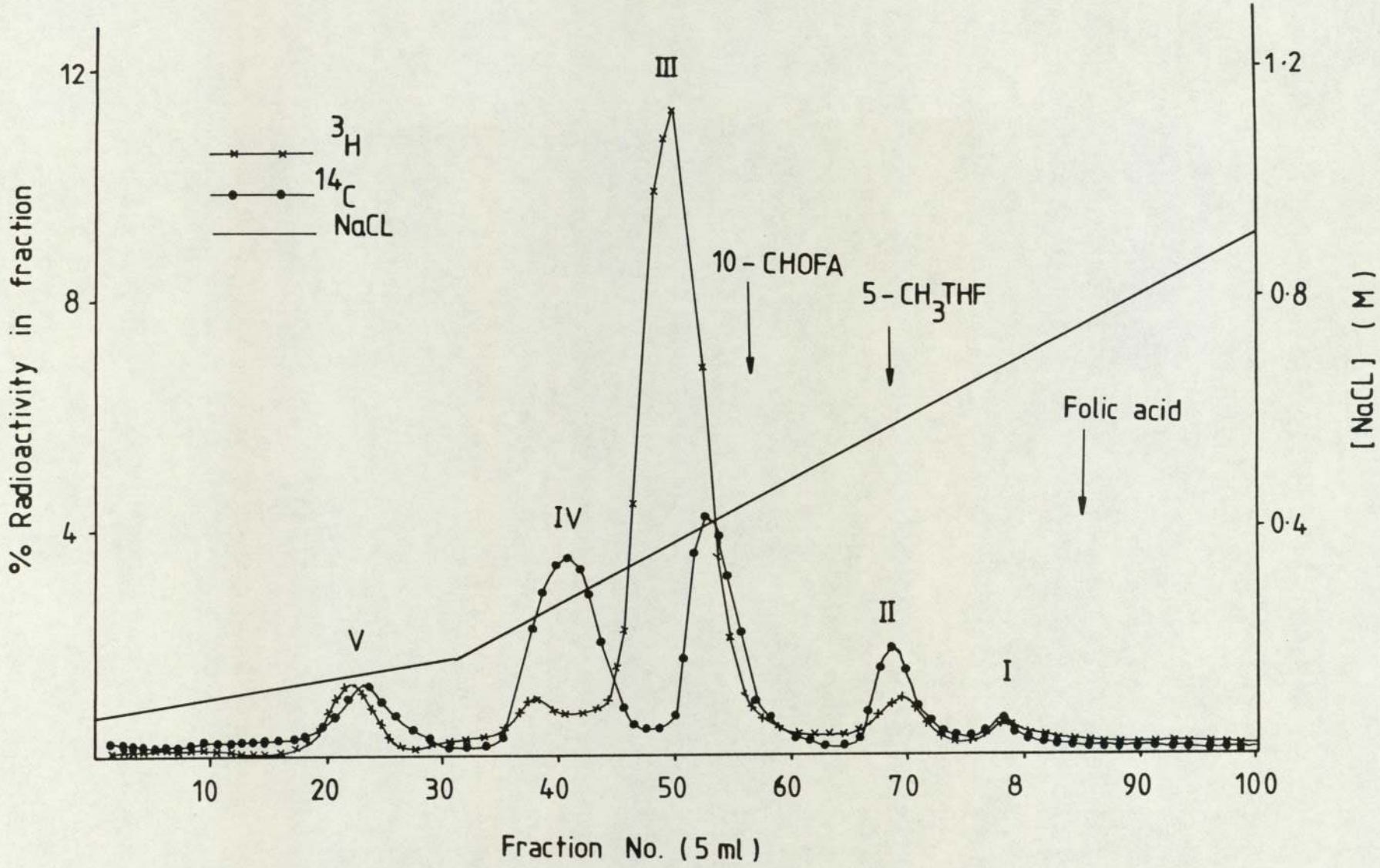


Fig. 4.5.3. DE-52 chromatography of the (48-72hr) urine sample of normal hamsters given dihydro-orotic acid and labelled folic acid (^{3}H + ^{14}C) orally.

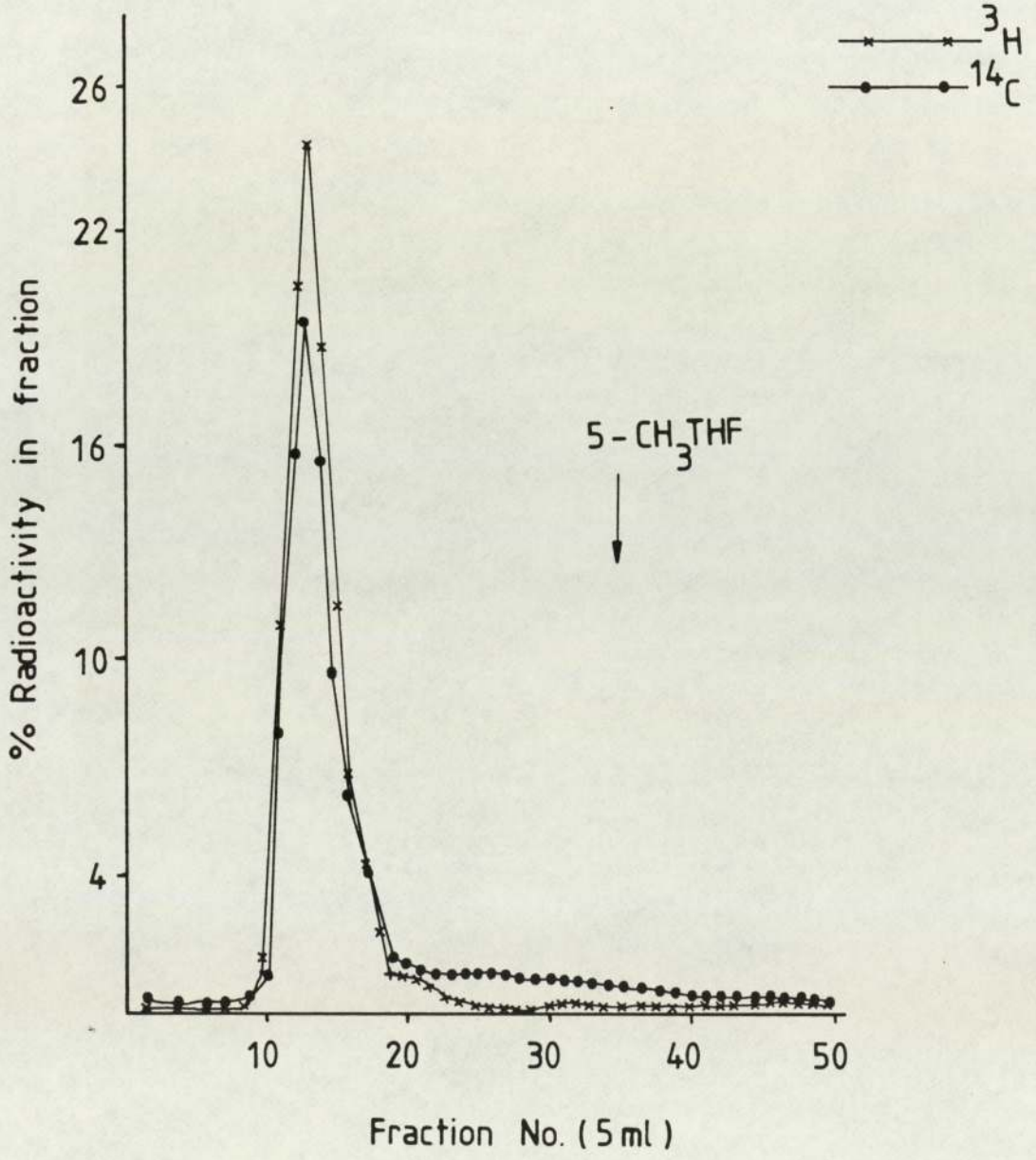


Fig. 4.5.4. Sephadex-G15 chromatography of (0-24hr) liver extracts of normal hamsters pre-treated with dihydro-orotic acid and labelled folic acid (^{3}H + ^{14}C) orally.

Table 4.1.1

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Urine	$\pm \frac{13.98}{3.21}$	$\pm \frac{9.04}{2.16}$	$\pm \frac{2.79}{1.29}$	$\pm \frac{1.76}{0.77}$	$\pm \frac{2.63}{1.19}$	$\pm \frac{1.48}{0.62}$	19.4	12.28
Faeces	$\pm \frac{15.28}{0.62}$	$\pm \frac{12.76}{4.62}$	$\pm \frac{5.7}{0.9}$	$\pm \frac{3.76}{1.42}$	$\pm \frac{3.84}{1.54}$	$\pm \frac{1.67}{0.31}$	24.82	18.19
Liver	$\pm \frac{10.89}{2.82}$	$\pm \frac{17.15}{6.2}$	$\pm \frac{13.11}{5.14}$	$\pm \frac{20.26}{5.81}$	$\pm \frac{11.61}{4.37}$	$\pm \frac{14.31}{3.75}$	11.61	14.31
Kidney	$\pm \frac{1.04}{0.13}$	$\pm \frac{1.3}{0.18}$	$\pm \frac{1.01}{0.34}$	$\pm \frac{1.38}{0.51}$	$\pm \frac{0.81}{0.11}$	$\pm \frac{1.11}{0.22}$	0.81	1.11
Total							56.63	45.89

Recovery of radioactivity in the urine, faeces and tissues, 24hr, 48hr, 72hr after the administration of a mixture of ^{3}H + ^{14}C folic acid to normal Syrian golden hamsters. The results are expressed as the percentage of the dose (mean $\pm$ standard deviation).

Table 4.1.2

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3THF	3.17	3.26	0.44	0.53	0.17	0.19	3.78	3.98
10-CHOFA	-	1.05	-	0.31	-	0.27	-	1.63
p-AcABG	1.83	-	0.57	-	0.43	-	2.83	-
p-AcABA	4.23	-	0.89	-	0.52	-	5.64	-
Folate X	1.11	1.12	0.92	0.95	0.05	0.06	2.08	2.13
Folate Z	-	-	-	-	0.05	0.06	0.05	0.06
Metabolite (A)	-	1.05	-	0.22	-	0.11	-	1.38
Metabolite (B)	0.94	1.10	0.43	0.50	0.63	0.33	2.0	1.93
Urea	-	0.47	-	0.50	-	0.12	-	1.09
$^{3}H_2O$	0.47	-	0.53	-	0.76	-	1.76	-
Total	11.75	8.05	3.78	3.01	2.63	1.14	18.14	12.20

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

Table 4.2.1

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Urine	$\pm \frac{11.53}{4.24}$	$\pm \frac{5.56}{2.13}$	$\pm \frac{4.03}{2.4}$	$\pm \frac{1.92}{1.13}$	$\pm \frac{2.28}{0.86}$	$\pm \frac{1.21}{0.37}$	17.84	8.69
Faeces	$\pm \frac{6.31}{3.69}$	$\pm \frac{6.1}{4.18}$	$\pm \frac{4.37}{2.39}$	$\pm \frac{3.48}{1.81}$	$\pm \frac{3.66}{1.68}$	$\pm \frac{3.49}{2.83}$	14.34	13.07
Liver	$\pm \frac{6.81}{1.63}$	$\pm \frac{11.3}{1.39}$	$\pm \frac{7.4}{1.81}$	$\pm \frac{11.0}{3.17}$	$\pm \frac{7.43}{3.72}$	$\pm \frac{8.24}{3.66}$	7.43	8.24
Kidney	$\pm \frac{0.85}{0.2}$	$\pm \frac{1.18}{0.19}$	$\pm \frac{0.84}{0.21}$	$\pm \frac{1.15}{0.18}$	$\pm \frac{0.89}{0.13}$	$\pm \frac{0.89}{0.07}$	0.89	0.89
Total							40.5	30.89

Recovery of radioactivity in the urine, faeces and tissues of normal Syrian hamsters after the administration i.p. of xanthopterin (2.5mg/kg body wt.) (as was explained in the text) and the administration of a mixture of ^{3}H + ^{14}C folic acid (100µg/body wt.) for 24hr, 48hr and 72hr. The results are expressed as a percentage of the dose. (Mean ± standard deviation.)

Table 4.2.2

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3THF	1.11	1.37	0.3	0.28	0.14	0.15	1.55	1.8
10-CHOFA	-	0.67	-	0.28	-	0.16	-	1.11
p-AcABG	1.69	-	0.6	-	0.24	-	2.53	-
p-AcABA	4.21	-	1.56	-	0.52	-	6.29	-
Folate X	0.6	1.04	0.34	0.37	0.17	0.15	1.11	1.56
Folate Z	-	-	-	-	-	-	-	-
Metabolite (A)	-	1.06	-	0.04	-	0.12	-	1.22
Metabolite (B)	0.07	0.072	0.13	0.16	0.17	0.19	0.37	0.42
Urea	-	0.51	-	0.13	-	0.12	-	0.76
$^{3}H_2O$	0.3	-	0.2	-	0.27	-	0.77	-
Total	7.98	4.72	3.13	1.26	1.51	0.89	12.62	6.87

Metabolites present in the urine of normal hamsters following the administration of ^{14}C + ^{3}H folic acid (100µg/kg body wt) after treatment with xanthopterin (i.p.) as the percentage of the dose.

Table 4.3.1

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Urine	$\pm \frac{18.86}{6.6}$	$\pm \frac{6.48}{2.95}$	$\pm \frac{7.48}{1.6}$	$\pm \frac{2.54}{0.41}$	$\pm \frac{3.04}{1.04}$	$\pm \frac{1.65}{0.81}$	29.38	10.67
Faeces	$\pm \frac{9.77}{3.83}$	$\pm \frac{16.81}{2.53}$	$\pm \frac{8.41}{2.4}$	$\pm \frac{10.08}{1.45}$	$\pm \frac{4.6}{1.4}$	$\pm \frac{6.7}{2.6}$	22.78	33.59
Liver	$\pm \frac{14.10}{6.17}$	$\pm \frac{18.59}{6.89}$	$\pm \frac{12.85}{4.15}$	$\pm \frac{19.02}{7.44}$	$\pm \frac{14.15}{2.39}$	$\pm \frac{19.02}{4.50}$	14.15	19.02
Kidney	$\pm \frac{1.75}{0.63}$	$\pm \frac{2.32}{0.58}$	$\pm \frac{1.42}{0.27}$	$\pm \frac{1.68}{0.21}$	$\pm \frac{1.42}{0.27}$	$\pm \frac{2.5}{0.89}$	1.42	2.5
Total							67.73	67.78

Recovery of radioactivity in the urine, faeces and tissues after the administration of 2mg of allopurinol to normal male hamsters and the administration of ^{3}H + ^{14}C folic acid.

The results are expressed as the percentage of the dose. (Mean ±standard deviation.)

Table 4.3.2

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3THF	0.72	0.89	0.17	0.21	0.16	0.13	1.05	1.23
10-CHOFA	-	0.82	-	0.56	-	0.31	-	1.69
p-AcABG	3.76	-	1.67	-	0.46	-	5.89	-
p-AcABA	9.97	-	3.53	-	0.75	-	14.25	-
Folate X	1.86	1.81	0.44	0.46	0.17	0.16	2.47	2.43
Folate Z	-	-	-	-	-	-	-	-
Metabolite (A)	-	1.09	-	0.59	-	0.17	-	1.85
Metabolite (B)	-	-	-	-	0.34	0.45	0.34	0.45
Urea	-	0.46	-	0.44	-	0.17	-	1.07
$^{3}H_2O$	0.55	-	0.85	-	0.35	-	1.75	-
Total	16.86	5.07	6.66	2.26	2.23	1.39	25.75	8.72

Metabolites present in the urine of Syrian hamsters following the administration of ^{14}C + ^{3}H folic acid (100µg/kg body wt) after treatment with allopurinol. The results are expressed as the percentage of the dose.

Table 4.4.1

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Urine	$\pm \frac{22.97}{6.91}$	$\pm \frac{10.26}{3.54}$	$\pm \frac{5.63}{1.82}$	$\pm \frac{2.95}{0.88}$	$\pm \frac{2.91}{0.99}$	$\pm \frac{1.68}{0.52}$	31.51	14.89
Faeces	$\pm \frac{8.42}{2.08}$	$\pm \frac{10.91}{1.86}$	$\pm \frac{4.76}{1.72}$	$\pm \frac{5.61}{3.56}$	$\pm \frac{3.3}{0.73}$	$\pm \frac{1.26}{0.17}$	16.48	17.78
Liver	$\pm \frac{13.76}{3.5}$	$\pm \frac{18.99}{2.58}$	$\pm \frac{9.46}{1.14}$	$\pm \frac{15.63}{3.31}$	$\pm \frac{10.72}{3.66}$	$\pm \frac{17.54}{3.39}$	10.72	17.54
Kidney	$\pm \frac{1.66}{0.16}$	$\pm \frac{2.09}{0.12}$	$\pm \frac{1.34}{0.14}$	$\pm \frac{1.67}{0.35}$	$\pm \frac{1.63}{0.32}$	$\pm \frac{1.84}{0.29}$	1.63	1.84
Total							60.34	52.05

Recovery of radioactivity in the urine, faeces and tissues after receiving 0.1 i.u. of somatotropin by normal male hamsters and the administration of a mixture of ^{14}C + ^{3}H folic acid (100µg/kg body wt) for 24hr, 48hr and 72hr. The results are expressed as the percentage of the dose. (Mean ± standard deviation.)

Table 4.4.2

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3 THF	6.13	5.23	0.51	0.48	0.28	0.31	6.92	6.02
10-CHOFA	-	0.94	-	0.77	-	0.21	-	1.92
p-AcABG	4.68	-	1.34	-	0.41	-	6.43	-
p-AcABA	8.36	-	2.13	-	0.58	-	11.07	-
Folate X	-	-	-	-	-	-	-	-
Folate Z	-	-	-	-	-	-	-	-
Metabolite (A)	-	1.27	-	1.77	-	0.25	-	3.29
Metabolite (B)	0.76	1.40	0.38	0.3	0.32	0.52	1.46	2.22
Urea	-	0.41	-	0.11	-	0.12	-	0.64
$^{3}H_2O$	0.62	-	0.25	-	0.63	-	1.5	-
Total	20.55	9.25	4.61	3.43	2.22	1.41	27.38	14.09

Metabolites present in the urine of Syrian hamsters following the administration of ^{14}C + ^{3}H folic acid (100µg/kg body wt) after treatment with somatotropin. The results are expressed as the percentage of the dose.

Table 4.5.1

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Urine	$\pm\frac{24.66}{14.96}$	$\pm\frac{8.68}{6.64}$	$\pm\frac{8.2}{4.93}$	$\pm\frac{3.36}{1.12}$	$\pm\frac{4.5}{2.1}$	$\pm\frac{2.04}{1.02}$	37.36	14.08
Faeces	$\pm\frac{13.02}{7.92}$	$\pm\frac{15.49}{10.57}$	$\pm\frac{1.96}{1.04}$	$\pm\frac{2.47}{1.92}$	$\pm\frac{1.8}{0.91}$	$\pm\frac{1.98}{0.53}$	16.78	19.94
Liver	$\pm\frac{17.17}{7.65}$	$\pm\frac{24.24}{10.22}$	$\pm\frac{12.38}{5.3}$	$\pm\frac{16.36}{3.38}$	$\pm\frac{10.01}{1.69}$	$\pm\frac{15.56}{3.94}$	10.01	15.65
Kidney	$\pm\frac{1.5}{0.49}$	$\pm\frac{1.83}{0.84}$	$\pm\frac{1.26}{0.1}$	$\pm\frac{1.37}{0.24}$	$\pm\frac{1.49}{0.23}$	$\pm\frac{1.3}{0.48}$	1.49	1.3
Total							65.64	50.97

Recovery of radioactivity in the urine, faeces and tissues after the administration of 2.0mg of dihydro-orotic acid to normal male hamsters and the receiving a mixture of ^{3}H + ^{14}C folic acid for 24hr, 48hr and 72hr. The results are revealed as the percentage of the dose. (Mean $\pm$ standard deviation.)

Table 4.5.2

	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3 THF	2.73	2.95	0.32	0.3	0.25	0.21	3.3	3.46
10-CHOFA	-	0.71	-	0.42	-	0.52	-	1.65
p-AcABG	5.96	-	2.03	-	0.84	-	8.83	-
p-AcABA	12.49	-	4.37	-	2.55	-	19.41	-
Folate X	1.00	0.80	0.21	0.15	-	-	1.21	0.95
Folate Z	-	-	-	-	-	-	-	-
Metabolite (A)	-	1.09	-	0.59	-	0.17	-	1.85
Metabolite (B)	1.36	0.98	0.40	0.99	0.31	0.72	2.07	2.69
Urea	-	0.63	-	0.56	-	0.23	-	1.42
$^{3}H_2O$	0.44	-	0.42	-	0.34	-	1.2	-
Total	23.98	7.16	7.75	3.01	4.29	1.85	36.02	12.02

Metabolites present in the urine of Syrian hamsters following the administration of ^{14}C + ^{3}H folic acid (100µg/kg body wt) after administered dihydro-orotic acid. The results are expressed as the percentage of the dose.

CHAPTER 5

The metabolism of [2-^{14}C] and [3',5',7,9-^{3}H] - folic acid in a control subject, cancer and alcoholic patients

5.1 Folic acid metabolism in control and cancer patients:

Earlier studies on the metabolism of folic acid in man have used various radiolabelled folate derivatives, such as, ^{3}H - labelled tracers (Johns, et al., 1961; Chanarin and Mclean, 1967), ^{14}C - labelled tracers (Krumdieck, et al., 1978) or a mixture of both ^{3}H and ^{14}C labelled tracers (Pheasant, et al., 1979; Saleh, et al., 1980). Folate absorption in man has also been studied by giving large doses and measuring blood or urine levels by microbiological assay (Chanarin, et al., 1958; Anderson, et al., 1960; Klipstein, 1963; Ratanasthien, 1975). Observations on the effect of malignant disease have been made in rats bearing different kinds of tumour (Barford and Blair, 1978; Connor and Blair, 1979; Pheasant and Blair, 1979 and Bates, et al., 1980) while the effect of malignant disease in man has been studied recently by Pheasant et al.,(1979)and Saleh, et al.,(1980).

In this chapter the study of the metabolism of mixtures of ^{14}C and ^{3}H labelled folic acid in a control subject and the malignant disease in man was carried on to extend the previous studies.

Materials and Methods:

Two groups of patients were obtained from the General Hospital.

Group (1) : One patient suffering from a non malignant disorder as a control.

Group (2) : Two patients suffering from malignant disease with different sizes of tumour (details in Table 5.1.1).

All patients received an oral dose of radiolabelled 5μCi [2-^{14}C] and 20μCi [3',5',7,9-^{3}H]-folic acid plus 5mg of unlabelled folic acid. Urine was collected for three time periods, 0-6hr, 6-12hr and 12-24hr after the administration.

The description of the collection of the urine, the determination of radioactivity and column chromatography is given in chapter 2.

Results:

The distribution of the urinary recovery of radioactivity for the three time periods of collection of urine is shown in Table 5.1.2. Following the administration of folic acid to a control patient and patients suffering from malignant disease, the urinary excretion over 24hr was 31.35% ^{3}H and 27.76% ^{14}C in control patient J.T.; 1.07% ^{3}H and 0.76% ^{14}C in patient F.K. suffering from extensive malignancy and was 19.05% ^{3}H and 17.99% ^{14}C in patient L.K. suffering from malignant disease with a small tumour. The recovery of the ^{3}H dose was slightly higher than ^{14}C dose at all times in the urine samples of all patients.

Urinary metabolites:

Qualitative analysis of urine samples at 6hr, 12hr, and 24hr of both control and cancer patients was carried out by sequential chromatography on both DE-52 and Sephadex-G15 columns. Figure 5.1.1 shows the DE-52 chromatography of the 0-6hr urine sample of control patient J.T. showing three radioactive species; the first two labelled with both ^{3}H and ^{14}C isotopes as intact folates which were identified by chromatography with authentic standards on both columns as folic acid (the major component) and 5-CH_3THF (a small species) (peak I, II). The third species, the ^{3}H-scission product was found to be p-AcABG by its position (tube 20-21) on Sephadex-G15 (Figure 5.1.2). The analysis of the 6-12hr urine sample of this patient on columns was not possible because of the shortage of urine and low radioactivity.

DE-52 chromatography and Sephadex-G15 of the 12-24hr urine sample of this control subject (J.T.) (Figure 5.1.3, 5.1.4) had a similar pattern to the 0-6hr urine sample.

DE-52 chromatography and Sephadex-G15 chromatography of the 0-6hr urine sample of cancer patient L.K. (Figure 5.1.5, 5.1.6) showed that the pattern of metabolites was similar to those found in a control subject (J.T.) in the 0-6hr urine sample, except a slight decrease in unchanged folic acid excretion and in addition, the appearance of another small ^{3}H metabolite (III) and another unidentified ^{14}C peak (metabolite A). Qualitative analysis of the 6-12hr urine sample of the cancer patient L.K. on both column systems had similar patterns of metabolites as in the 0-6hr except the appearance of a small amount of p-AcABA, as well as p-AcABG (Figure 5.1.7), while DE-52 chromatography of the 12-24hr urine sample (Figure 5.1.8) showed an apparently different pattern from both 0-6hr and 6-12hr urine sample of the same patient and the control subject. The unchanged folic acid (I) was sharply decreased with increasing 5-CH_3THF (II). However, the ^{3}H-scission product (III) was shown to be two catabolites, p-AcABG and p-AcABA (Figure 5.1.9).Tritiated water was detected in the control and cancer patients after chromatographed on Sephadex-G15.

Qualitative analysis of the urine samples of the cancer patient F.K. suffering from a massive size of tumour for all times of urine collection was not possible because of low radioactivity which did not allow chromatography on both column systems.

Table 5.1.3 summarizes the distribution of the metabolites in both control and malignant subjects as the percentage of dose. The percentage of unchanged folic acid was decreased in patient suffering from malignant disorders, while the percentage of 5-CH_3THF was slightly increased. The percentage of the scission product appeared approximately similar in both conditions.

Table 5.1.4 shows the individual ratios of folic acid: 5-CH_3 THF in both the control patient and patients with malignancy. The ratio decreased sharply in cancer case during 0-6hr, 6-12hr and 12-24hr while the decrease was lower in the control during those times of urine sample. The ratio of urinary scission products to intact folates is shown in the same table and shows the change in this ratio in the cancer subject with the increasing time.

5.2 The effect of a low dose of folic acid in a control subject:

Materials and Methods:

One control patient (B.F.) suffering from a disease other than malignancy (details in Table 5.2.1) received orally 5μCi [2-^{14}C] and 20μCi [3',5',7,9-^{3}H]-folic acid (50μg folic acid). The urine was collected for three times 0-6hr, 6-12hr and 12-24hr. The determination of radioactivity and column chromatography were described previously in chapter 2.

Results:

Quantitative analysis of 0-6hr, 6-12hr and 12-24hr urine sample is shown in Table 5.2.2. After the low dose of folic acid, the recovery of radioactivity of 0-24hr urine sample was 3.03% ^{3}H and 2.01% ^{14}C compared with the control subject who received a high dose of folic acid (31.35% ^{3}H and 27.76% ^{14}C of the dose). A low dose gives a lower percentage of radioactivity in the urine. As before the percentage of the ^{3}H dose was slightly higher than of the ^{14}C dose.

Urinary metabolites:

DE-52 chromatography and Sephadex-G15 chromatography of the 0-6hr urine sample (Figure 5.2.1, 5.2.2) showed a different pattern of metabolites to that of the control subject who received a high dose of folic acid. No folic acid was observed and a small amount

of 5-CH_3THF (I) appeared. A large tritiated-scission product (II) was composed of p-AcABG as shown by Sephadex-G15 column. There was also a new metabolite B labelled with ^{14}C only as shown on DE-52 chromatography. Figure 5.2.3, 5.2.4 shows the qualitative analysis of the 6-12hr urine sample which had similar patterns, but lacked metabolite B on DE-52 chromatography. The radioactivity appearing in the 12-24hr urine sample was too low to be chromatographed.

Table 5.2.3 shows the distribution of the metabolites as the percentage of the dose. The amount of 5-CH_3THF and scission products appeared similar to the control subject who received a high dose of folic acid. The ratio of folic acid: 5-CH_3THF was not measured because of the absence of unchanged folic acid. The ratio of scission product to 5-CH_3THF is shown in Table 5.1.4 showing an increase of this ratio with the fall of total urinary folate amount. The percentage of the ^{3}H dose was slightly higher than that of the ^{14}C in the urine.

5.3 The metabolism of folic acid in alcoholism:

Many investigations in man and animals have demonstrated a relationship between the concomitant ingestion of alcohol and folate. Alcohol exerts a toxic effect on folate homeostasis by its impairment of dietary folate intake and by interfering with folate metabolism (Hillman and Steinberg, 1982 & Steinberg, et al., 1980). Moreover, studies of alcohol toxicity in man and animal models have demonstrated that acute alcohol ingestion results in a dramatic fall in serum folate level and bile folate level as well as its effect on the folate enterohepatic cycle (Hillman, et al., 1978). Recently Halsted (1980) suggested that folate deficiency, the most common sign of malnutrition in chronic alcoholism and acute administration

of ethanol severely impairs the hepatic metabolism of folate to polyglutamate derivatives in rat (Brown, et al., 1973b and Steinberg, et al., 1980). Extensive kinetic studies using various isotopes of folate were carried out to determine the impact of alcohol on hepatic uptake of folates, rates of reduction and methylation of oxidized congeners and synthesis of folate polyglutamate and the mobilization of folate stores for the release of methyltetrahydrofolate into bile for transport to tissues (Hillman and Steinberg, 1982). Using an intramuscular injection of ^{3}H-folic acid on the monkey treated with folate supplement plus ethanol, Halsted, et al., (1975) demonstrated the decreased hepatic retention of labelled folate and increased urinary and faecal excretion of the label. The acute ingestion of alcohol had no apparent effect on the tissue distribution of ^{3}H-folic acid in man.

In this chapter the effect of alcoholism on the rate of the breakdown of the folate molecule in man using a mixture of ^{3}H + ^{14}C folic acid was investigated.

Materials and Methods:

One subject (S.C) suffering from alcoholism (details in Table 5.3.1) received a mixture of 5μCi [2-^{14}C] and 20μCi [3',5',7,9-^{3}H]-folic acid plus 5mg unlabelled folic acid orally. Urine was collected for three time periods 0-6hr, 6-12hr and 12-24hr. The determination of radioactivity, the technique of collection of urine and column chromatography have been previously described in chapter 2.

Results:

The urinary recovery of radioactivity after the oral dose of folic acid to the alcoholic patient during 0-6hr, 6-12hr and 12-24hr

was distributed as shown in Table 5.3.2. The recovery of radioactivity of 0-24hr urine sample was (56.03% ^{3}H and 50.3% ^{14}C) higher than in a control patient (non alcoholic) (31.35% ^{3}H and 27.76% ^{14}C of the dose). Thus it appears that alcoholism has increased the radioactivity excreted in urine.

Urinary metabolites:

Urine samples were sequentially chromatographed on both DE-52 and Sephadex-G15 columns. DE-52 chromatography of 0-6hr urine sample (Figure 5.3.1) showed similar pattern to that which appeared in a control subject (non alcoholic), despite the appearance of metabolite B with ^{14}C only (IV). The Sephadex-G15 pattern of ^{3}H-scission product peaks also matched that of in control, except the appearance of metabolite A (Figure 5.3.2). DE-52 chromatography of 6-12hr urine sample of this patient had similar pattern as of 0-6hr urine sample on the same column system. However separation in Sephadex-G15 showed a small amount of p-AcABA as well as the p-AcABG and metabolite A (Figure 5.3.3). DE-52 chromatography and Sephadex-G15 column of 12-24hr urine sample of this alcoholic patient (Figure 5.3.4, 5.3.5) showed decreasing unchanged folic acid and ^{3}H-scission product, while an increase of 5-CH_3THF was observed. However the Sephadex-G15 pattern was similar to those of other urine samples from the alcoholic. The distribution of the metabolites as the percentage of the dose is summarized in Table 5.3.3. The percentage of folic acid falls with the increasing time of urine sample. Similar considerations applied to both 5-CH_3THF and scission products. Table 5.3.4 shows the ratio of folic acid: 5-CH_3THF for the three times of urine sample, as well as the ratio of scission product: intact folates.

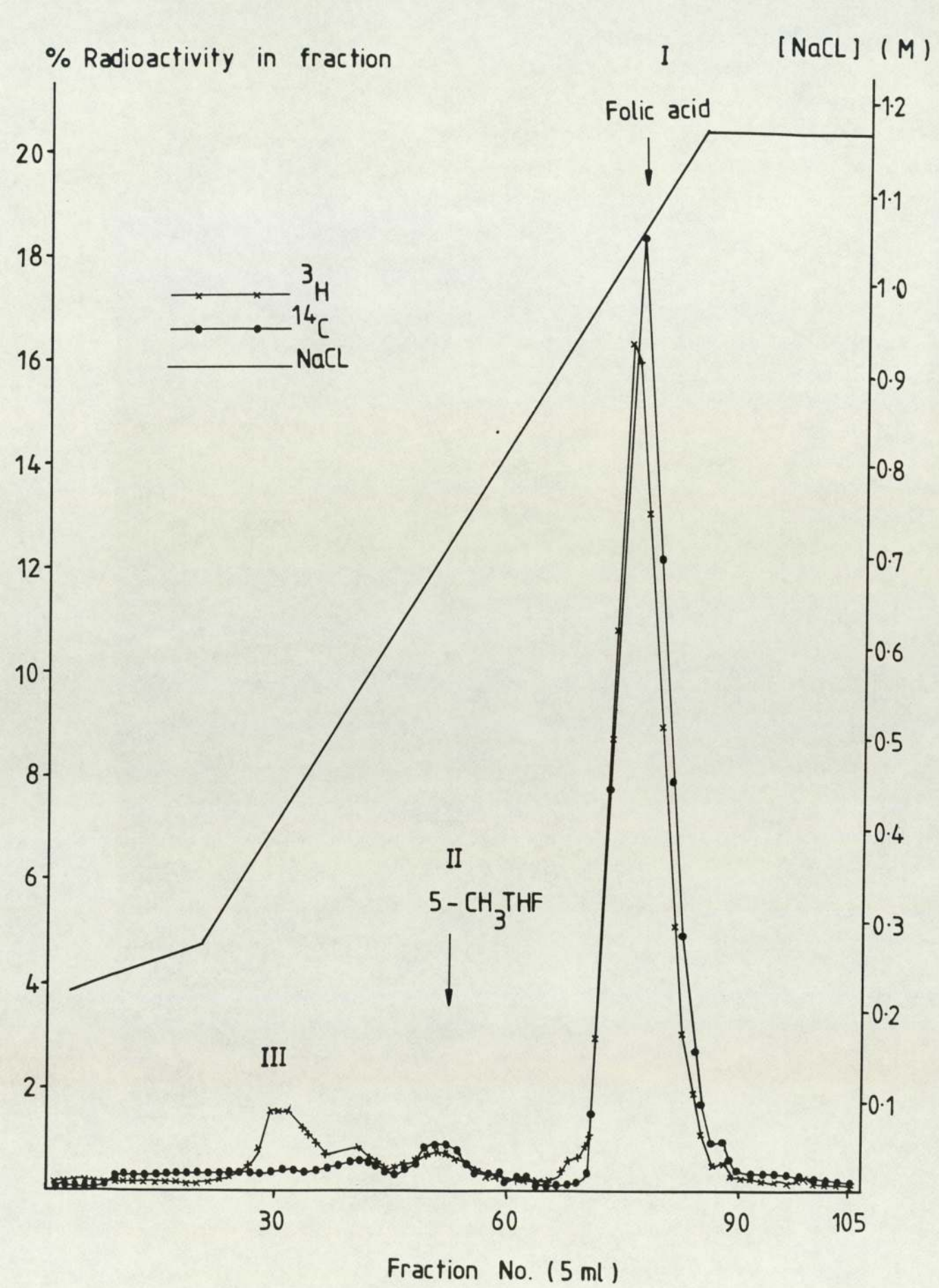


Fig. 5.1.1 DE-52 chromatography of 0-6 hr urine sample of a control patient (J.T.) after the administration of a mixture of ^3H + ^{14}C folic acid (5 mg).

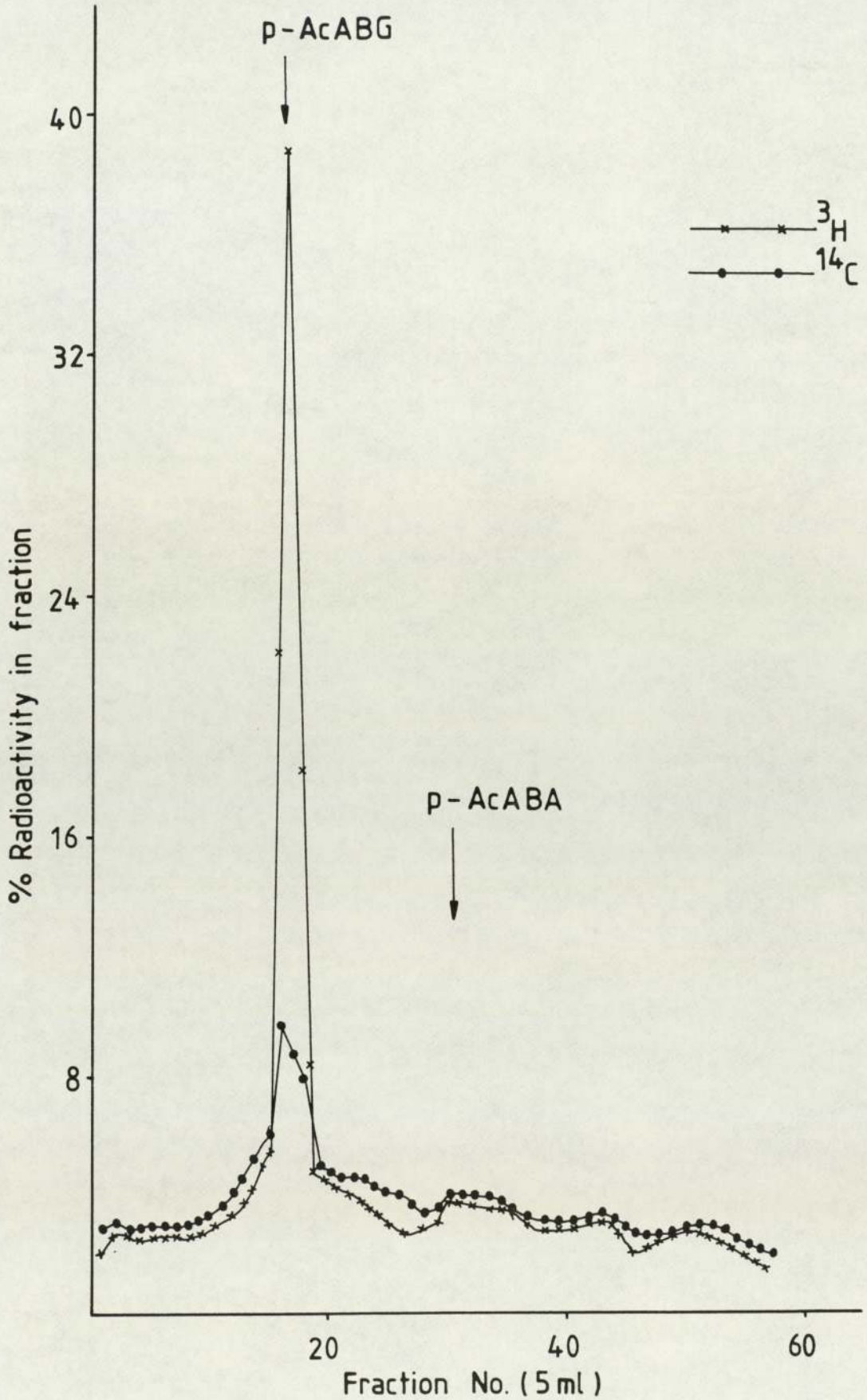


Fig. 5.1.2 Sephadex-G15 chromatography of the tritiated peak (III) from DE-52 chromatography of 0-6 hr urine sample of a control patient (J.T.) receiving a mixture of ^{3}H + ^{14}C folic acid (5 mg).

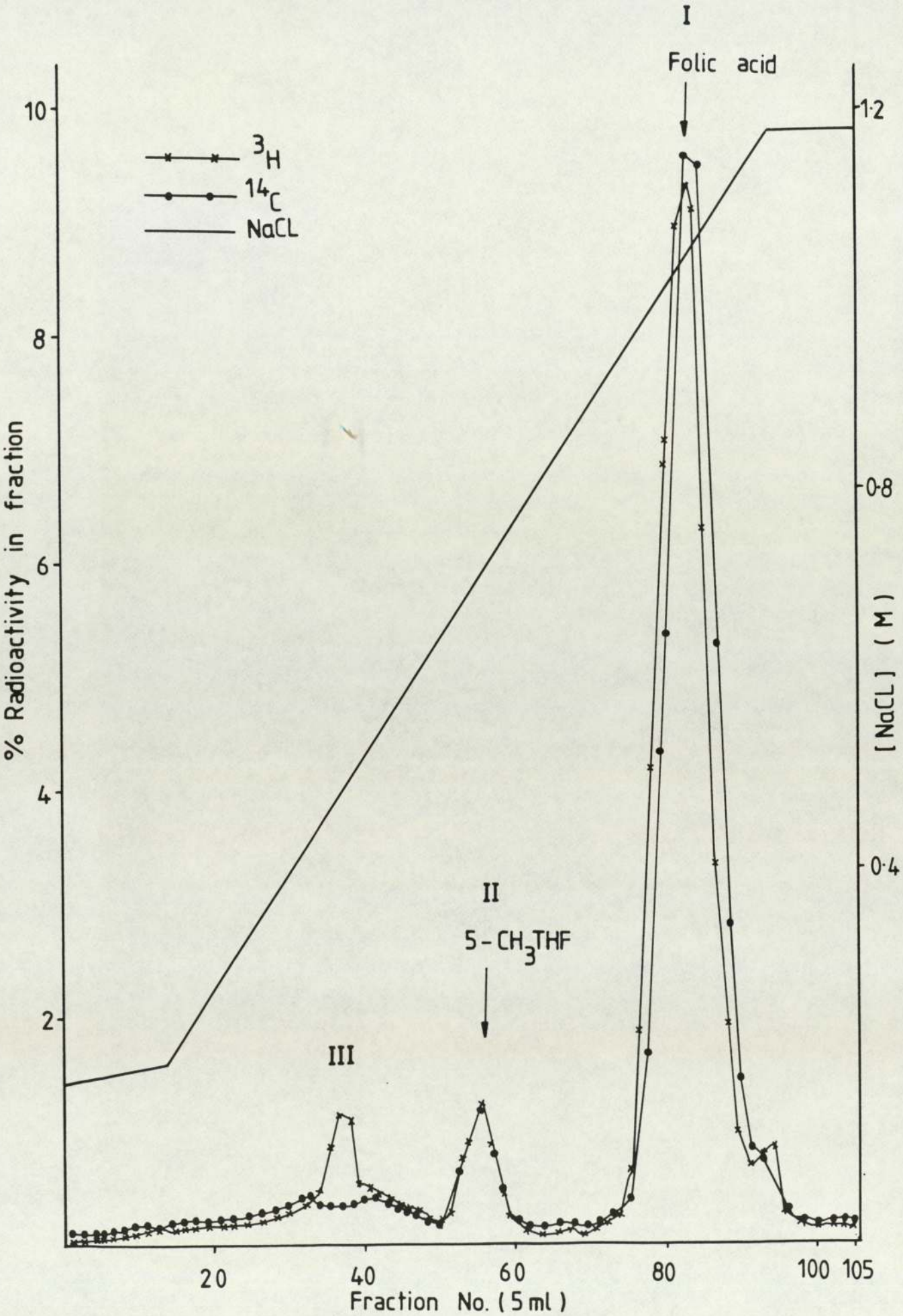


Fig. 5.1.3 DE-52 chromatography of 12-24 hr urine sample of a control patient (J.T.) receiving high dose of ^{3}H + ^{14}C folic acid (5 mg).

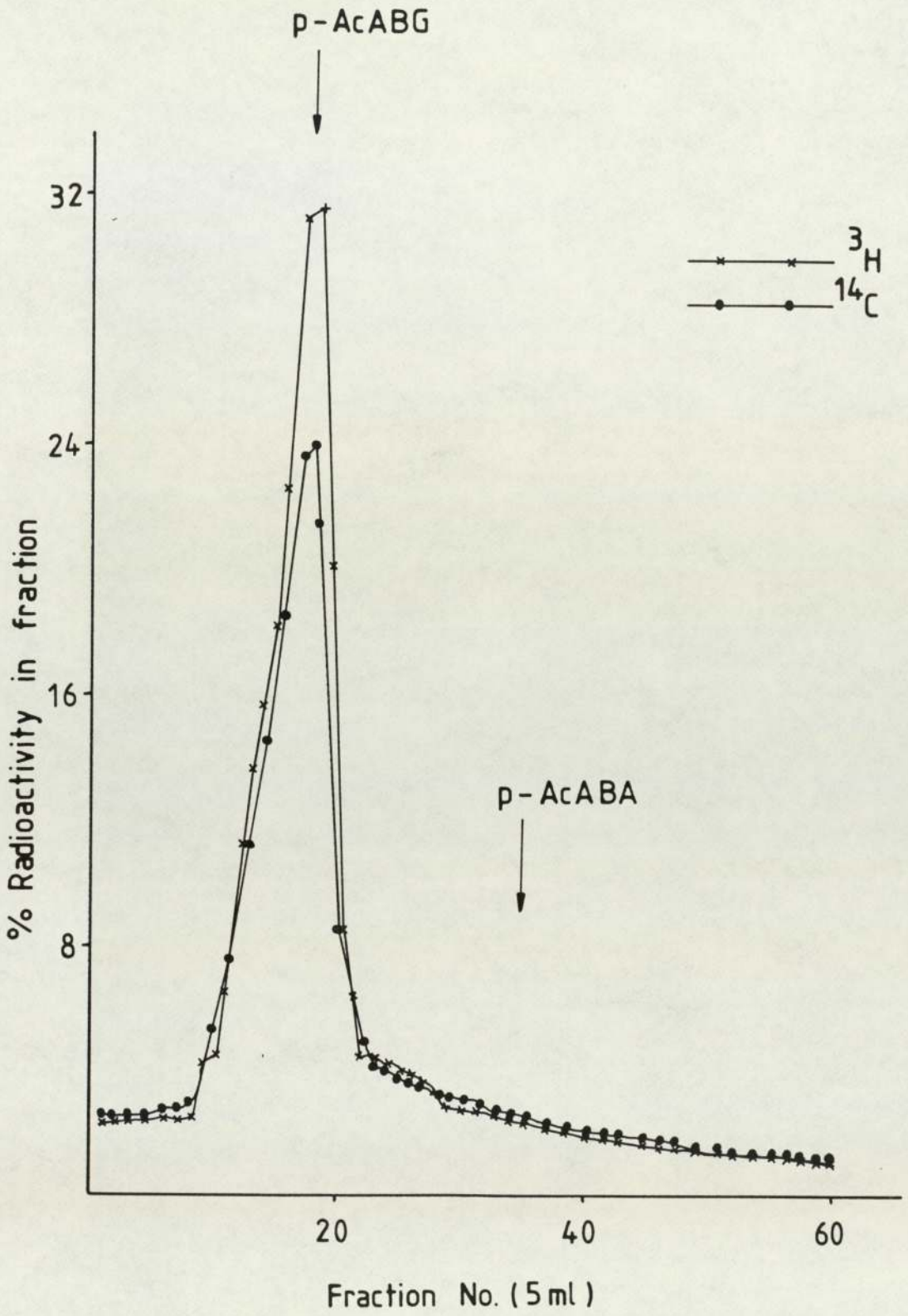
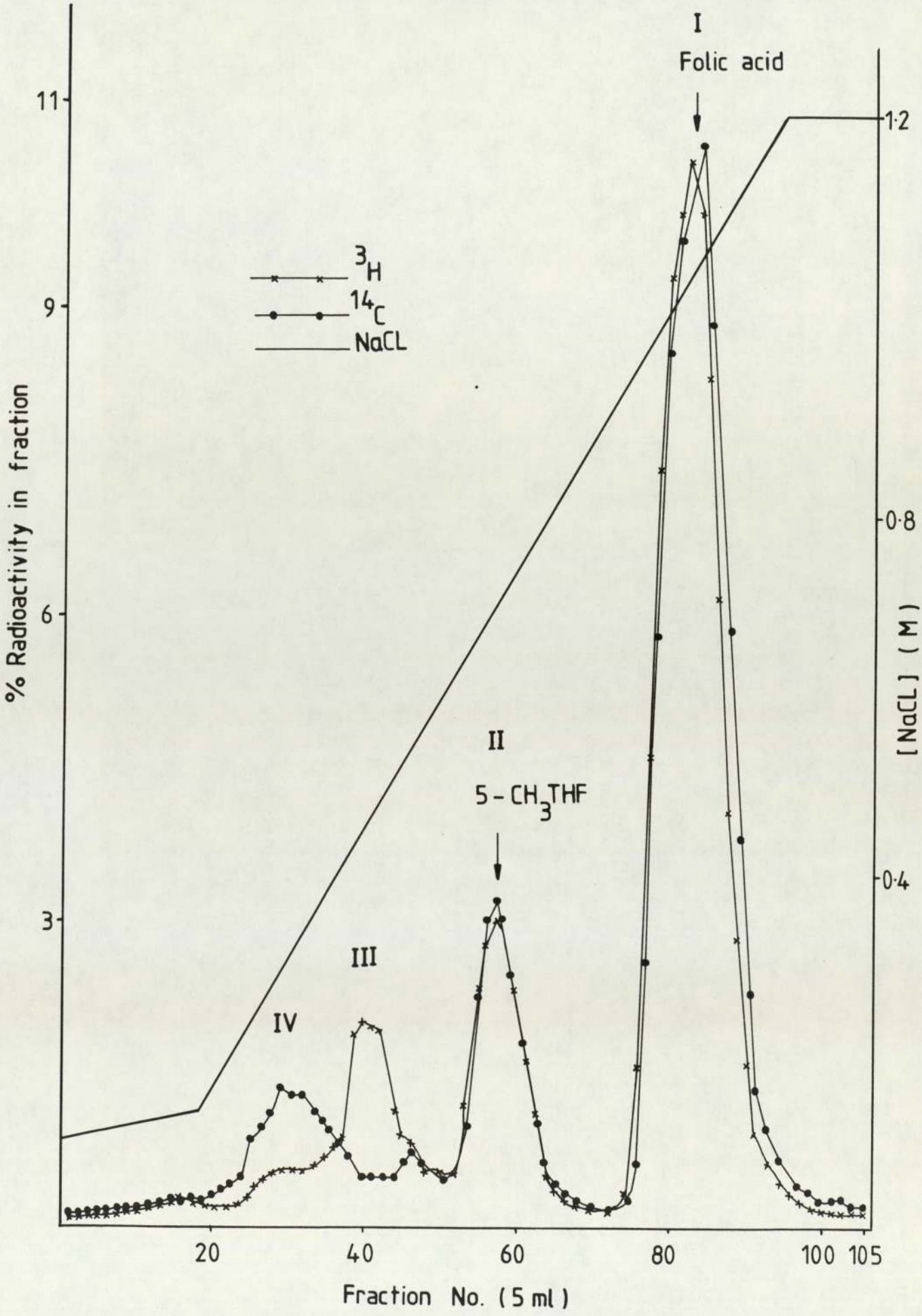


Fig. 5.1.4 Sephadex-G15 chromatography of the tritiated peak (III) from DE-52 chromatography of 12-24 hr urine sample of a control patient (J.T.) after the administration of a mixture of ^{3}H + ^{14}C folic acid (5 mg).


I
Folic acid
II
5 - CH3THF
III
IV
3H
14C
NaCL
% Radioactivity in fraction
[NaCL] (M)
Fraction No. (5 ml)
11
9
6
3
1·2
0·8
0·4
20
40
60
80
100
105

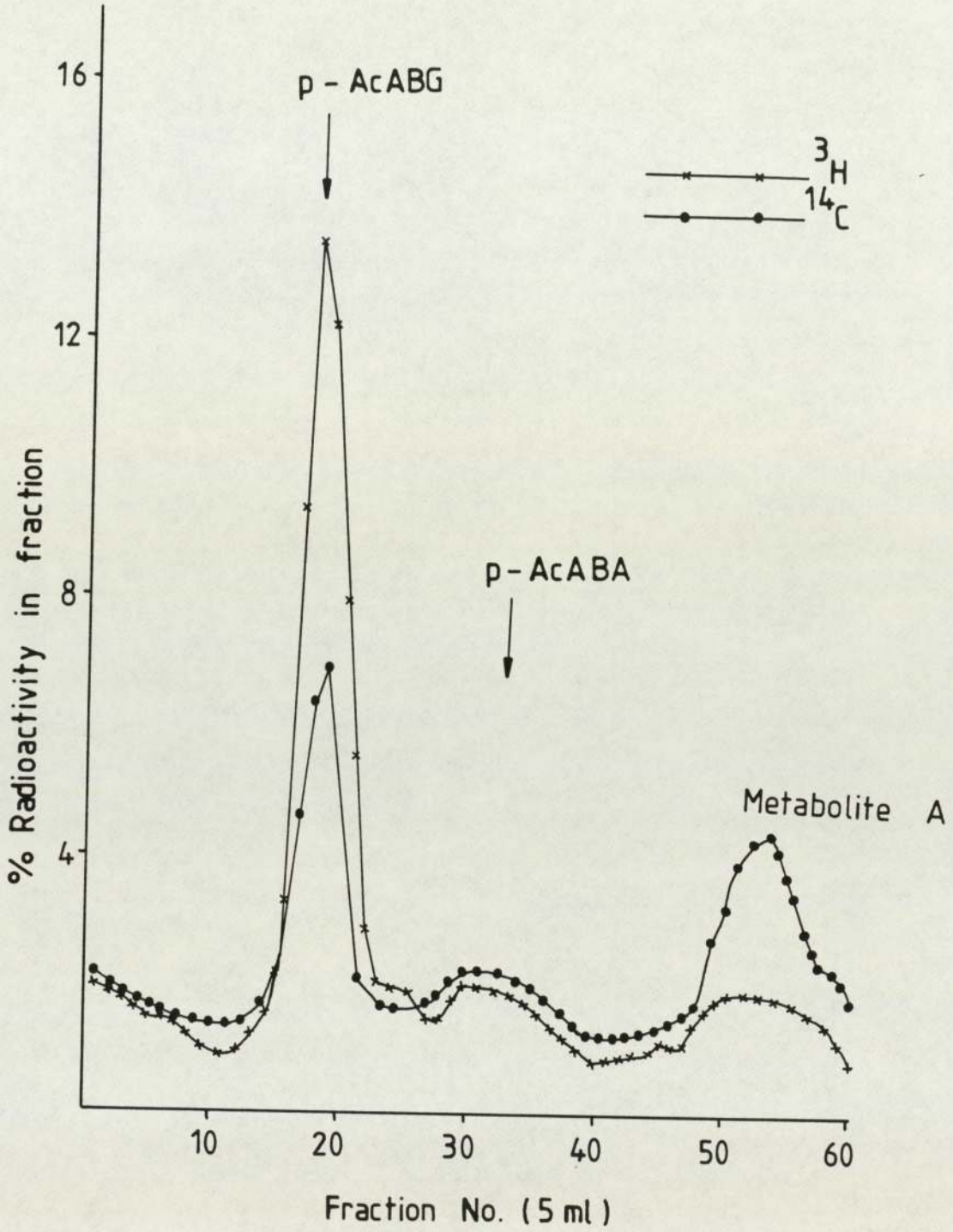


Fig. 5.1.6 Sephadex-G15 chromatography of peak III from DE-52 chromatography of 0-6 hr urine sample of a cancer patient (L.K.).

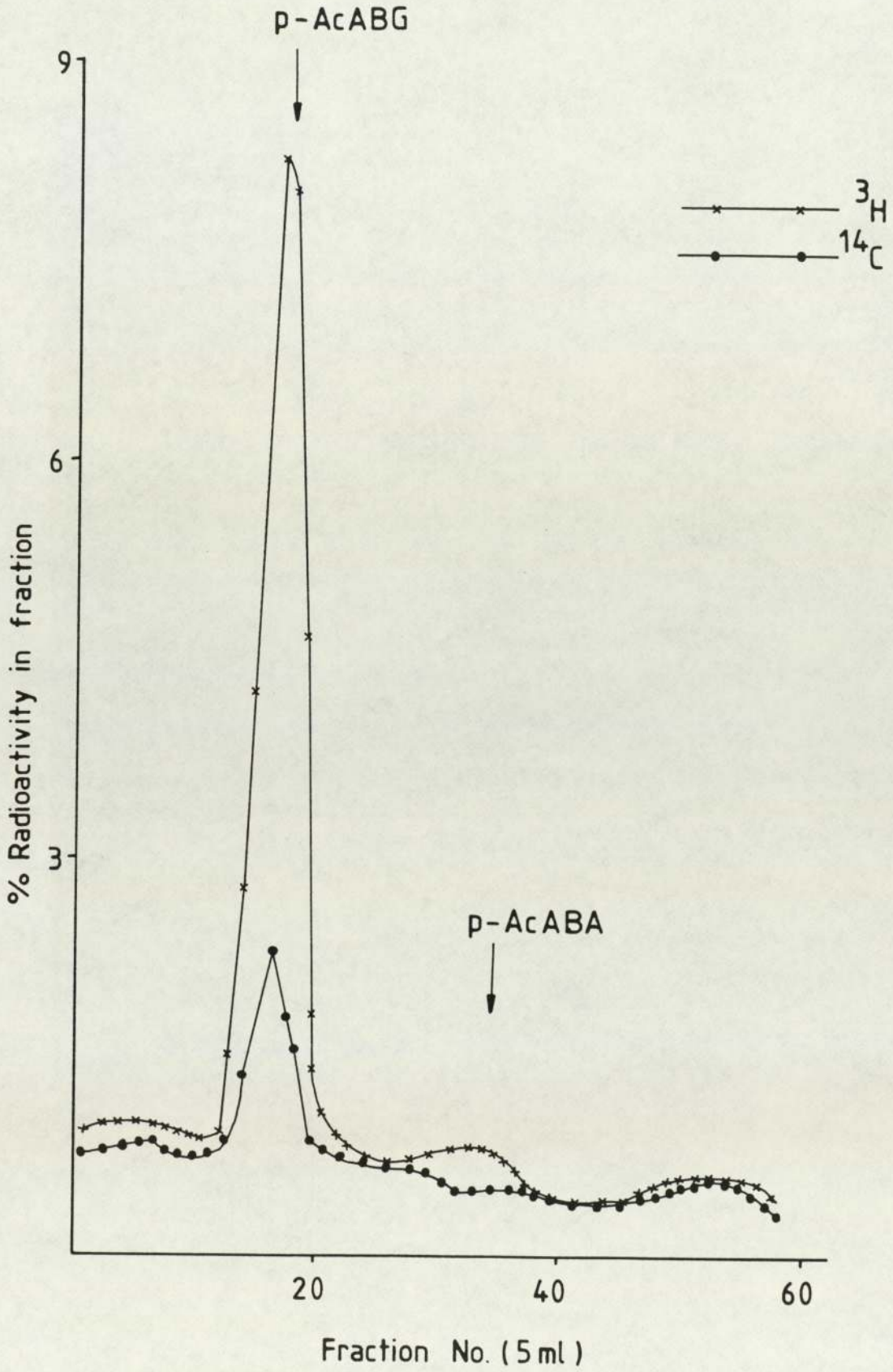


Fig. 5.1.7 Sephadex-G15 chromatography of the tritiated peak (III) from DE-52 chromatography of 6-12 hr urine sample of a cancer patient (L.K.).

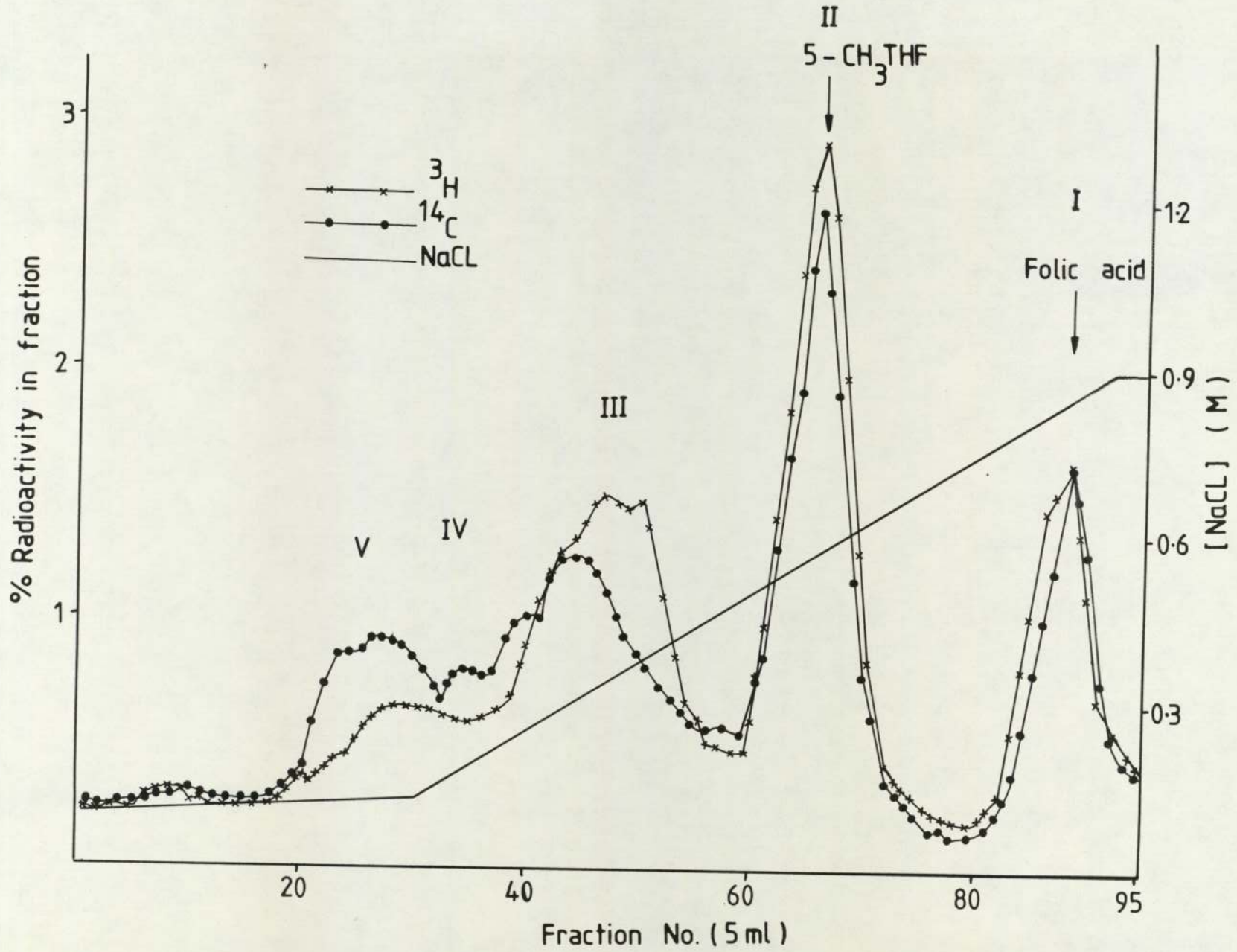

II
5 - CH3THF
I
Folic acid
III
IV
V
3H
14C
NaCL
% Radioactivity in fraction
[NaCL] (M)
Fraction No. (5 ml)
1
2
3
0·3
0·6
0·9
1·2
20
40
60
80
95

Fig. 5.1.8 DE-52 chromatography of 12-24 hr urine sample of a cancer patient (L.K.) after administration of a mixture of ^{3}H + ^{14}C folic acid (5 mg).

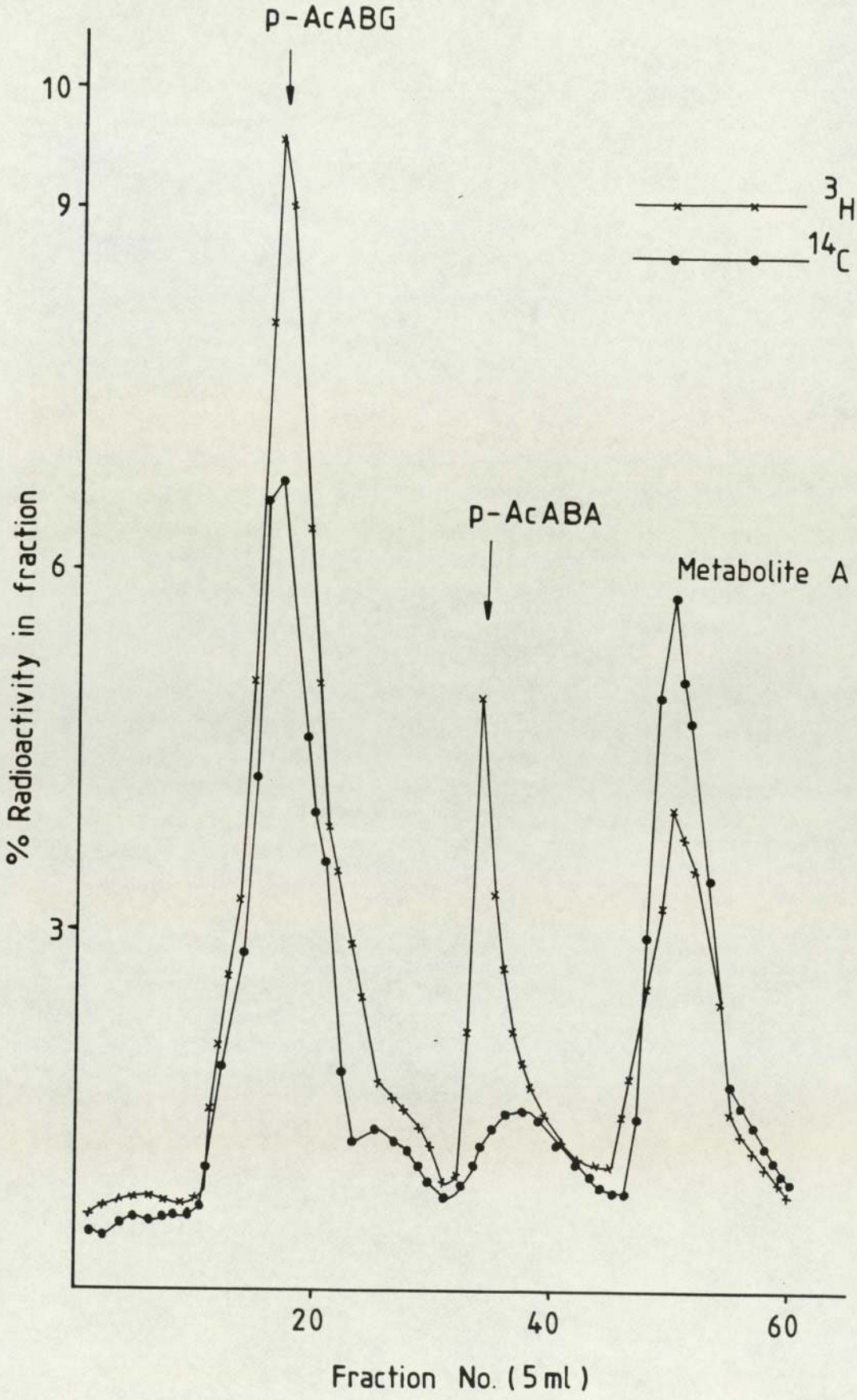


Fig. 5.1.9 Sephadex-G15 chromatography of the tritiated peak (III) from DE-52 chromatography of 12-24 hr urine sample of a cancer patient (L.K.).

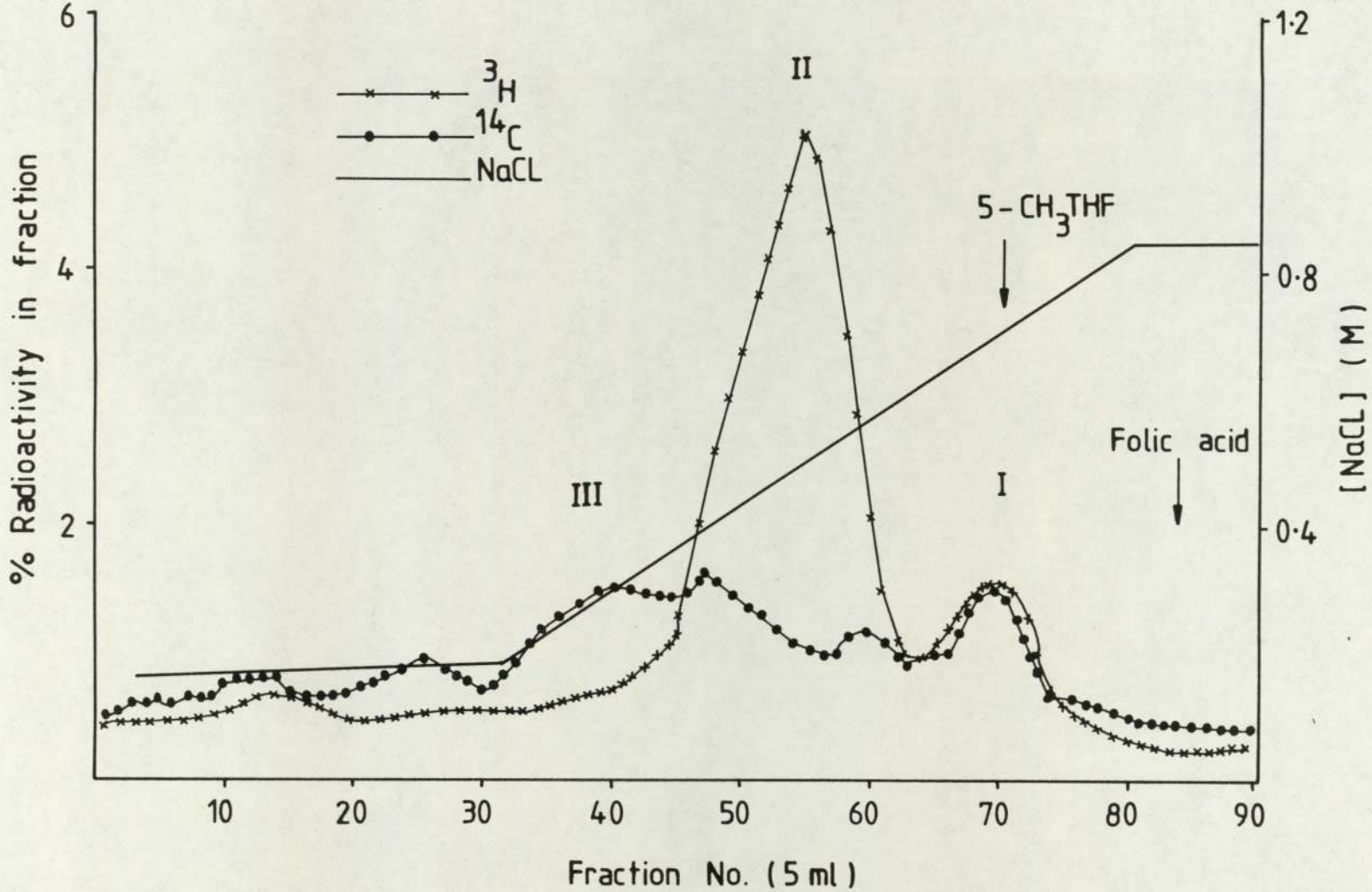


Fig. 5.2.1 DE-52 chromatography of 0-6 hr urine sample of a control patient (B.F.) after the administration of a mixture of ^3H + ^{14}C folic acid (50 µg).

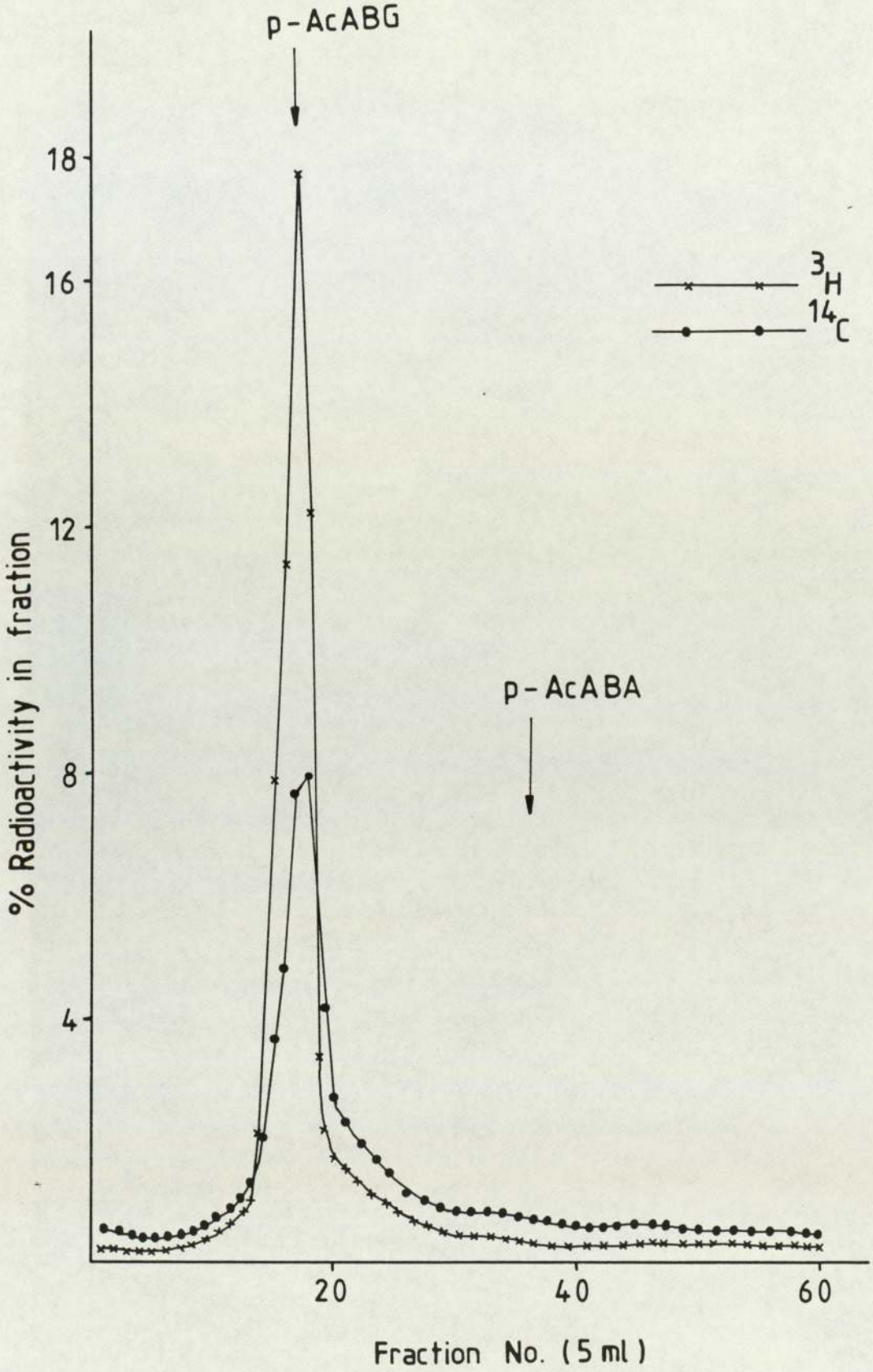


Fig. 5.2.2 Sephadex-G15 chromatography of the tritiated peak (II) from DE-52 chromatography of 0-6 hr urine sample of a control patient (B.F.) receiving low dose of a mixture of ^3H + ^{14}C folic acid (50 µg).

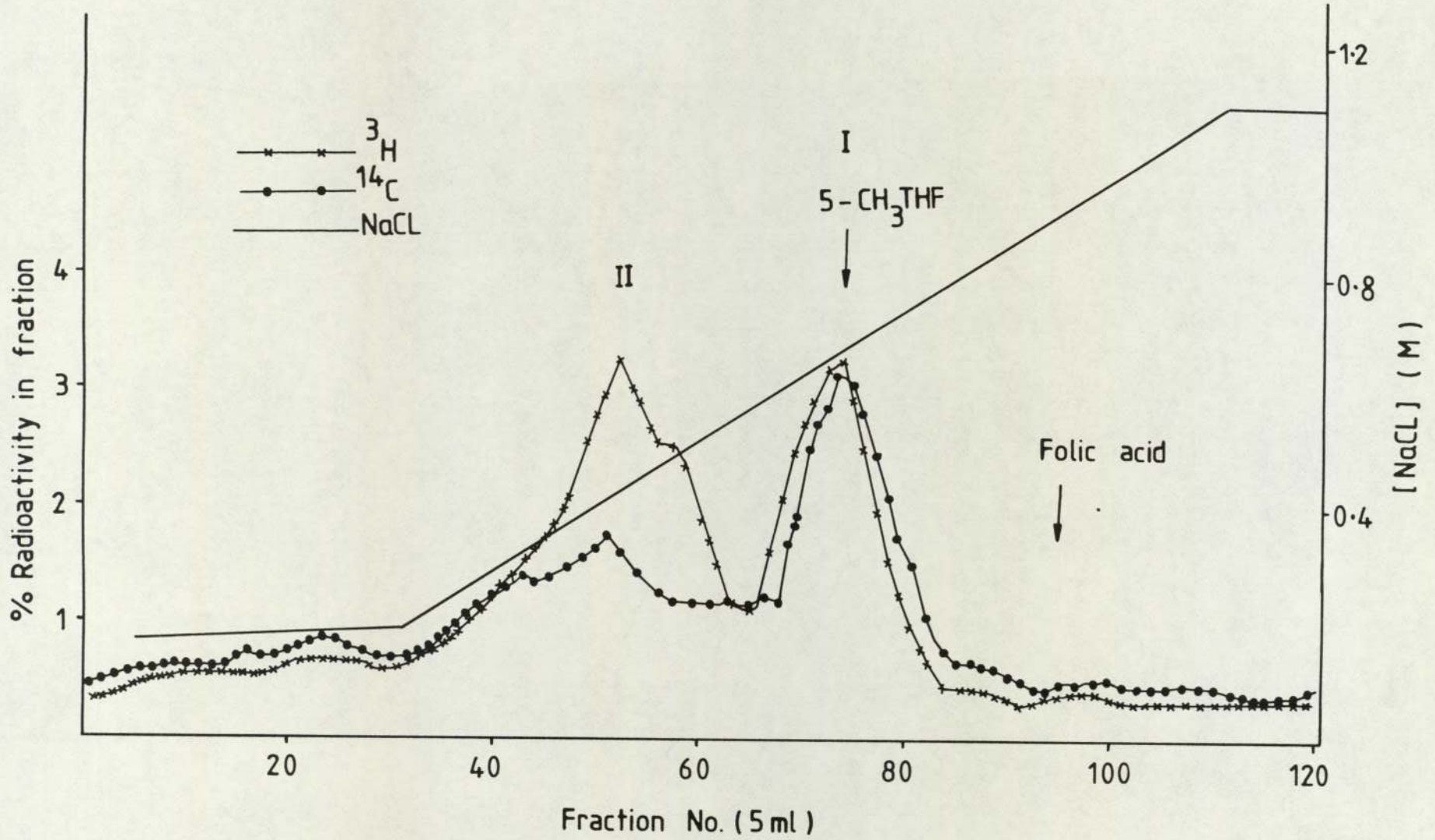


Fig. 5.2.3 DE-52 chromatography of 6-12 hr urine sample of a control patient (B.F.) after the oral administration of a mixture of ^{3}H + ^{14}C folic acid (50 ug).

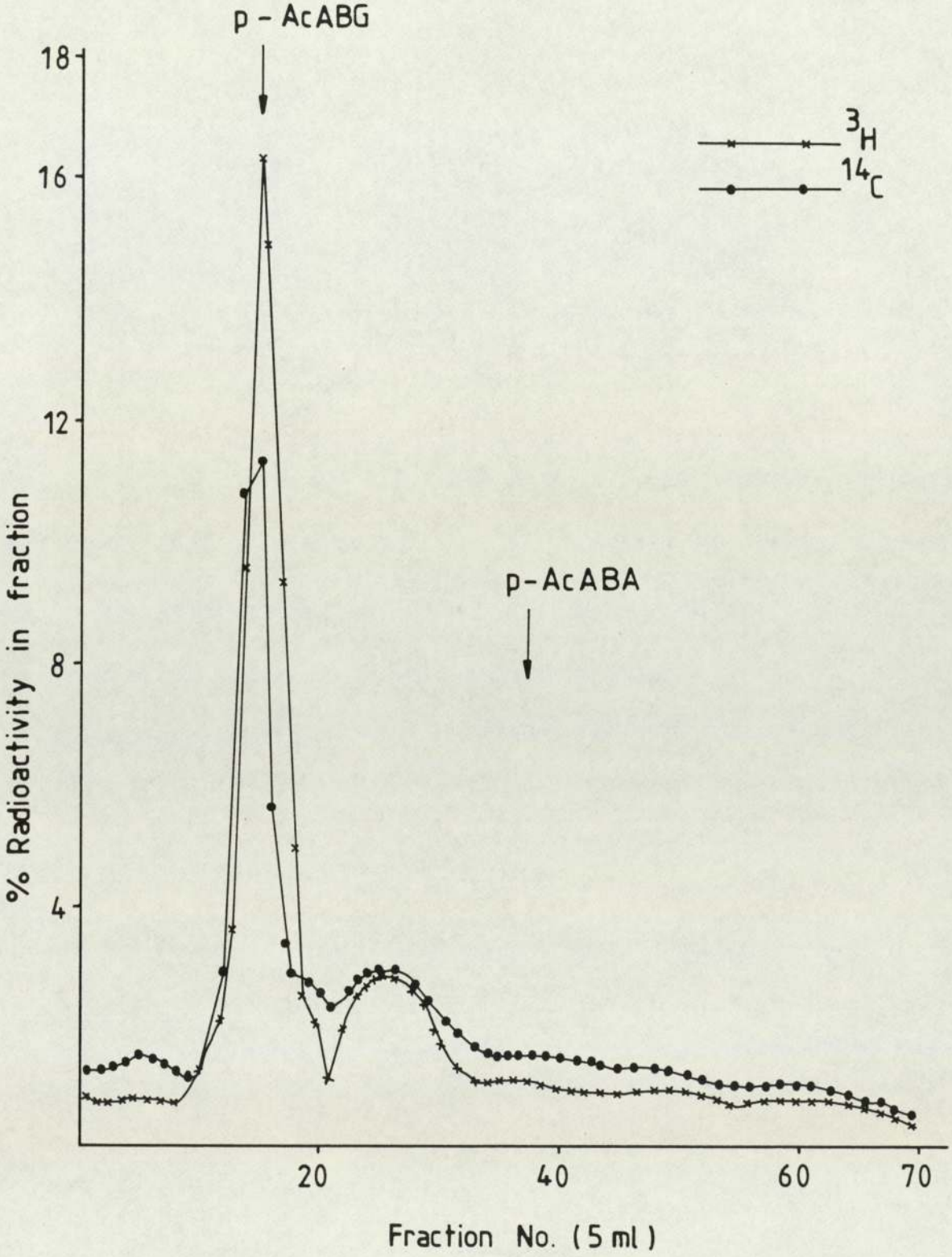
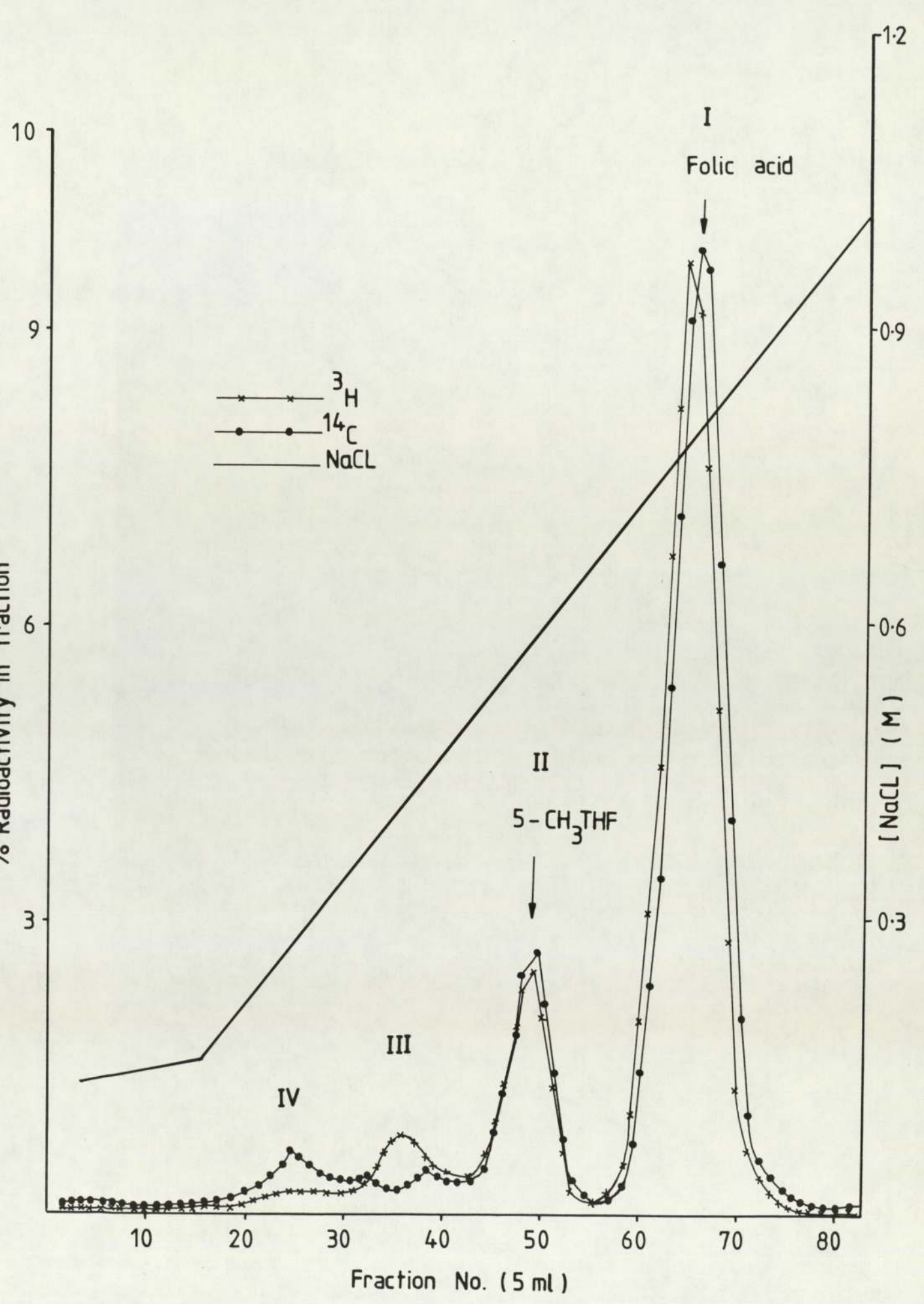


Fig. 5.2.4 Sephadex-G15 chromatography of the tritiated peak (II) from DE-52 chromatography of 6-12 hr urine sample of a control patient (B.F.) receiving low dose of ^{3}H + ^{14}C folic acid (50 µg).


I
Folic acid
II
5-CH3THF
III
IV
3H
14C
NaCL
% Radioactivity in Fraction
[NaCL] (M)
Fraction No. (5 ml)
10
9
6
3
1·2
0·9
0·6
0·3
10
20
30
40
50
60
70
80

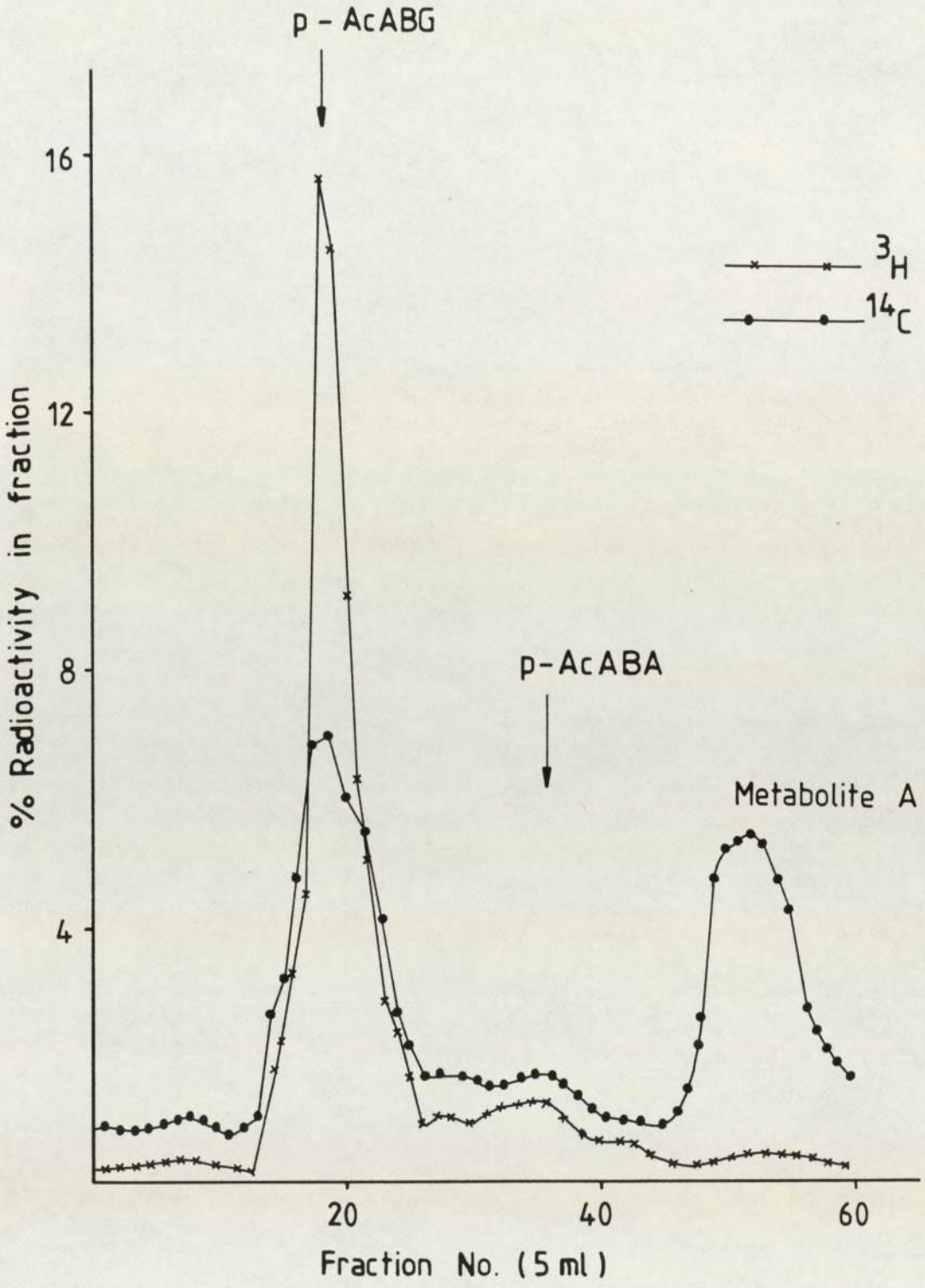


Fig. 5.3.2 Sephadex-G15 chromatography of the tritiated peak (III) from DE-52 chromatography of 0-6 hr urine sample of alcoholic patient (S.C.).

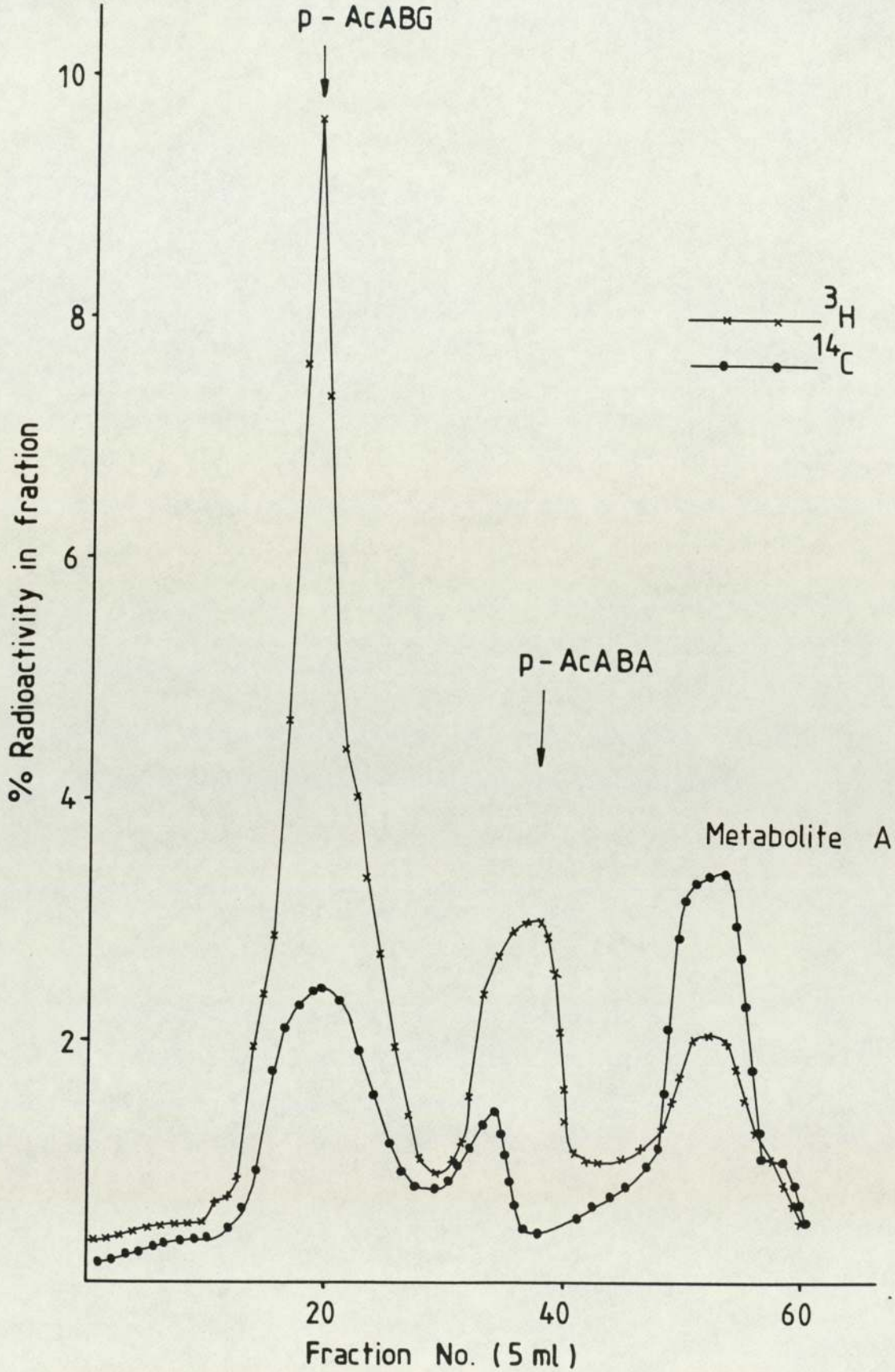


Fig. 5.3.3 Sephadex-G15 chromatography of the tritiated peak (III) from DE-52 chromatography of 6-12 hr urine sample of alcoholic patient (S.C.).

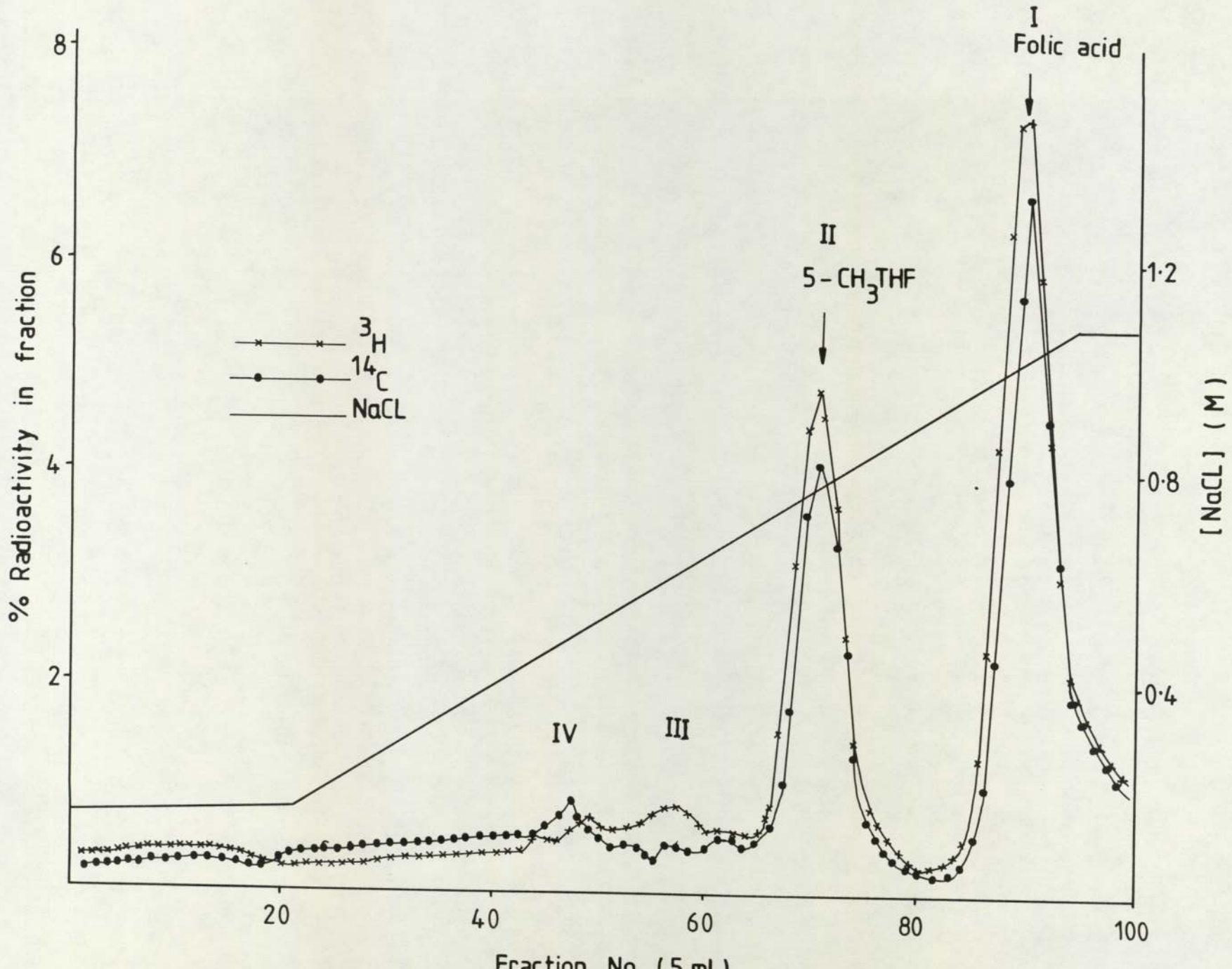

I
Folic acid
II
5 - CH3THF
IV
III
3H
14C
NaCL
% Radioactivity in fraction
[NaCL] (M)
Fraction No. (5 mL)
8
6
4
2
1·2
0·8
0·4
20
40
60
80
100

Fig. 5.3.4 DE-52 chromatography of 12-24 hr urine sample of alcoholic patient (S.C.) after the administration of ^{3}H + ^{14}C folic acid (5 mg).

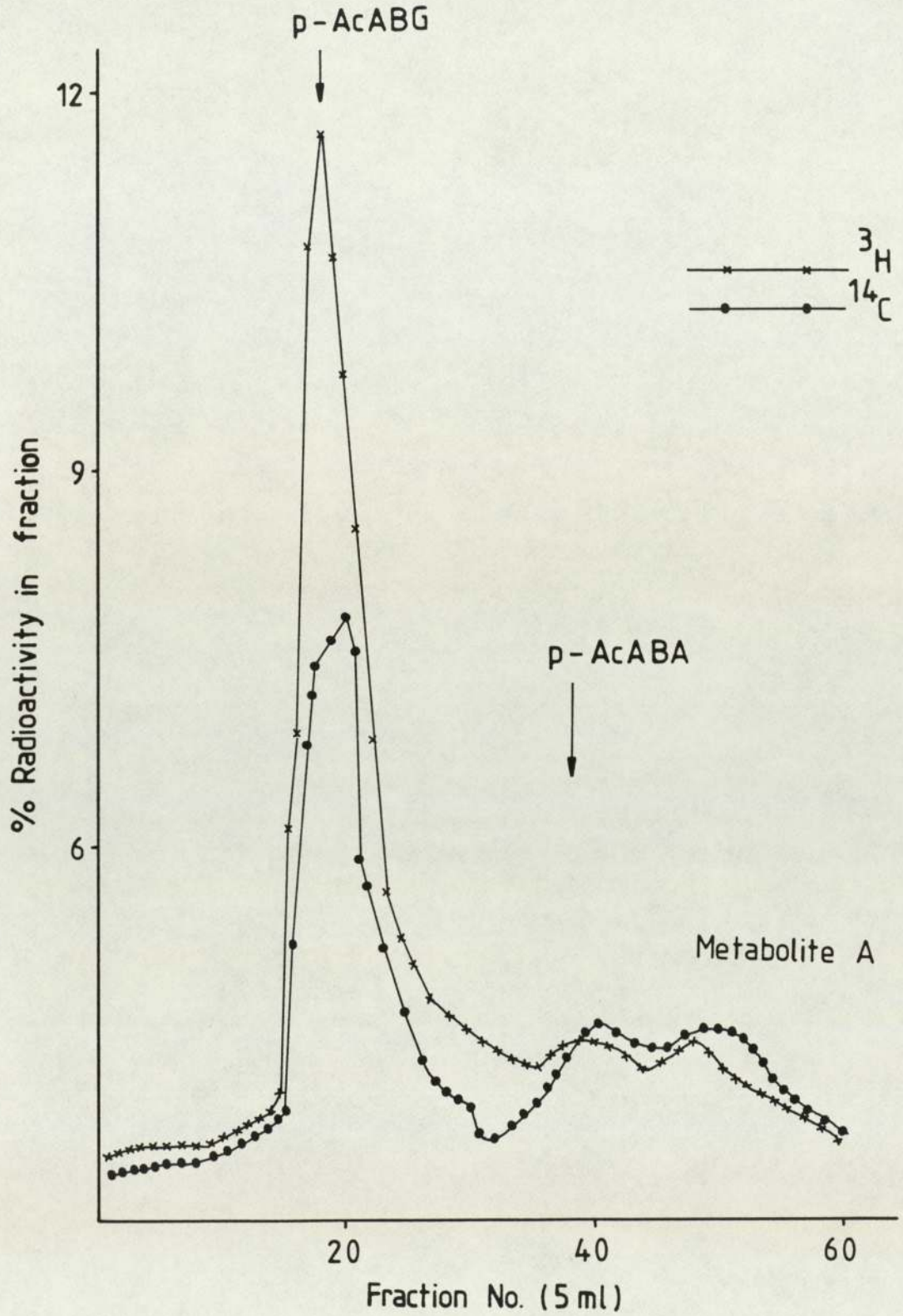


Fig. 5.3.5 Sephadex-G15 chromatography of the tritiated peak from DE-52 chromatography of 12-24 hr urine sample of alcoholic patient (S.C.).

Table 5.1.1

Name	Age	Sex	Diagnosis	Therapy
J.T.	74	F	Iron-deficiency (anemia), parkinsonian tremor	Diazepam, Sinemet, Ferrous-sulphate, Cimetidine
L.K.	54	M	Weight loss, anorexia, anaplastic retroperitonal-carcinoma	Paracetamol, Triazocam
F.K.	72	F	primary hepato cellular-carcinoma, cryptogenic-cirrhosis, hypertension	Sotacol, Methyl Dopa, Spironolactone, vitamin K, Diazepam, Paracetamol

Clinical details of patients studied

Table 5.1.2

Patient	Case	0-6hr		6-12hr		12-24hr		0-24hr	
		^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
J.T.	Control	12.12	10.33	0.43	0.34	18.80	17.09	31.35	27.76
L.K.	Cancer	11.50	10.50	5.59	5.38	1.96	2.11	19.05	17.99
F.K.	Cancer	0.01	0.01	0.01	.0.01	1.05	0.74	1.07	0.76

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

Table 5.1.3

Patient		Sample	Folic acid ^{3}H	Folic acid ^{14}C
J.T.	Control	0-6hr	9.13	8.36
		6-12hr	N.d.	N.d.
		12-24hr	14.88	13.73
L.K.	Cancer	0-6hr	7.46	6.80
		6-12hr	3.17	2.81
		12-24hr	0.31	0.31
F.K.	Cancer	0-6hr		
		6-12hr	N.d.	
		12-24hr		

Patient		Sample	5-CH_3THF ^{3}H	5-CH_3THF ^{14}C	p-AcABG ^{3}H	p-AcABA ^{3}H
J.T.	Control	0-6hr	0.57	0.48	1.61	0.0
		6-12hr	N.d.	N.d.	N.d.	N.d.
		12-24hr	1.08	0.96	2.04	0.00
L.K.	Cancer	0-6hr	1.90	1.71	1.81	0.0
		6-12hr	1.04	1.29	1.34	0.3
		12-24hr	0.6	0.91	0.85	0.7
F.K.	Cancer	0-6hr				
		6-12hr	N.d.		N.d.	
		12-24hr				

The relative distribution of the major labelled metabolites appearing in the various urine samples in both control and cancer patients after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (5mg). The results are expressed as the percentage of the dose present as each metabolite.

N.d. Not detected.

Table 5.1.4

Folic acid: 5-CH_3THF

Patient	Dose mg	0-6hr ^3H	0-6hr ^{14}C	6-12hr ^3H	6-12hr ^{14}C	12-24hr ^3H	12-24hr ^{14}C
J.T.+	5	16.1:1	17.42:1	N.d.		13.78:1	14.30:1
L.K.*	5	3.9:1	4.0:1	3.0:1	2.2:1	0.52:1	0.34:1
F.K.*	5	N.d.		N.d.		N.d.	
B.F+	0.05	-	-	-	-	N.d.	

Scission product: intact folate

Patient	Dose mg	0-6hr ^3H	0-6hr ^{14}C	6-12hr ^3H	6-12hr ^{14}C	12-24hr ^3H	12-24hr ^{14}C
J.T.+	5	0.17:1	0.08:1	N.d.		0.13:1	0.10:1
L.K.*	5	0.19:1	0.18:1	0.32:1	0.32:1	0.93:1	0.79:1
F.K.*	5	N.d.		N.d.		N.d.	
B.F+	0.05	5.67:1	6.1:1	2.55:1	2.17:1	N.d.	

The ratio of folic acid:5-CH_3THF and the scission product:intact folate excreted from the various urine samples after the administration of either low dose (50μg) or high dose (5mg) of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid to control and cancer patients.

N.d. : Not detected

\+ : Control patient

* : Cancer patient

Table 5.2.1

Name	Age	Sex	Diagnosis	Therapy
B.F.	62	M	Myocardial infarction (previously well)	Frusemide

Clinical details of patient studied

Table 5.2.2

Patient		Dose µg	0-6hr		6-12hr		12-24hr		0-24hr	
			^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
B.F.	Control	50	1.23	0.6	1.02	0.79	0.78	0.62	3.03	2.01

Urinary recovery of radioactivity after the oral dose of[2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (50µg) during three collection periods. The results are expressed as the percentage of the dose.

Table 5.2.3

Patient		Sample	Folic acid ^{3}H	Folic acid ^{14}C
B.F.	Control	0-6hr	-	-
		6-12hr	-	-
		12-24hr	N.d.	

Patient		Sample	5-CH_3THF ^{3}H	5-CH_3THF ^{14}C	p-AcABG ^{3}H	p-AcABA ^{3}H
B.F.	Control	0-6hr	0.18	0.08	1.02	0.0
		6-12hr	0.27	0.23	0.69	0.0
		12-24hr	N.d.		N.d.	

The relative distribution of the major labelled metabolites in the three collection periods of urine sample after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (50μg) to the control patient. The results are expressed as the percentage of the dose present as each metabolite.

N.d. Not detected.

Table 5.3.1

Name	Age	Sex	Diagnosis	Therapy
S.C.	65	M	Alcoholic-probably cirrhosis drinks 10 pt beer/day and/ or 3 bottles whisky/week Alocoholic macrocytosis (serum-folate 2.4mg/ml)	Folic acid, Frusemide, Spironolactone

Clinical details of patient studied

Table 5.3.2

Patient	Case	0-6hr		6-12hr		12-24hr		0-24hr	
		^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
S.C.	Control (Alcoholic)	31.36	27.79	20.42	18.66	4.25	3.94	56.03	50.3

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

Table 5.3.3

Patient		Sample	Folic acid ^{3}H	Folic acid ^{14}C
S.C.	Control	0-6hr	23.09	19.97
		6-12hr	14.92	13.38
	(Alcoholic)	12-24hr	2.03	1.83

Patient		Sample	5-CH_3THF ^{3}H	5-CH_3THF ^{14}C	p-AcABG ^{3}H	p-AcABA ^{3}H
S.C.	Control	0-6hr	5.25	4.68	2.88	0.0
		6-12hr	3.03	2.82	2.03	0.78
	(Alcoholic)	12-24hr	1.16	1.07	0.54	0.05

The relative distribution of the major labelled metabolites appearing in the various samples of urine after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (5mg) to alcoholic patient. The results are expressed as the percentage of the dose present as each metabolite.

Table 5.3.4

		Folic acid: 5-CH_3THF					
		0-6hr		6-12hr		12-24hr	
Patient		^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
S.C.	Control (Alcoholic)	4.4:1	4.3:1	4.9:1	4.7:1	1.75:1	1.7:1

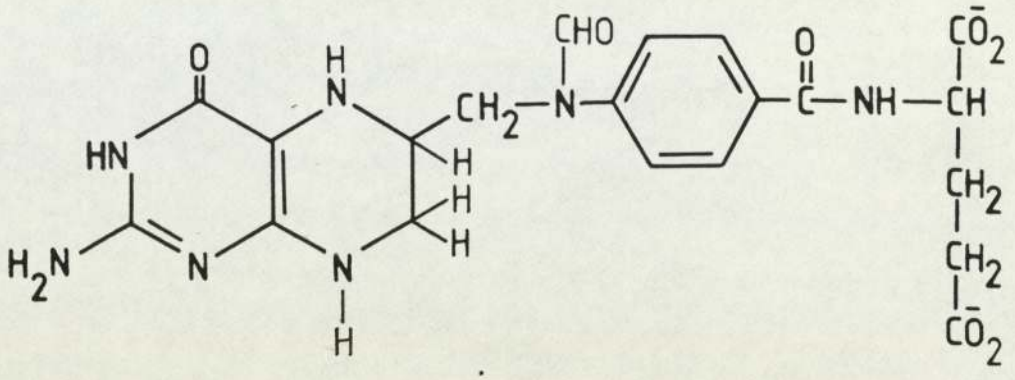
		Scission product: intact folate					
		0-6hr		6-12hr		12-24hr	
Patient		^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
S.C.	Control (Alcoholic)	0.1:1	0.01:1	0.11:1	0.13:1	0.17:1	0.17:1

The ratio of folic acid : 5-CH_3THF and the ratio of scission product:intact folate excreted after the administration of an oral dose of [2-^{14}C] and[3',5',7,9-^{3}H]-folic acid (5mg) to alcoholic patient from 0-6hr, 6-12hr and 12-24hr period of collecting urine.

CHAPTER 6

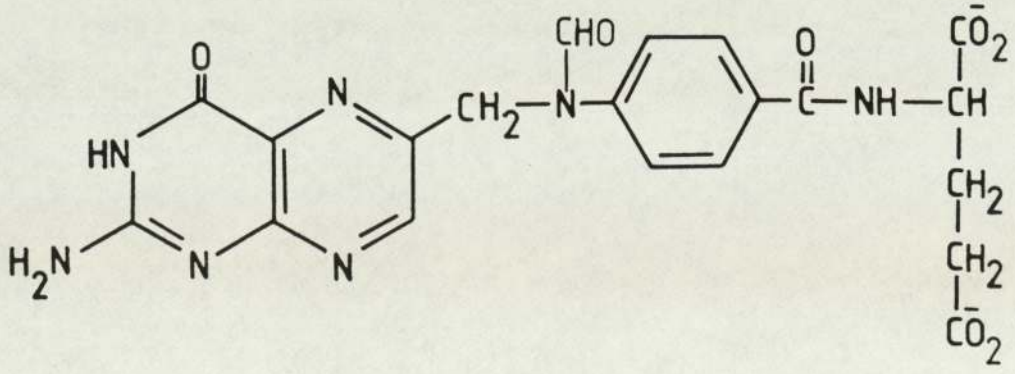
The effect of a low dose of 10-formylfolate on folic acid metabolism

10-formylfolate (10-CHOFA) an oxidized folate, is an important constituent of food folate where it is likely to be derived from a reduced form of folate conjugate (Butterworth, et al., 1963; Santini, et al., 1964). 10-CHOTHF is very readily oxidized like tetrahydrofolate (Rowe, 1978). However this oxidation of 10-CHOTHF, unlike THF, does not occur with scission but with the retention of the whole structure to give 10-CHOFA (Figure 6.A). Studies in vitro using dihydrofolate reductase from various mammalian sources showed that 10-CHOFA was not reduced by either dihydrofolate reductase from Erlich ascites cell (Bertino, et al., 1965) or bovine dihydrofolate reductase (A. Sahota, personal communication). Thus it has no function in the folate cycle. 10-CHOFA is a powerful inhibitor of mammalian dihydrofolate reductase from bovine liver and rat liver with Ki = 10^{-8}M (d'Urso-Scott, et al., 1974 and Friedkin, et al., 1975). 10-CHOFA derived from the oxidation of 10-CHOTHF could act as a natural inbuilt regulator of the normal folate metabolic cycle in man (J. Blair, personal communication). However, it may be a weak inhibitor of dihydrofolate reductase from human breast tumour and from human gut tumour. Detailed in vivo studies have been carried out by many workers. Beavon and Blair (1975) using [2-^{14}C] - 10-CHOFA, suggested that it was only slowly metabolised by rats and thus unavailable to the folate pool. Pheasant and co-workers (1981), using mixed labelled 10-CHOFA, detected that this compound was reduced and incorporated into folate pool. In man using radiolabelled 10-formylfolate, Saleh (1981) showed that very limited metabolism of this compound occurred in vivo. Other studies using microbiological assay by Ratanasthien, et al., (1974) showed no metabolism of orally administered 10-CHOFA.



10-Formyltetrahydrofolate

Oxidation



10-formylfolate

Figure 6.A : Oxidation of 10-CHOTHF

In this chapter, the first in vivo study of the effect of low dose of 10-formylfolate on the metabolism of folic acid was investigated.

Materials and Methods:

Two control patients J.B. and D.C. suffering from non malignant diseases (details in Table 6.1) were studied. The first patient (J.B.) was given a mixture of 5μCi [2-^{14}C] and 20μCi [3',5',7,9-^{3}H]-folic acid (50μg folic acid) plus 57μg of unlabelled 10-formylfolic acid. The second patient (D.C.) was given a mixture of 5μCi [2-^{14}C] and 20μCi [3',5',7,9-^{3}H] - folic acid plus 50μg of unlabelled 10-formylfolicacid. The urine of both patients was collected for 0-6hr, 6-12hr, 12-24hr time periods as described in chapter 2. The determination of radioactivity and the column chromatography were also described in chapter 2.

Results:

Table 6.2 shows the recovery of ^{3}H and ^{14}C in the 0-6hr, 6-12hr and 12-24hr urine sample of patients who received the low dose of 10-formyfolic acid. The amount of urinary radioactivity was similar to those having a low dose of folic acid. The urinary excretion of radioactivity in the 0-24hr urine sample of patient J.B. was 4.73% ^{3}H and 0.42% ^{14}C of the dose and was 1.31% ^{3}H and 1.31% ^{14}C of the dose in patient D.C. The percentage of the ^{3}H dose was higher than that the ^{14}C dose in the patient J.B., whereas this ratio was unity in patient D.C.

Urinary metabolites:

The urine samples were chromatographed sequentially on DE-52 chromatography and Sephadex-G15 column.

DE-52 chromatography of 0-6hr urine sample of patient J.B. is illustrated in Figure 6.1 and showed four radioactive components; an ^{3}H-scission product (I) identified on Sephadex-G15 (Figure 6.2) as p-AcABG and ^{14}C metabolite A. Two other ^{14}C species (II, III) on DE-52 chromatography were also seen. The 6-12hr urine sample was not chromatographed because of its low radioactivity. DE-52 chromatography of 12-24hr urine sample (Figure 6.3) showed a different composition with unchanged folic acid and 5-CH_3THF (I,II). Other small amounts of radioactive species with both ^{14}C and ^{3}H isotopes were seen. No ^{3}H-scission product appeared. Tritiated water was also detected. DE-52 chromatography of 6-12hr urine sample of patient D.C. (Figure 6.4) showed similar pattern as that of 12-24hr urine sample of patient J.B. with the addition of a ^{3}H-scission product (III) identified as p-AcABG on Sephadex-G15 column (Figure 6.5). Qualitative analysis of both 0-6hr and 12-24hr urine samples of this patient (D.C.) was not done because there was not enough to be chromatographed.

Table 6.3 shows the radioactivity of each metabolite as the percentage of the dose. Table 6.4 summarizes the ratio of folic acid: 5-CH_3THF and in addition, the ratio of scission product: intact folate.

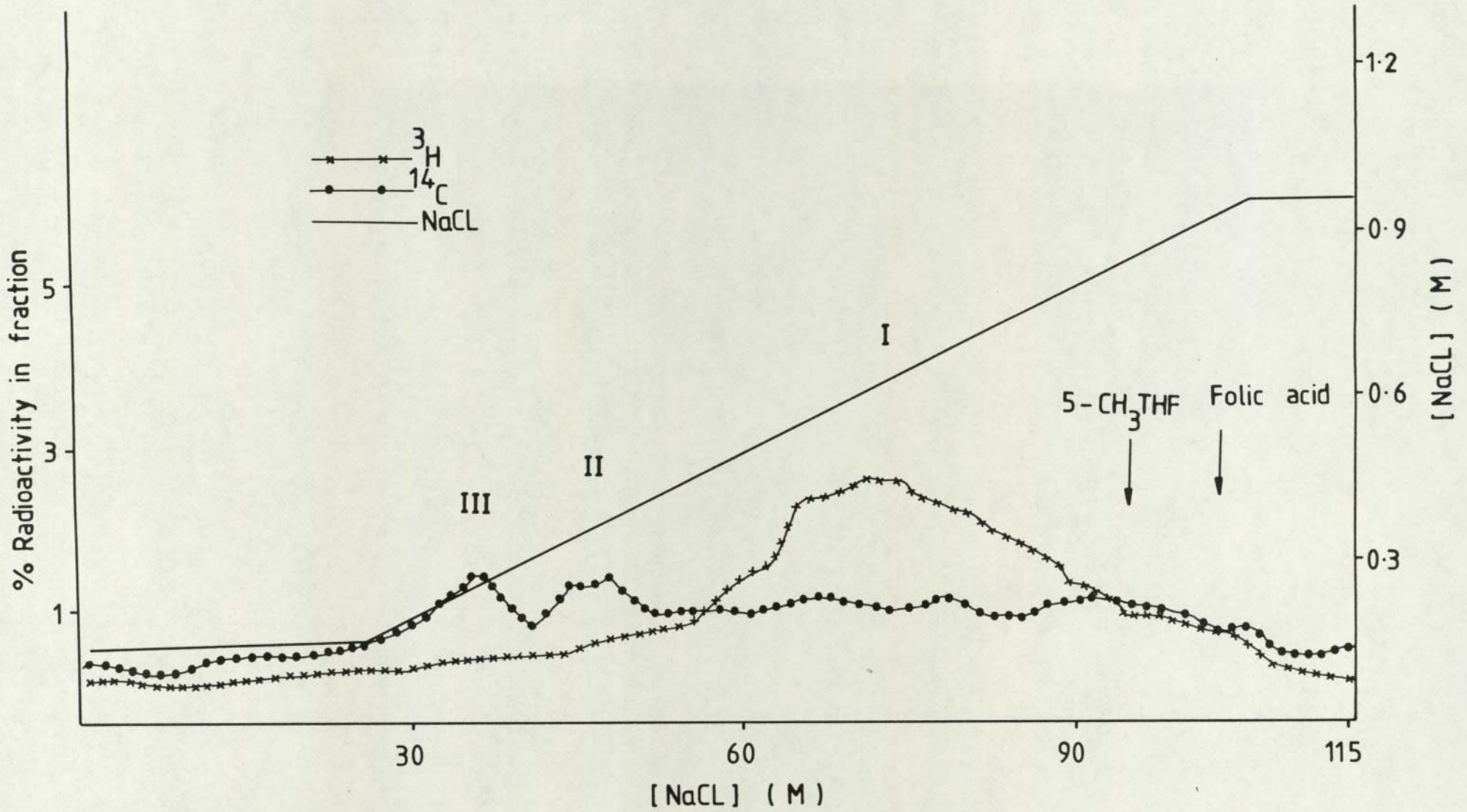


Fig. 6.1. DE-52 chromatography of 0-6hr urine sample of control patient (J.B.) after the oral administration of a mixture of ^{3}H + ^{14}C folic acid (50µg) plus the unlabelled 10-formylfolic acid (57µg).

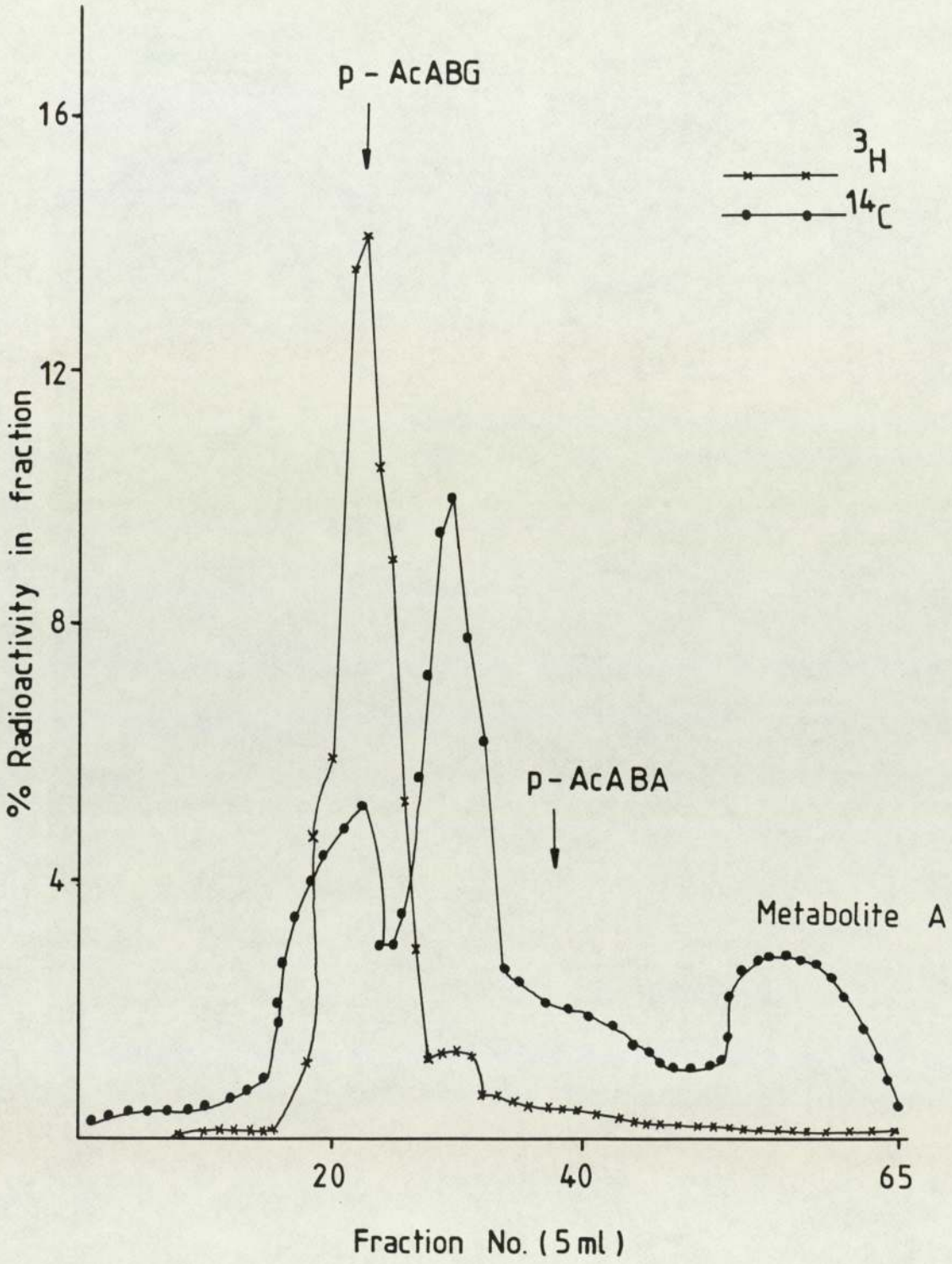


Fig. 6.2. Sephadex-G15 chromatography of the tritiated peak (I) from DE-52 chromatography of 0-6hr urine sample of a control patient (J.B.).

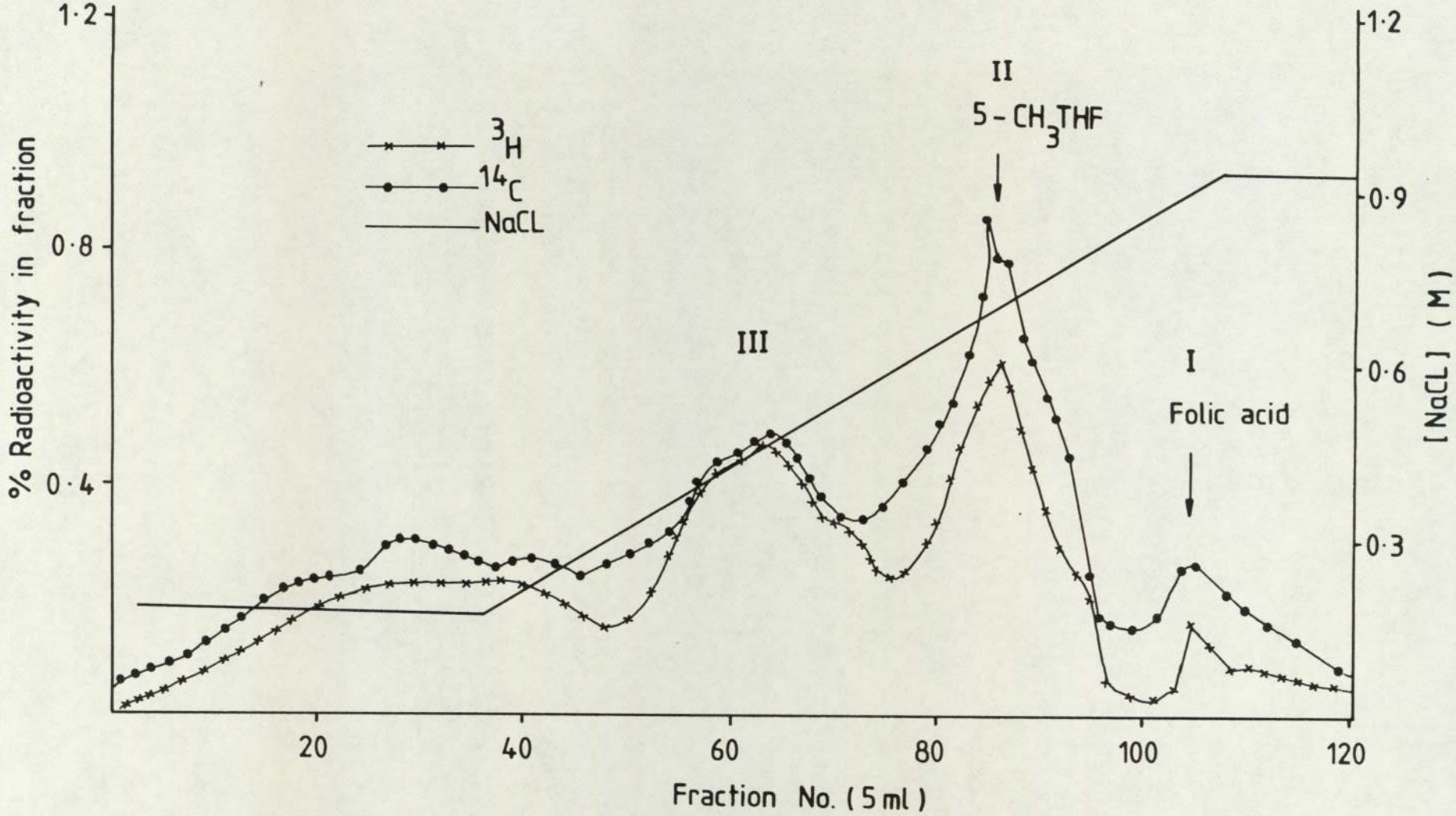


Fig. 6.3. DE-52 chromatography of 12-24hr urine sample of a control patient (J.B.) after the oral administration of a mixture of ^{3}H + ^{14}C folic acid (50µg) plus the unlabelled 10-formylfolic acid (57µg).

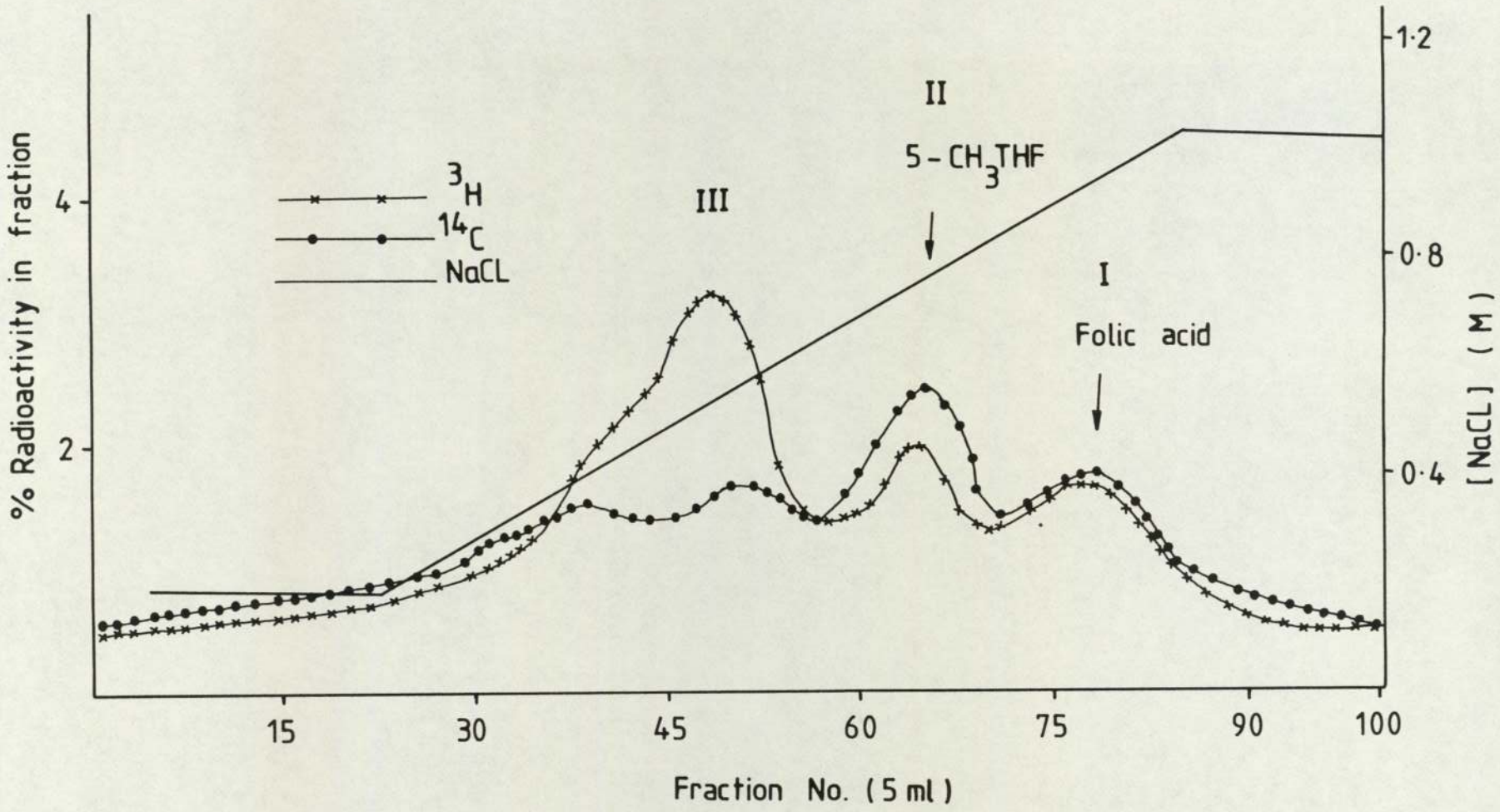


Fig. 6.4. DE-52 chromatography of the (6-12hr)urine sample of a control patient (D.C.) after the oral administration of a mixture of ^{3}H + ^{14}C folic acid (50µg) plus unlabelled 10-formylfolic acid (50µg).

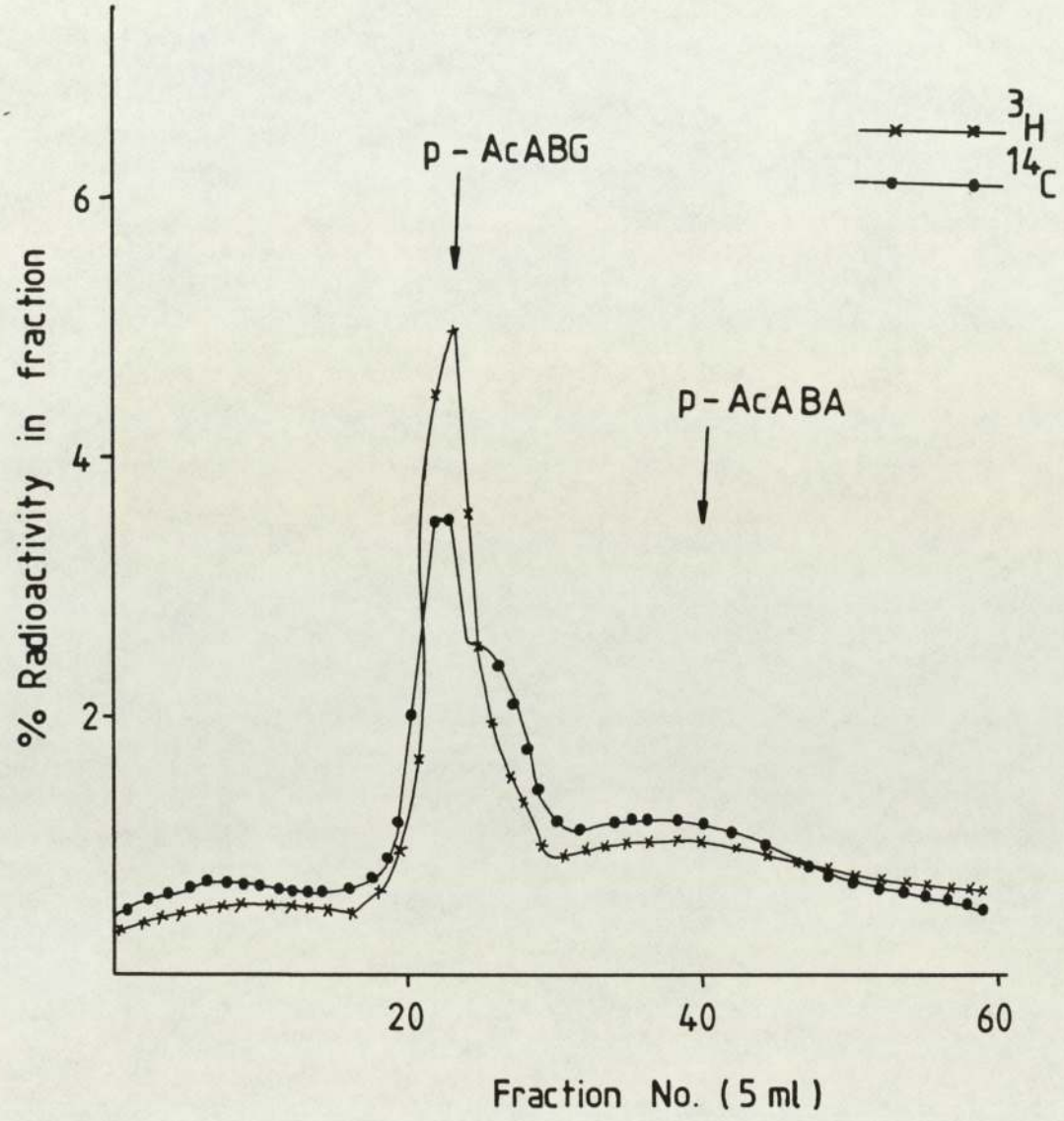


Fig. 6.5. Sephadex-G15 chromatography of the tritiated peak (III) from DE-52 chromatography of 6-12hr urine sample of a control patient (D.C.).

Table 6.1

Name	Age	Sex	Diagnosis	Therapy
J.B.	36	M	Acute myocardial infarction	None
D.C.	83	F	Stroke	None

Clinical details of patients studied

Table 6.2

Patient		Dose µg	0-6 hr		6-12 hr	
			^{3}H	^{14}C	^{3}H	^{14}C
J.B.	Control	50	1.75	0.18	0.52	0.07
D.C	Control	57	0.13	0.13	0.94	1.06

Patient		Dose µg	12-24 hr		0-24 hr	
			^{3}H	^{14}C	^{3}H	^{14}C
J.B.	Control	50	2.46	0.17	4.73	0.42
D.C.	Control	57	0.24	0.12	1.31	1.31

Urinary recovery of radioactivity after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (50µg) plus unlabelled (50µg, 57µg) 10-formylfolic acid. The results are expressed as the percentage of the dose recovered during three collection periods.

Table 6.3

Patient	Sample	Folic acid			
		^{3}H		^{14}C	
J.B. (Control)	0-6 hr	-		-	
	6-12 hr		N.d.		
	12-24 hr	0.10		0.08	
D.C. (Control)	0-6 hr		N.d.		
	6-12 hr	0.06		0.06	
	12-24 hr		N.d.		

Patient	Sample	5-CH_3THF			p-AcABG	p-AcABA	
		^{3}H		^{14}C	^{3}H		^{3}H
J.B. (Control)	0-6 hr	-		-	0.79		0.0
	6-12 hr		N.d.			N.d.	
	12-24 hr	0.62		0.04	-		-
D.C. (Control)	0-6 hr		N.d.			N.d.	
	6-12 hr	0.14		0.19	0.30		0.0
	12-24 hr		N.d.			N.d.	

The relative distribution of the major labelled metabolites appearing during three collection periods of urine sample after an oral administration of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid dose plus (50µg, 57µg) 10-formylfolic acid to control patients. The results are expressed as the percentage of the dose.

N.d. Not detected.

Table 6.4

Patient	Folic acid: 5-CH_3THF					
	0-6 hr		6-12 hr		12-24 hr	
	^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
J.B. (Control)	-	-	N.d.		0.62:1	2.0:1
D.C (Control)	N.d.		0.43:1	0.32:1	N.d.	

Patient	Scission product: Intact folate					
	0-6 hr		6-12 hr		12-24 hr	
	^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
J.B. (Control)	-	-	N.d.		-	-
D.C (Control)	N.d.		1.5:1	0.84:1	N.d.	

The ratio of folic acid: 5-CH_3THF and the ratio of scission product: intact folates excreted from three collection periods of urine sample after the administration of oral dose (50µg) of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid plus low dose (50µg , 57µg) of 10-formylfolic acid to control patients.

N.d = Not detected

CHAPTER 7

General discussion

This thesis describes studies of folate metabolism in man and the Syrian golden hamster and compares the results with those in other small mammals.

Considerable catabolism of folates into non-folate scission products is found in the hamster (this thesis), the rat, the man (Saleh, 1981) and guinea pig (Choolun, 1982). This could have a significant effect on the folate status of these mammals. In all these animals, folic acid is absorbed and reduced to tetrahydrofolates, a proportion is retained by the body, a proportion exchanges with tissue folate, a small amount passes into bile and a large amount into urine including scission products (figure 7.1). Pheasant, et al., (1981) suggested that two pools of folate are found in the rat; a short-term pool of folate monoglutamate and long-term pool as folate polyglutamate. Present evidence suggests that scission of folates most likely proceeds by oxidative scission of labile folate derivatives produced during the normal metabolic pathways (Saleh, et al., 1981) and does not involve specific enzymes.

Following the administration of mixed labelled [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid to normal Syrian hamsters, radioactivity was recovered in the urine, faeces, liver and kidney during 0-48hr and 0-72hr (Table 7.1), (Table 7.2). In all cases the radioactivity was recovered in the urine with an excess of excretion of ^{3}H over ^{14}C species during 0-72hr (19.4% ^{3}H, 12.28% ^{14}C) or in 0-48hr (23.79% ^{3}H, 11.24% ^{14}C). A similar excess of ^{3}H over ^{14}C radioactivity also was found in the faeces (0-48hr) in contrast to that of previous work on the rat. Several metabolites were detected in the hamster urine (Table 7.3). Several intact folates with a slightly higher ^{3}H/^{14}C ratio

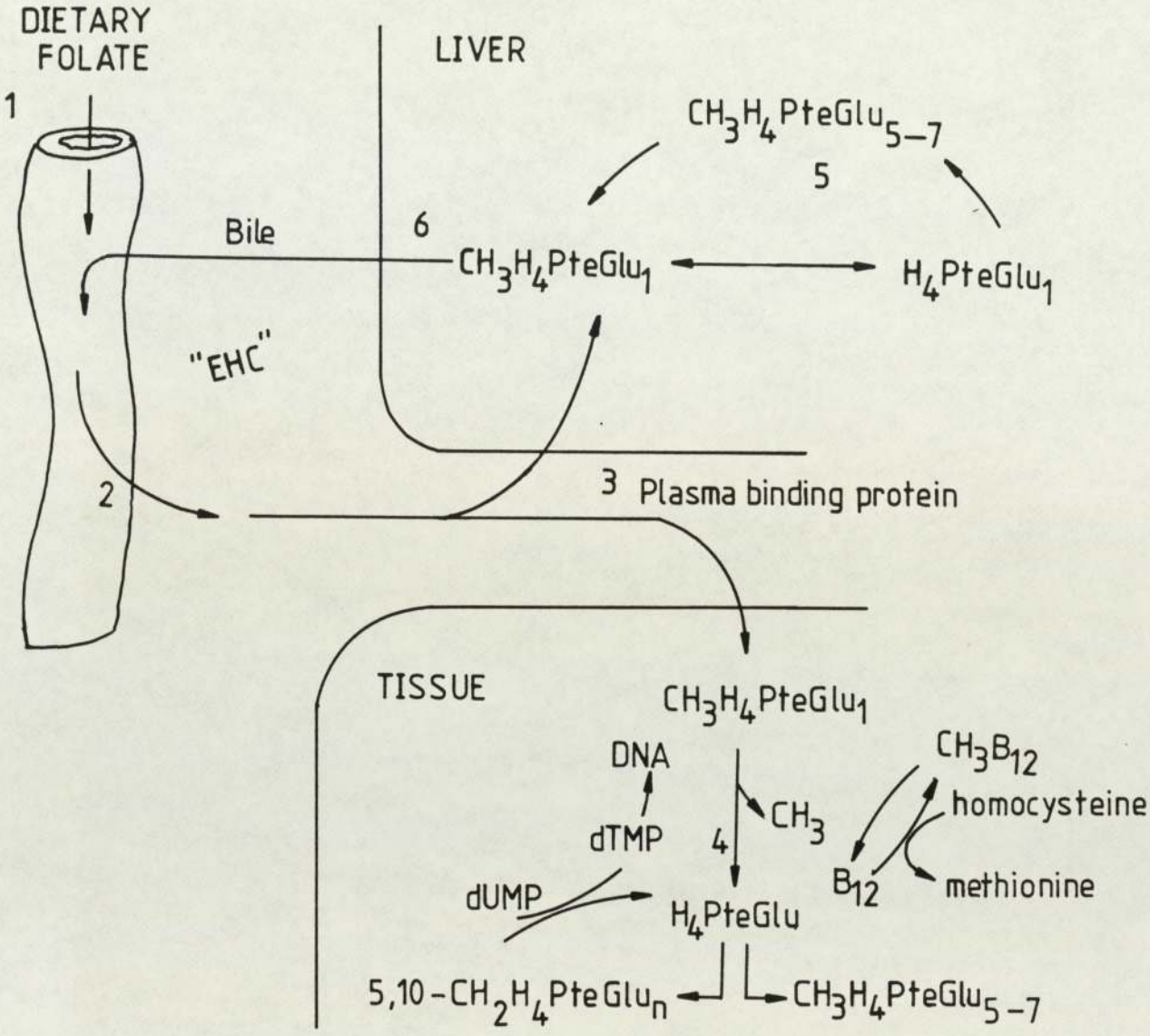


Fig. 7.1 Important elements in normal folate homeostasis include: (1) the level of folate in the diet, (2) intestinal absorption, (3) transport to liver and other tissues, (4) the intracellular metabolic steps involved in methionine and DNA synthesis, (5) liver uptake and incorporation into an intracellular folate polyglutamate ($CH_3H_4pteGlu_{5-7}$) pool, and (6) the transport of methyltetrahydrofolate monoglutamate ($CH_3H_4pteGlu_1$) in to bile for reabsorption and supply to tissues (the folate enterohepatic cycle - EHC).

Table 7.1

Animals No	Urine				Faeces	
	0-24hr		24-48hr		0-48hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters 7	17.59 ± 5.86	6.79 ± 4.08	6.03 ± 4.78	2.16 ± 1.44	22.12 ± 8.98	20.35 ± 7.79
Hamsters 7	20.53 ± 0.48	11.65 ± 1.86	3.42 ± 1.64	1.87 ± 0.87	25.81 ± 6.7	26.70 ± 4.57
Average	19.06 ± 3.17	9.22 ± 2.97	4.73 ± 3.21	2.02 ± 1.12	23.97 ± 7.84	23.53 ± 6.18

Animal No	Liver		Kidney		Total	
	0-48hr		0-48hr		0-48hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters 7	16.82 ± 5.99	15.41 ± 5.28	1.84 ± 0.70	1.72 ± 0.72	64.4	39.64
Hamsters 7	11.69 ± 5.99	10.24 ± 5.32	0.92 ± 0.22	0.74 ± 0.15	62.37	51.2
Average	14.26 ± 5.99	12.83 ± 5.3	1.38 ± 0.46	1.23 ± 0.44	63.39	45.42

Recovery of radioactivity in the urine, faeces and tissues for 0-48hr following the administration of 100µg/kg body wt of a mixture of labelled folic acid (^{14}C + ^{3}H) to each normal hamster as the control assay. The results are expressed as the percentage of the dose (mean ± standard deviation).

Table 7.2

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Urine	13.98 ± 3.21	9.04 ± 2.16	2.79 ± 1.29	1.76 ± 0.77	2.63 ± 1.19	1.48 ± 0.62	19.4	12.28
Faeces	15.28 ± 0.62	12.76 ± 4.62	5.7 ± 0.9	3.76 ± 1.42	3.84 ± 1.54	1.48 ± 0.31	24.82	18.19
Liver	10.89 ± 2.82	17.15 ± 6.2	13.11 ± 5.14	20.26 ± 5.81	11.61 ± 4.37	14.31 ± 3.75	11.61	14.31
Kidney	1.04 ± 0.13	1.3 ± 0.18	1.01 ± 0.34	1.38 ± 0.51	0.81 ± 0.11	1.11 ± 0.22	0.81	1.11
Total							56.63	45.89

Recovery of radioactivity in the urine, faeces and tissues, 24hr, 48hr, 72hr, after the administration of a mixture of ^{3}H + ^{14}C folic acid to normal Syrian golden hamsters. The results are expressed as percentage of the dose (mean ± standard deviation).

Table 7.3

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3 THF	3.17	3.26	0.44	0.53	0.17	0.19	3.78	3.98
10-CHOFA	-	1.05	-	0.31	-	0.27	-	1.63
p-AcABG	1.83	-	0.57	-	0.43	-	2.83	-
p-AcABA	4.23	-	0.89	-	0.52	-	5.64	-
Folate X	1.11	1.12	0.92	0.95	0.05	0.06	2.08	2.13
Folate Z	-	-	-	-	0.05	0.06	0.05	0.06
Metabolite (A)	-	1.05	-	0.22	-	0.11	-	1.38
Metabolite (B)	0.94	1.10	0.43	0.50	0.63	0.33	2.0	1.93
Urea	-	0.47	-	0.50	-	0.12	-	1.09
$^{3}H_2O$	0.47	-	0.53	-	0.76	-	1.76	-
Total	11.75	8.05	3.78	3.01	2.63	1.14	18.14	12.20

Metabolites present in the urine of normal hamsters following the administration of ^{14}C + ^{3}H folic acid. The results are expressed as the percentage of the dose.

than the folate administered were found. Scission products appeared labelled with ^{3}H only and with ^{14}C only as well as a metabolite with a much reduced ratio ^{3}H:^{14}C. The increased $^{3}H/^{14}C$ ratio in the intact folates may be due to differences in the handling of the two radiolabelled species in vivo, possibly, during absorption across the gut (Connor, 1979; Connor, et al., 1980). The intact folates included 5-CH_3THF (3.78%) and a small amount of 10-CHOTHF (1.63%) and an unidentified metabolite which chromatographed close to 5-CH_3THF on DE-52 column during 0-72hr. No folic acid was detected, presumably because of its complete reduction to tetrahydrofolates. The tritiated scission products consisted of two catabolites; p-acetamidobenzoyl-L-glutamate (2.83%) and p-acetamidobenzoic acid (5.64%) during 0-72hr. The formation of these folate catabolites suggested that, a possible catabolic route of folate would be cleavage of the $C_9 - N_{10}$ bond in tetrahydrofolate in the tissue to give pterin and p-aminobenzoyl-L-glutamate (Blair, 1958; Murphy, et al., 1976). p-Aminobenzoyl-L-glutamate has been shown to be metabolised to p-acetamidobenzoyl-L-glutamate (p-AcABG) in vivo (Murphy, et al., 1976) by addition of an acetyl group and to p-acetamidobenzoic acid (p-AcABA) (Pheasant, et al., 1981) by acetylation and loss of the glutamate moiety.

The considerable liver radioactivity consists solely of a ^{14}C and ^{3}H labelled species after 24hr which has a similar chromatographic behaviour to polyglutamates. This result agrees with many studies, for example, the early work of Noronha and Silverman (1962) and Bird et al., (1965) who found that in the liver a major portion of folate compounds is present as α-glutamyl

peptides (about 75% in rat liver). The radioactivity in the hamster liver reached a maximum in 72hr. The breakdown of liver folate may be by a pathway that involves chemical oxidation and fragmentation of the folate molecule (Blair, 1958; Murphy, et al., 1976; Pheasant, et al., 1981) to give p-AcABG in the urine. The production of radiolabelled p-AcABG in the urine is therefore related to the amount of radiolabelled folate polyglutamate retained in the liver. p-AcABA, the other ^{3}H metabolite found in the urine, is the catabolite of the monoglutamate pool (Pheasant, et al., 1981; Connor, 1979) (figure 7.2). Measurement of p-AcABG can be used to assess the breakdown of tissue polyglutamate (Saleh, et al., 1982). A good estimate of the extent of folate catabolism may be obtained using formula : $\frac{x}{y} \times 100$, where, x = the percentage of the dose excreted in the late urine sample (24-48hr) as p-AcABG, while y = the percentage of the dose retained in the liver at 24hr. The calculated rate of folate polyglutamate breakdown in the normal hamster is shown in Table 7.4. Since the distribution of ^{3}H-label in [3',5',7,9-^{3}H]-folate was 42.5% of tritium at the glutamyl containing fragment (3', 5' position), appropriate correction must, therefore, be made for this (Table 7.5).

In man, following the administration of a high dose of both labelled and unlabelled folic acid, similar results of urinary excretion radioactivity were found compared to the previous work on man (Pheasant, et al., 1979; Saleh, et al.,1980) (Table 7.6). Several metabolites were detected, folic acid, 5-CH_3THF and scission products in the three urine samples collected (0-6hr), (6-12hr) and (12-24hr) (Table 7.7). This confirmed that, the folic acid had undergone reduction as in the rat (Pheasant, et al., 1981). The major scission product was p-AcABG which particularly

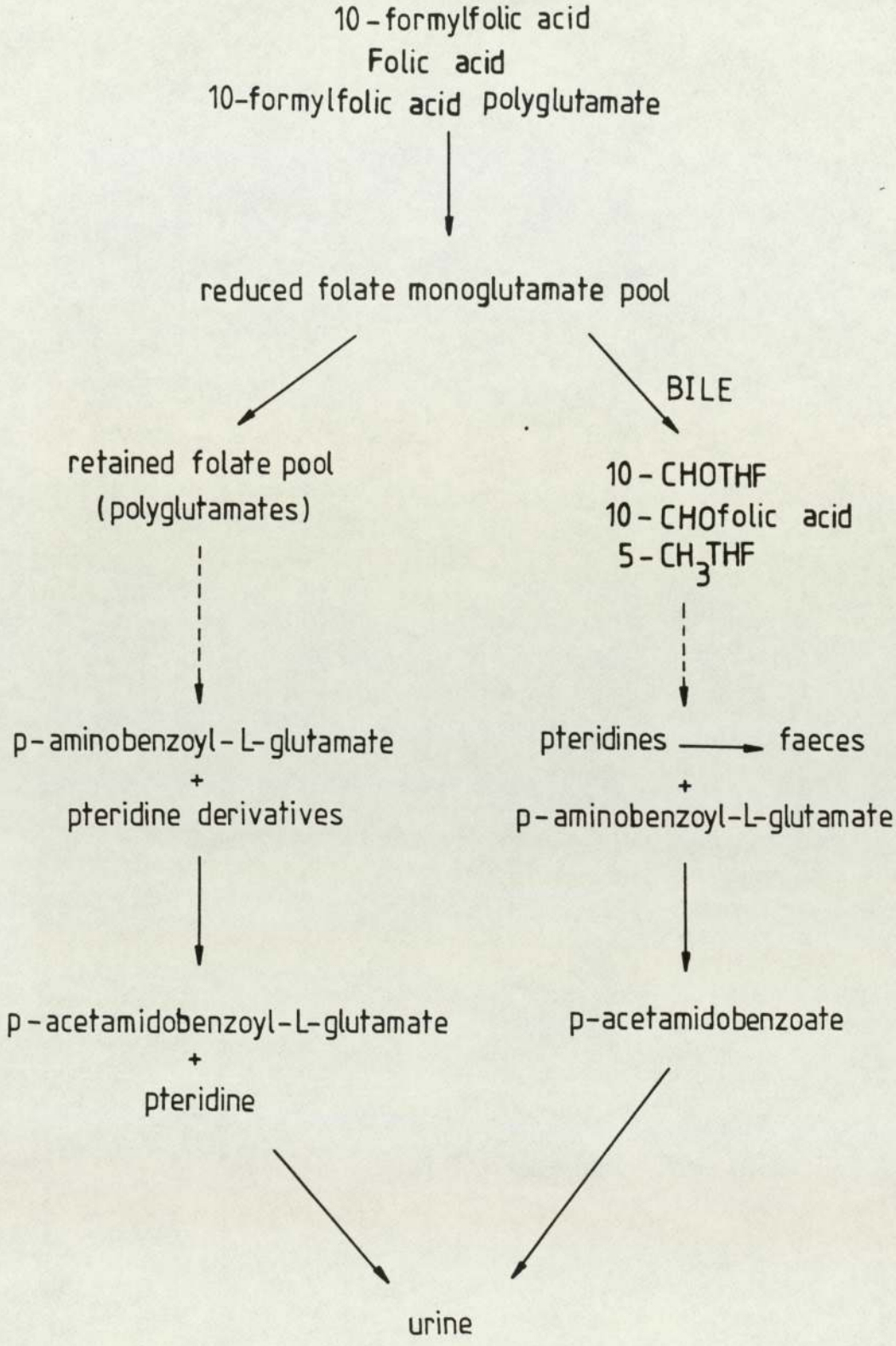


figure 7-2 : Suggested routes of catabolism in vivo

Table 7.4

Animal group	%^{3}H of the dose found in the liver after 24hr Y	%^{3}H dose excreted as p-AcABG at 24-48hr X	% retained radioactivity excreted as p-AcABG $\frac{X}{Y} \times 100$
Control hamster	10.89	0.57	5.23

Animal group	%^{3}H of dose found in the liver after 48hr Y	%^{3}H dose excreted as p-AcABG at 48-72hr X	% retained radioactivity excreted as p-AcABG $\frac{X}{Y} \times 100$
Control hamster	13.11	0.43	3.28

Excretion of catabolites of polyglutamate. The results are given as the percentage of liver radioactivity excreted as p-AcABG in normal golden hamsters receiving $^3H + ^{14}C$ folic acid orally during 24-72hr.

Table 7.5

Group	0-24hr (1)	0-24hr (2)	24-48hr (1)	24-48hr (2)	48-72hr (1)	48-72hr (2)	0-72hr (1)	0-72hr (2)
Control hamster	1.83 (4.3)	4.23 (10.0)	0.57 (1.3)	0.89 (2.1)	0.43 (1.0)	0.52 (1.2)	2.83 (6.6)	5.64 (13.3)
Xanthopterin treated hamster	1.69 (4.0)	4.21 (9.91)	0.6 (1.4)	1.56 (3.7)	0.24 (0.6)	0.52 (1.2)	2.53 (6.0)	6.29 (14.81)
Allopurinol treated hamster	3.76 (8.8)	9.97 (23.5)	1.67 (3.9)	3.53 (8.3)	0.46 (1.1)	0.75 (1.8)	5.89 (13.8)	14.25 (33.6)
Somatotropin treated hamster	4.68 (11.0)	8.36 (19.7)	1.34 (3.2)	2.13 (5.0)	0.41 (1.0)	0.58 (1.36)	6.43 (15.2)	11.07 (26.06)
Dihydro-orotic acid treated hamster	5.96 (14.0)	12.49 (29.4)	2.03 (4.8)	4.37 (10.3)	0.84 (2.0)	2.55 (6.0)	8.83 (20.8)	19.41 (45.7)

(1) = p-AcABG

(2) = p-AcABA

The excretion of the tritiated catabolites (p-AcABG and p-AcABA) in the urine of hamsters dosed with a mixture of ^{3}H + ^{14}C folic acid. The results are expressed as a percentage of the dose. (Values in parenthesis refer to the estimated % of the dose catabolised.)

Table 7.6

Patient	Case	0-6hr		6-12hr		12-24hr		0-24hr	
		^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
J.T.	Control	12.12	10.33	0.43	0.34	18.80	17.09	31.35	27.76
+ S.R.	Control	19.6	17.3	5.3	4.6	8.4	7.3	33.3	29.2
* N.R.	Control	8.6	8.4	24.0	22.2	4.7	3.1	37.3	33.7

\+ Data supplied by A.M. Saleh (1981)

* Data supplied by A.E. Pheasant

Urinary recovery of radioactivity of control patients after an oral administration of a mixture dose of ^{3}H + ^{14}C folic acid (5mg). The results are expressed as the percentage of the dose recovered during the three collection periods.

Table 7.7

Patient	Sample	Folic acid		5-CH_3 THF		p-AcABG	p-AcABA
		^3H	^{14}C	^3H	^{14}C	^3H	^3H
J.T.	0-6hr	9.13	8.36	0.57	0.48	1.61	0.00
(control)	6-12hr	N.d.	N.d.	N.d.	N.d.	N.d.	N.d.
	12-24hr	14.88	13.73	1.08	0.96	2.04	0.00
+S.R.	0-6hr	13.9	12.4	3.7	3.5	1.2	0.0
(control)	6-12hr	3.6	3.3	0.7	0.7	1.0	0.0
	12-24hr	4.6	4.3	1.3	1.3	1.0	0.9

The relative distribution of the major labelled metabolites appearing in the various of urine samples in control patients after the oral administration of a mixture of ^3H + ^{14}C folic acid (5 mg). The results are expressed as the percentage of the dose.

+ Data supplied by A.M. Saleh (1981)

N.d. = Not detected

appears during the first urine sample (0-6hr) and decreased with increasing time, while, p-AcABA was delayed in its excretion and was not always detected. Table 7.8 compares folate metabolism in the hamster with that in man and in the rat, guinea pig and scorbutic guinea pig while Table 7.9 summarizes the urinary recovery in total during 0-24hr.

Mechanism of scission

The mechanism of formation of the scission products is yet not known. However, Guest, et al., (1983) suggested that the oxidative cleavage of the C_9-N_{10} bond may be achieved by a chemical process and found no evidence for catalysis by the microsomal mixed function oxidase. Because the cleavage of folate monoglutamate may occur in the gut lumen following biliary excretion (Pheasant, et al., 1981; Hillman, et al., 1977), the urinary and faecal isotopic imbalances may result from the pterin derived fragment being less well absorbed than ^{3}H labelled fragment (Saleh, 1981).

7.1 The effect of xanthopterin on folic acid metabolism in hamsters

Table 7.1.1 shows the distribution of radioactivity in urine, faeces and tissues of the hamsters after the administration of xanthopterin by intraperitoneal injection. Urinary radioactivity excretion (17.84% ^{3}H, 8.69% ^{14}C) as percentage of the dose for 72hr was similar to the control hamsters (19.4% ^{3}H, 12.28% ^{14}C). Oral administration of xanthopterin also gave similar results (15.76% ^{3}H, 7.68% ^{14}C) (Table 7.1.2). Xanthopterin was probably poorly absorbed through the intestinal membrane and stomach. No kidney hypertrophy could be seen in either experiment. There was very little change in the scission products which consisted of both p-AcABA (6.29%) and p-AcABG (2.53%) (Table 7.1.3).

Table 7.8

Human and Animals	Condition	5-CH_3THF % of dose		^{3}H-p-AcABG % of dose	^{3}H-p-AcABA % of dose
		^{3}H	^{14}C		
* Man	normal	5.0	4.2	5.0	0.0
* Rat	normal	7.8	7.1	2.7	3.2
Hamster	normal	3.17	3.26	1.83	4.23
+ Guinea pig	normal	N.d		0.3	9.8
+ Guinea pig	scorbutic	N.d		0.7	7.7

* : Data supplied by A.M. Saleh (1981)

+ : Data supplied by R. Choolun (1982)

N.d = Not detected

The metabolites in urine of man, rat, hamster, guinea pig and scorbutic guinea pig after administering a mixture of ^{3}H + ^{14}C folic acid during 0-24hr. The results represent the percentage of the dose.

Table 7.9

Human and Animals	Condition	Dose	0-24hr	
			^{3}H	^{14}C
* Man	normal	5mg	39.2	31.8
* Rat	normal	100µg	25.9 ± 3.5	20.5 ± 2.6
Hamster	normal	100µg	13.98 ± 3.21	9.04 ± 2.16
+ Guinea pig	normal	400µg	24.1 ± 5.8	3.5 ± 1.5
+ Guinea pig	scorbutic	400µg	17.5 ± 8.7	4.7 ± 1.9

Recovery of radioactivity in urine of man, rat, hamster, guinea pig and scorbutic guinea pig after administering a mixture of ^{3}H + ^{14}C folic acid during 0-24hr. The results are expressed as the percentage of the dose (mean ± standard deviation).

* : Data supplied by A.M. Saleh (1981)

+ : Data supplied by R. Choolun (1982)

Table 7.1.1

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Urine	± 11.53 4.24	± 5.56 2.13	± 4.03 2.4	± 1.92 1.13	± 2.28 0.86	± 1.21 0.37	17.84	8.69
Faeces	± 6.31 3.69	± 6.1 4.18	± 4.37 2.39	± 3.48 1.81	± 3.66 1.68	± 3.49 2.83	14.64	13.07
Liver	± 6.81 1.63	± 11.3 1.39	± 7.4 1.81	± 11.0 3.17	± 7.43 3.72	± 8.24 3.66	7.43	8.24
Kidney	± 0.85 0.2	± 1.18 0.19	± 0.84 0.21	± 1.15 0.18	± 0.89 0.13	± 0.89 0.07	0.89	0.89
Total							40.8	30.89

Recovery of radioactivity in the urine, faeces and tissues of Syrian hamsters after the administration i.p. of xanthopterin (2.5mg/kg body wt.) following the administration of a mixture of ^{3}H + ^{14}C folic acid (100µg/Kg body wt.) for 24hr, 48hr and 72hr. The results are expressed as percentage of the dose. (Mean ± standard deviation.)

Table 7.1.2

Animals No	Urine				Faeces	
	0-24hr		24-48hr		0-48hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters 3	16.09 ± 1.4	5.88 ± 0.14	3.70 ± 0.36	1.67 ± 0.6	20.13 ± 0.36	14.65 ± 1.05
Hamsters 7	10.16 ± 3.12	6.37 ± 2.44	4.95 ± 1.90	3.68 ± 2.0	17.7 ± 11.60	20.3 ± 11.46
Hamsters 5	10.71 ± 7.24	4.44 ± 2.7	1.68 ± 0.35	1.00 ± 0.3	10.04 ± 1.17	10.09 ± 1.99
Average	12.32 ± 3.92	5.56 ± 1.76	3.44 ± 0.87	2.12 ± 0.97	15.96 ± 4.38	15.01 ± 4.84

Animal No	Liver		Kidney		Total	
	0-48hr		0-48hr		0-48hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Hamsters 3	15.06 ± 6.51	13.44 ± 0.61	2.17 ± 1.32	1.17 ± 0.67	57.15	36.81
Hamsters 7	14.52 ± 4.60	12.55 ± 3.69	2.01 ± 0.36	0.99 ± 0.10	49.34	43.89
Hamsters 5	11.27 ± 3.87	8.58 ± 3.75	1.51 ± 0.56	0.8 ± 0.34	35.21	24.91
Average	13.62 ± 4.99	11.52 ± 2.68	1.90 ± 0.75	0.99 ± 0.37	47.23	35.20

Recovery of radioactivity in the urine, faeces and tissues 0-48hr after the administration of 100mg/kg body wt of xanthopterin and 100µg/kg body wt of a mixture of labelled folic acid (^{3}H + ^{14}C) orally to normal Syrian hamsters. The results are expressed as the percentage of the dose. (Mean ± Standard deviation)

Table 7.1.3

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3 THF	1.11	1.37	0.3	0.28	0.14	0.15	1.55	1.8
10-CHOFA	-	0.67	-	0.28	-	0.16	-	1.11
p-AcABG	1.69	-	0.6	-	0.24	-	2.53	-
p-AcABA	4.21	-	1.56	-	0.52	-	6.29	-
Folate X	0.6	1.04	0.34	0.37	0.17	0.15	1.11	1.56
Folate Z	-	-	-	-	-	-	-	-
Metabolite (A)	-	1.06	-	0.04	-	0.12	-	1.22
Metabolite (B)	0.07	0.072	0.13	0.16	0.17	0.19	0.37	0.42
Urea	-	0.51	-	0.13	-	0.12	-	0.76
$^{3}H_2O$	0.3	-	0.2	-	0.27	-	0.77	-
Total	7.98	4.72	3.13	1.26	1.51	0.89	12.62	6.87

Metabolites present in the urine of normal hamsters following the administration of ^{14}C + ^{3}H folic acid after pretreatment with xanthopterin (i.p.). The results are expressed as the percentage of the dose.

The excretion of intact folates was similar to the control, except the level of 5-CH_3THF was lower than normal in the first urine sample. The percentage of p-AcABG scission product appearing in the urine related to the liver folate polyglutamate in the hamster after xanthopterin pretreatment was slightly higher (8.5%) compared to that of the control hamster (5%) (Table 7.1.4).

These findings demonstrate that, although xanthopterin has been claimed to cause rapid increase in kidney mass due to hyperplasia, by increasing the DNA and RNA content of the kidney at about four days after injection (Haddow, et al., 1972), it seems to have little effect on folate metabolism and slightly increases catabolism. In previous work Haddow, and co-workers (1972) suggested that xanthopterin induces hypertrophy of the kidney by inhibition of xanthine oxidase, so leading to the accumulation of abnormal amounts of xanthine and hypoxanthine and possibly increasing synthesis of nucleic acids. The failure to reduce folate catabolism by xanthopterin induced xanthine oxidase inhibition suggests xanthine oxidase is not directly involved in folate catabolism. Xanthopterin is oxidized by molecular oxygen and xanthine oxidase to yield leucopterin and superoxide anion ($O_2^{\cdot-}$) (figure 7.1.1). The latter may be involved in folate catabolism.

7.2 Effect of allopurinol on folate metabolism in hamsters

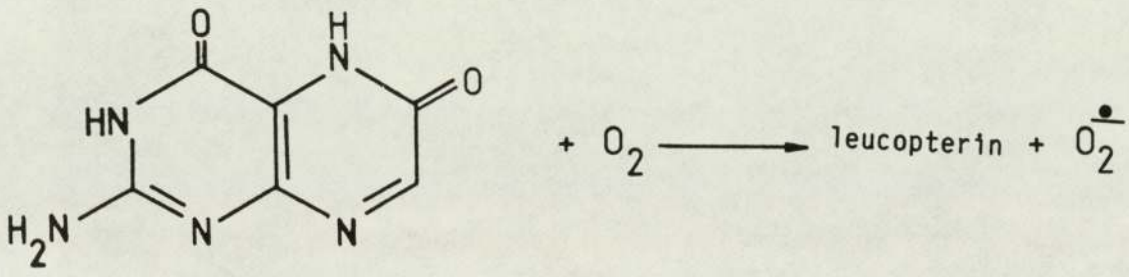
The administration of allopurinol greatly changed folic acid metabolism. The excretion of urinary radioactivity increased particularly in the first (18.86 ±6.6% ^3H, 6.48 ±2.95% ^{14}C) and second (7.48 ±1.6% ^3H, 2.54 ±0.41% ^{14}C) urine samples (Table 7.2.1). There was an increase in breakdown products and a decrease of intact folate species (Table 7.2.2) (14.25% p-AcABA and 5.89% p-AcABG of the dose) compared with the control hamsters (5.64% p-AcABA and 2.83% p-AcABG of the dose).

Table 7.1.4

Animal group	%^{3}H of the dose found in the liver after 24hr Y	%^{3}H dose excreted as p-AcABG at 24-48hr X	% retained radioactivity excreted as p-AcABG $\frac{X}{Y} \times 100$
Xanthopterin treated hamster	6.81	0.60	8.8

Animal group	%^{3}H of dose found in the liver after 48hr Y	%^{3}H dose excreted as p-AcABG at 48-72hr X	% retained radioactivity excreted as p-AcABG $\frac{X}{Y} \times 100$
Xanthopterin treated hamster	7.40	0.24	3.24

Excretion of catabolites of polyglutamate. The results are given as the percentage of liver radioactivity excreted as p-AcABG during 24-72hr of $^{3}H + ^{14}C$ folic acid and xanthopterin administration.



Xanthopterin

Figure 7-1-1 : Formation of superoxide anion ($O_2^{\bullet-}$) during the Xanthopterin oxidation

Table 7.2.1

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Urine	± 18.86 6.6	± 6.48 2.95	± 7.48 1.6	± 2.54 0.41	± 3.04 1.04	± 1.65 0.81	29.38	10.67
Faeces	± 9.77 3.83	± 16.81 2.53	± 8.41 2.4	+ 10.08 1.45	+ 4.6 1.4	± 6.7 2.6	22.78	33.59
Liver	± 14.10 6.17	± 18.59 6.89	± 12.85 4.15	± 19.02 7.44	± 14.15 2.39	± 19.02 4.50	14.15	19.02
Kidney	± 1.75 0.63	± 2.32 0.58	± 1.42 0.27	± 1.68 0.21	± 1.42 0.27	+ 2.5 0.89	1.42	2.5
Total							67.73	67.78

Recovery of radioactivity in the urine, faeces and tissues after the administration of 2mg of allopurinol to normal Syrian hamsters following the administration of ^{3}H + ^{14}C folic acid for 24hr, 48hr, 72hr. The results are expressed as the percentage of the dose. (Mean ± standard deviation.)

Table 7.2.2

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3THF	0.72	0.89	0.17	0.21	0.16	0.13	1.05	1.23
10-CHOFA	-	0.82	-	0.56	-	0.31	-	1.69
p-AcABG	3.76	-	1.67	-	0.46	-	5.89	-
p-AcABA	9.97	-	3.53	-	0.75	-	14.25	-
Folate X	1.86	1.81	0.44	0.46	0.17	0.16	2.47	2.43
Folate Z	-	-	-	-	-	-	-	-
Metabolite (A)	-	1.09	-	0.59	-	0.17	-	1.85
Metabolite (B)	-	-	-	-	0.34	0.45	0.34	0.45
Urea	-	0.46	-	0.44	-	0.17	-	1.07
$^{3}H_2O$	0.55	-	0.85	-	0.35	-	1.75	-
Total	16.86	5.07	6.66	2.26	2.23	1.39	25.75	8.72

Metabolites present in the urine of Syrian hamsters following the administration of (^{14}C + ^{3}H) folic acid after treated with allopurinol. The results are expressed as the percentage of the dose.

This increase was most marked in the first 24hr and returned to nearer normal on the later times. Allopurinol is rapidly absorbed after oral ingestion, and is a powerful inhibitor of xanthine oxidase enzyme (X.O.) in vitro and also in vivo as indicated by the accumulation of oxypurines in the urine (Rundles, et al., 1963) and blood (Klinenberg, et al., 1963). The inhibition of X.O. by allopurinol was found here not to reduce folate catabolism, thus the interaction of allopurinol with folate metabolism in Syrian hamster was not mediated by xanthine oxidase. As allopurinol is a substrate of X.O., it will also generate superoxide anion ($O_2^{\div}$). The increase of p-AcABG in 24-48hr urine sample had returned to normal in the third urine sample (48-72hr). Table 7.2.3 shows the rate of catabolism calculated by using the radioactivity retained in the liver at 24hr and 48hr. The hamster pre-treated with allopurinol shows a higher breakdown of polyglutamate than the normal hamsters.

7.3 Effect of dihydro-orotic acid on folate metabolism in hamsters

In the dihydro-orotic acid treated hamster, the excretion of ^{3}H-labelled fragments is even higher (28.24%) than in allopurinol treated hamster (20.14%) and normal hamster (8.47%) (Table 7.3.1). An increase in the excretion of radioactivity was recovered in the urine samples (Table 7.3.2). The rate of the breakdown of tissue polyglutamate calculated as before is given in Table 7.3.3. Dihydro-orotic acid appears to increase the rate of folate degradation. Dihydro-orotic acid is oxidized to orotate with oxygen as the electron acceptor (Reynolds, et al., 1955) in the presence of the enzyme dihydroorotic acid dehydrogenase (Webster, 1981; Lieberman and Kornberg, 1953). This enzyme has been found to produce superoxide anion (Forman and Kennedy,1975b) which could increase the formation of scission product.

Table 7.2.3

Animal group	%^{3}H of the dose found in the liver after 24hr Y	%^{3}H dose excreted as p-AcABG at 24-48hr X	% retained radioactivity excreted as p-AcABG $\frac{X}{Y} \times 100$
Allopurinol treated hamster	14.10	1.67	11.84

Animal group	%^{3}H of the dose found in the liver after 48hr Y	%^{3}H dose excreted as p-AcABG at 48-72hr X	% retained radioactivity excreted as p-AcABG $\frac{X}{Y} \times 100$
Allopurinol treated hamster	12.85	0.46	3.58

Excretion of catabolites of polyglutamate. The results are given as the percentage of liver radioactivity excreted as p-AcABG during 24-72hr of oral administration of allopurinol plus $^{3}H + ^{14}C$ folic acid in normal Syrian hamsters.

Table 7.3.1

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3THF	2.73	2.95	0.32	0.3	0.25	0.21	3.3	3.46
10-CHOFA	-	0.71	-	0.42	-	0.52	-	1.65
p-AcABG	5.96	-	2.03	-	0.84	-	8.83	-
p-AcABA	12.49	-	4.37	-	2.55	-	19.41	-
Folate X	1.00	0.80	0.21	0.15	-	-	1.21	0.95
Folate Z	-	-	-	-	-	-	-	-
Metabolite (A)	-	1.09	-	0.59	-	0.17	-	1.85
Metabolite (B)	1.36	0.98	0.40	0.99	0.31	0.72	2.07	2.69
Urea	-	0.63	-	0.56	-	0.23	-	1.42
$^{3}H_2O$	0.44	-	0.42	-	0.34	-	1.2	-
Total	23.98	7.16	7.75	3.01	4.29	1.85	36.02	12.02

Metabolites present in the urine of Syrian hamsters following the administration of (^{14}C + ^{3}H) folic acid after receiving dihydro-orotic acid. The results are expressed as the percentage of the dose.

Table 7.3.2

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Urine	24.66 ± 14.96	8.68 ± 6.64	8.2 ± 4.93	3.36 ± 1.12	4.5 ± 2.1	2.04 ± 1.02	37.36	14.08
Faeces	13.02 ± 7.92	15.49 ± 10.57	1.96 ± 1.04	2.47 ± 1.92	1.8 ± 0.91	1.98 ± 0.53	16.78	19.94
Liver	17.17 ± 7.65	24.24 ± 10.22	12.38 ± 5.3	16.36 ± 3.38	10.01 ± 1.69	15.56 ± 3.94	10.01	15.65
Kidney	1.5 ± 0.49	1.83 ± 0.84	1.26 ± 0.1	1.37 ± 0.24	1.49 ± 0.23	1.3 ± 0.48	1.49	1.3
Total							65.64	50.97

Recovery of radioactivity in the urine, faeces and tissues after the administration of 2.0mg of dihydro-orotic acid to normal Syrian hamsters following their receiving a mixture of ^{3}H + ^{14}C folic acid (100µg/kg body wt) for 24hr, 48hr and 72hr. The results are expressed as the percentage of the dose. (Mean ± standard deviation.)

Table 7.3.3

Animal group	%^{3}H of the dose found in the liver after 24hr Y	%^{3}H dose excreted as p-AcABG at 24-48hr X	% retained radio-activity excreted as p-AcABG $\frac{X}{Y} \times 100$
Dihydro-orotic acid treated hamster	17.17	2.03	11.82

Animal group	%^{3}H of the dose found in the liver after 48hr Y	%^{3}H dose excreted as p-AcABG at 48-72hr X	% retained radio-activity excreted as p-AcABG $\frac{X}{Y} \times 100$
Dihydro-orotic acid treated hamster	12.38	0.84	6.79

Excretion of catabolites of polyglutamate. The results are given as the percentage of liver radioactivity excreted as p-AcABG in normal Syrian hamsters receiving dihydro-orotic acid plus $^3H + ^{14}C$ folic acid orally.

7.4 Effect of growth hormone (somatotropin) on folate metabolism in hamsters

The action of somatotropin on folate metabolism was observed after the administration of this hormone to normal Syrian hamsters. It increased the urinary excretion of radioactivity in the first (0-24hr) (22.97 ±6.91% ^{3}H, 10.26 ±3.54% ^{14}C) and second (24-48hr) (5.63 ±1.82% ^{3}H, 2.95 ±0.88% ^{14}C) urine samples (Table 7.4.1).

The ^{3}H-labelled fragments were increased (17.5%) similarly to the other agents (allopurinol and dihydro-orotic acid) (Table 7.4.2). p-AcABA was the major catabolite during first (8.36%) and second (2.13%) urine samples. p-AcABG was increased in the second urine sample (24-48hr) and was related to folate polyglutamate radioactivity retained in the liver (Table 7.4.3). The rate of folate polyglutamate breakdown measured as the rates at 24-48hr and 48-72hr were 9.74% and 4.33% respectively in hamsters pre-treated with somatrotropin and 5.23% and 3.28% in control hamsters. Somatotropin may increase purine formation and therefore could increase superoxide anion formation from the xanthine oxidase enzyme system and increase breakdown.

Mechanism of scission

These findings show that xanthopterin, allopurinol, dihydro-orotic acid and somatotropin all cause an increase in the degradation of folate molecule through the oxidative cleavage of C_9-N_{10} bond. Xanthine oxidase may be the source of $O_2^{\cdot-}$ which can then oxidize the labile folate derivatives, dihydrofolate (DHF) and tetrahydrofolate (THF) (Figure 7.4.1) (see chapter 1 for more details).

Table 7.4.1

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Urine	± 22.97 ± 6.91	± 10.26 ± 3.54	± 5.63 ± 1.82	± 2.95 ± 0.88	± 2.91 ± 0.99	± 1.68 ± 0.52	31.51	14.89
Faeces	± 8.42 ± 2.08	± 10.91 ± 1.86	± 4.76 ± 1.72	± 5.61 ± 3.56	± 3.3 ± 0.73	± 1.26 ± 0.17	16.48	17.78
Liver	± 13.76 ± 3.5	± 18.99 ± 2.58	± 9.46 ± 1.14	± 15.63 ± 3.31	± 10.72 ± 3.66	± 17.54 ± 3.39	10.72	17.54
Kidney	± 1.66 ± 0.16	± 2.09 ± 0.12	± 1.34 ± 0.14	± 1.67 ± 0.35	± 1.63 ± 0.32	± 1.84 ± 0.29	1.63	1.84
Total							60.34	52.05

Recovery of radioactivity in the urine, faeces and tissues after receiving 0.1 i.u. of somatotropin by normal Syrian hamsters and the administration of a mixture of ^{14}C + ^{3}H folic acid for 24hr, 48hr and 72hr. The results are expressed as the percentage of the dose. (Mean ± standard deviation.)

Table 7.4.2

	0-24hr		24-48hr		48-72hr		0-72hr	
	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%	^{3}H%	^{14}C%
Folic acid	-	-	-	-	-	-	-	-
5-CH_3 THF	6.13	5.23	0.51	0.48	0.28	0.31	6.92	6.02
10-CHOFA	-	0.94	-	0.77	-	0.21	-	1.92
p-AcABG	4.68	-	1.34	-	0.41	-	6.43	-
p-AcABA	8.36	-	2.13	-	0.58	-	11.07	-
Folate X	-	-	-	-	-	-	-	-
Folate Z	-	-	-	-	-	-	-	-
Metabolite (A)	-	1.27	-	1.77	-	0.25	-	3.29
Metabolite (B)	0.76	1.40	0.38	0.3	0.32	0.52	1.46	2.22
Urea	-	0.41	-	0.11	-	0.12	-	0.64
$^{3}H_2O$	0.62	-	0.25	-	0.63	-	1.5	-
Total	20.55	9.25	4.61	3.43	2.22	1.41	27.38	14.09

Metabolites present in the urine of Syrian hamsters following the administration of (^{14}C + ^{3}H) folic acid after treated with somatotropin. The results are expressed as the percentage of the dose.

Table 7.4.3

Animal group	%^{3}H of the dose found in the liver after 24hr Y	%^{3}H dose excreted as p-AcABG at 24-48hr X	% retained radioactivity excreted as p-AcABG $\frac{X}{Y} \times 100$
Somatotropin treated hamster	13.76	1.34	9.74

Animal group	%^{3}H of the dose found in the liver after 48hr Y	%^{3}H dose excreted as p-AcABG at 48-72hr X	% retained radioactivity excreted as p-AcABG $\frac{X}{Y} \times 100$
Somatotropin treated hamster	9.46	0.41	4.33

Excretion of catabolites of polyglutamate. The results are given as the percentage of liver radioactivity excreted as p-AcABG in normal Syrian hamsters receiving somatotropin (growth hormone) plus folic acid orally.

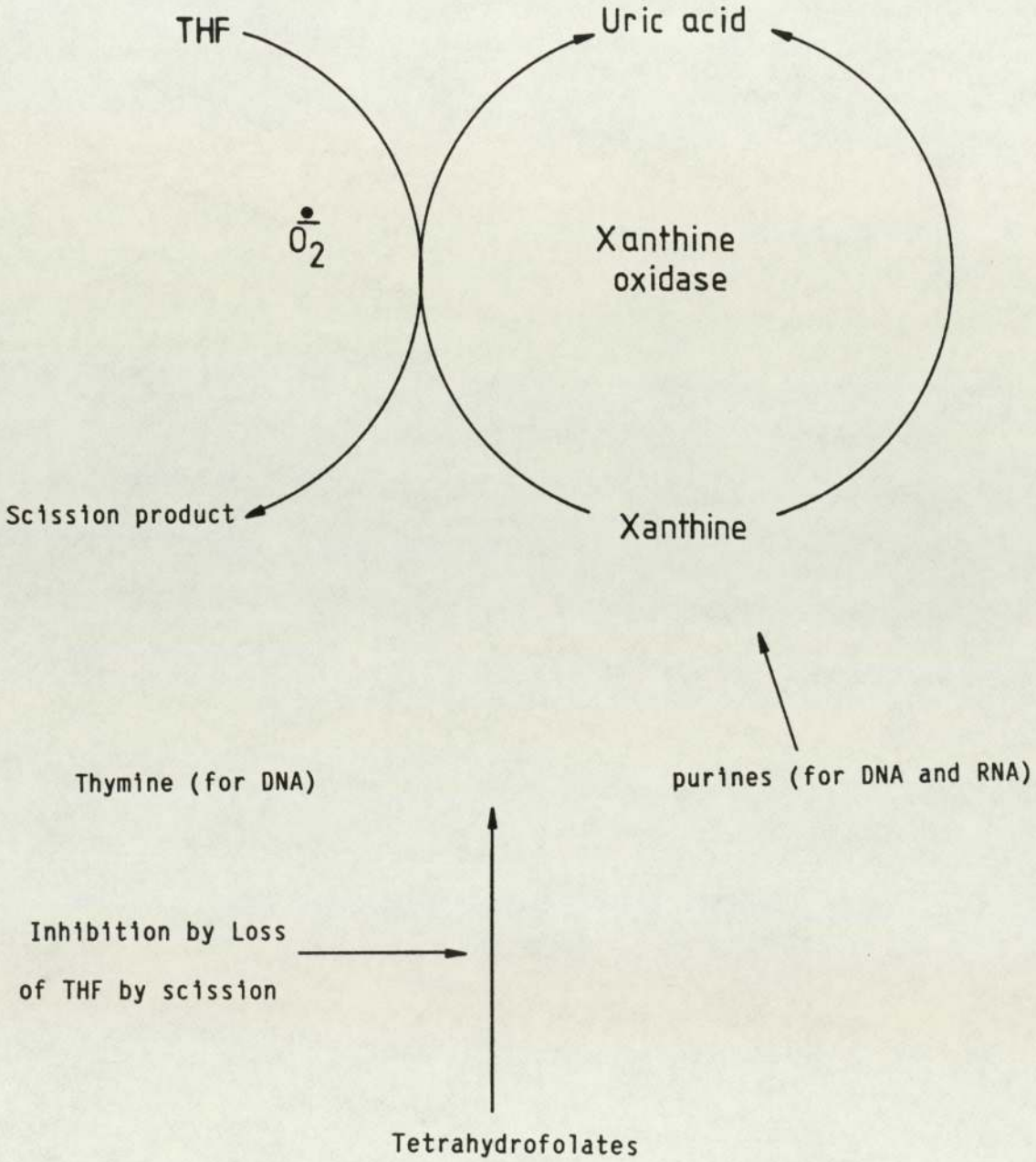


Figure 7-4-1 : The mechanism of the breakdown <u>in vivo</u>

Table 7.4.4 shows a higher breakdown of polyglutamate in hamsters pre-treated with all four agents related to control hamsters. p-AcABA was a major catabolite excreted particularly in the first urine sample (0-24hr) of hamsters pre-treated with xanthopterin, allopurinol, dihydro-orotic acid and somatotropin, and it was excreted in the second urine sample (24-48hr) also in higher amounts compared with control hamsters. There are several explanations for this appearance of p-AcABA in the later urine sample. It is thought to be due to the formation of p-AcABA from p-AcABG (the major breakdown of folate polyglutamate of the liver) before the latter excreted in the urine, therefore, p-AcABA excretion can also be used as a measure of liver polyglutamate scission (Table 7.4.5). The results presented in this thesis support the hypothesis of an oxidative breakdown process of tissue folates and suggested that the rate at which this breakdown process occurs could have a significant effect on the folate status in the animals.

7.5 Effect of malignant disease on folate metabolism in man

The urinary recovery of patients suffering from malignant disease is shown in Table 7.5.1 after the administration of a high dose of labelled and unlabelled folic acid. It was found that the excretion of urinary radioactivity in patient F.K. was much lower (1.07% ^{3}H, 0.76% ^{14}C) than in patient L.K. (19.05% ^{3}H, 17.9% ^{14}C) during 0-24hr urine samples. The decrease of urinary radioactivity may be due to the uptake of folate and formation of polyglutamate by a large tumour mass (Saleh, et al., 1980; Saleh, 1981). The folate present in the urine had higher $^{3}H/^{14}C$ ratio than the administered folic acid. This could be due to the secondary isotope effect seen in the handling of labelled folic acid by rat (Connor, et al., 1980) which may also occur in man.

Table 7.4.4

Animal group	%^{3}H of the dose found in the liver after 24hr Y	%^{3}H dose excreted as p-AcABG at 24-48hr X	% retained radio-activity excreted as p-AcABG $\frac{X}{Y} \times 100$
Control hamster	10.89	0.57	5.23
Xanthopterin treated hamster	6.81	0.60	8.8
Allopurinol treated hamster	14.10	1.67	11.84
Somatotropin treated hamster	13.76	1.34	9.74
Dihydro-orotic acid treated hamster	17.17	2.03	11.82

Animal group	%^{3}H of the dose found in the liver after 48hr Y	%^{3}H dose excreted as p-AcABG at 48-72hr X	% retained radio-activity excreted as p-AcABG $\frac{X}{Y} \times 100$
Control hamster	13.11	0.43	3.28
Xanthopterin treated hamster	7.40	0.24	3.24
Allopurinol treated hamster	12.85	0.46	3.58
Somatotropin treated hamster	9.46	0.41	4.33
Dihydro-orotic acid treated hamster	12.38	0.84	6.79

Excretion of catabolites of polyglutamate. The results are given as the percentage of liver radioactivity excreted as p-AcABG.

Table 7.4.5

Animal group	%^{3}H of the dose found in the liver after 24hr Y	%^{3}H dose excreted as p-AcABA at 24-48hr X	% retained radio-activity excreted as p-AcABA $\frac{X}{Y} \times 100$
Control hamster	10.89	0.89	8.17
Xanthopterin treated hamster	6.81	1.56	22.91
Allopurinol treated hamster	14.10	3.53	25.04
Somatotropin treated hamster	13.76	2.13	15.48
Dihydro-orotic acid treated hamster	17.17	4.37	25.45

Animal group	%^{3}H of the dose found in the liver after 48hr Y	%^{3}H dose excreted as p-AcABA at 48-72hr X	% retained radio-activity excreted as p-AcABA $\frac{X}{Y} \times 100$
Control hamster	13.11	0.52	3.97
Xanthopterin treated hamster	7.40	0.52	7.03
Allopurinol treated hamster	12.85	0.72	5.37
Somatotropin treated hamster	9.46	0.58	6.13
Dihydro-orotic acid treated hamster	12.38	2.55	20.60

Excretion of the catabolites of folate polyglutamate. The results are given as the percentage of liver radioactivity excreted as P-AcABA.

Table 7.5.1

Patient	Case	0-6hr		6-12hr	
		^{3}H	^{14}C	^{3}H	^{14}C
L.K.	Cancer	11.50	10.50	5.59	5.38
F.K.	Cancer (large tumour mass)	0.01	0.01	0.01	0.01
*H.P.	Cancer	14.7	13.2	3.6	3.1

Patient	Case	12-24hr		0-24hr	
		^{3}H	^{14}C	^{3}H	^{14}C
L.K.	Cancer	1.96	2.11	19.05	17.9
F.K.	Cancer (large tumour mass)	1.05	0.74	1.07	0.76
*H.P.	Cancer	0.9	0.5	19.2	16.8

* Data supplied by A.M. Saleh (1981)

Urinary recovery of radioactivity of cancer patients after an oral dose of ^{3}H + ^{14}C folic acid (5 mg). The results are expressed as the percentage of the dose recovered during the three collection periods (0-6hr), (6-12hr) and (12-24hr).

The level of the metabolites were varied compared with control patient (Table 7.5.2). The decrease in unchanged folic acid was probably due to increased tumour metabolism because of the large requirement of these cells for folate (Poirier, 1973; Taguchi, et al., 1974). The major catabolite was p-AcABG; in addition p-AcABA was sometimes detected and may be derived from p-AcABG by further catabolism. These findings confirmed previous results on man by Saleh, et al., (1980) and on rat by Connor and Blair (1979). The lower level of labelled p-AcABG excreted in the urine of rat in the presence of tumours has also been observed in this case in man with malignant disease. This could be due to the anoxia of solid tumours (Saleh, et al., 1982). Table 7.5.3 shows the comparison between normal man and normal rat with man with tumour masses and rat with tumour masses. The interesting feature of these observations is, in both cases, the amount of scission product is substantially reduced.

7.6 Effect of low dose in man

Table 7.6.1 shows the decrease in the urinary radioactivity excretion in man receiving low dose (50μg) of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid. This level is probably insufficient to exceed the renal threshold value for the folates (Johns, et al., 1961). The scission products excreted have much lower renal threshold value, hence, their dominance of these urine samples (Pheasant, et al., 1979). Table 7.6.2 shows the absence of unchanged folic acid and a little excretion of 5-CH_3THF appeared which apparently increased during the later urine samples. These results confirmed the observation of Pheasant, et al., (1979) and Saleh, (1981) on man. p-AcABG, the only catabolite which appeared may be due to the breakdown of folate polyglutamate in the tissues.

Table 7.5.2

Patient	Case	Sample	Folic acid ^{3}H	Folic acid ^{14}C	5-CH_3THF ^{3}H	5-CH_3THF ^{14}C
L.K.	Cancer	0-6hr	7.46	6.80	1.90	1.71
		6-12hr	3.17	2.81	1.04	1.29
		12-24hr	0.31	0.31	0.6	0.91
F.K.	Cancer (with large tumour mass)	0-6hr 6-12hr 12-24hr	N.d.	N.d.	N.d.	N.d.
*H.P.	Cancer	0-6hr	11.3	10.4	1.4	1.1
		6-12hr	1.7	1.7	0.6	0.7
		12-24hr	0.1	0.1	0.2	0.1

Patient	Case	Sample	p-AcABG ^{3}H	p-AcABA ^{3}H
L.K.	Cancer	0-6hr	1.81	0.0
		6-12hr	1.34	0.3
		12-24hr	0.85	0.7
F.K.	Cancer (with large tumour mass)	0-6hr 6-12hr 12-24hr	N.d.	N.d.
*H.P.	Cancer	0-6hr	0.7	0.0
		6-12hr	0.3	0.3
		12-24hr	0.2	0.1

The relative distribution of the major labelled metabolites appearing in the various of urine samples in cancer patients after the oral dose of a mixture of ^{3}H + ^{14}C folic acid and unlabelled folic acid (5 mg). The results are expressed as the percentage of the dose.

N.d. = Not detected
* : Data supplied by A.M. Saleh (1981)

* Table 7.5.3

Human and Animals	Condition	5-CH_3THF % of dose		^{3}H-p-AcABG % of dose	^{3}H-p-AcABA % of dose
		^{3}H	^{14}C		
Man	Control	5.0	4.2	5.0	0.0
Man	With tumour	3.8	3.7	0.9	0.1
Rat	Normal	7.8	7.1	2.7	3.2
Rat	With tumour	3.5	3.0	1.4	2.0

The percentage of dose as each metabolite in control patient and patient with tumour plus normal rat and rat with turmour during 0-24hr after administering a mixture of ^{3}H + ^{14}C folic acid.

* Data supplied by A.M. Saleh (1981)

Table 7.6.1

Patient	Case	Dose	0-6hr		6-12hr	
			^{3}H	^{14}C	^{3}H	^{14}C
B.F.	Control	50µg	1.23	0.6	1.02	0.79
+A.G.	Control	57µg	1.0	0.6	0.9	0.6
+P.H.	Control	57µg	1.8	1.1	0.6	0.5

Patient	Case	Dose	12-24hr		0-24hr	
			^{3}H	^{14}C	^{3}H	^{14}C
B.F.	Control	50µg	0.78	0.62	3.03	2.01
+A.G.	Control	57µg	0.3	0.2	2.2	1.4
+P.H.	Control	57µg	0.7	0.5	3.1	2.1

+ Data supplied by A.M. Saleh (1981)

Urinary recovery of radioactivity of control patients after an oral dose administration of low dose of a mixture of ^{3}H + ^{14}C folic acid (50µg, 57µg). The results are expressed as the percentage of the dose recovered during 0-24 hours.

Table 7.6.2

Patient	Dose	Sample	Folic acid ^{3}H	Folic acid ^{14}C
B.F. (Control)	50µg	0-6hr	0.0	0.0
		6-12hr	0.0	0.0
		12-24hr	N.d.	

Patient	Dose	Sample	5-CH_3THF ^{3}H	5-CH_3THF ^{14}C	p-AcABG ^{3}H	p-AcABA ^{3}H
B.F. (Control)	50µg	0-6hr	0.18	0.08	1.02	0.0
		6-12hr	0.27	0.23	0.69	0.0
		12-24hr	N.d.		N.d.	

The relative distribution of the major labelled metabolites appearing on the various urine samples in a control patient receiving low dose of a mixture of ^{3}H + ^{14}C folic acid (50µg) orally during 0-24hr. The results are expressed as the percentage of the dose.

N.d. = Not detected

7.7 Effect of alcohol on the folate metabolism in man

In this study the effect of alcohol was investigated in a patient who commonly drank 10 pint/day of beer and 3 bottles of whisky/week. Following the administration of high dose of labelled and unlabelled folic acid, there was a marked increase in the excretion of urinary radioactivity recovered (56.03% ^{3}H, 50.3% ^{14}C) compared with non-alcoholic patient (31.35% ^{3}H, 27.76% ^{14}C) (Table 7.7.1). An increase in the excretion of intact folate metabolites and scission products in the urine was observed (Table 7.7.2) indicating, that, the alcohol has an impact on folate metabolism *in vivo*.

7.8 Effect of 10-formylfolate on the folate metabolism in man

Table 7.8.1 shows the low excretion of radioactivity recovered in the urine of non-malignant patients receiving low dose (57µg, 50µg) of unlabelled 10-formylfolate (10-CHOFA) plus 50µg of (^{3}H, ^{14}C)-folic acid. Little intact folates were excreted with the scission product p-AcABG (Table 7.8.2). The appearance of some unmetabolised folic acid after low dose suggests that 10-CHOFA, a potent inhibitor of dihydrofolate reductase *in vitro*, is effective *in vivo* at low concentration. The catabolism can evidently occur possibly by gut microflora or gut enzyme (Connor and Blair, 1979). Studies on man by Saleh, *et al*., (1982) have found that a high dose of 10-CHOFA also effectively blocks the reduction of folic acid *in vivo*. Figure 7.8.1 summarizes the inhibition of dihydrofolate reductase by formylfolate after the conversion of 10-formyltetrahydrofolate in the presence of superoxide anion ($O_2^{\cdot-}$).

Table 7.7.1

Patient	Case	0-6hr ^{3}H	0-6hr ^{14}C	6-12hr ^{3}H	6-12hr ^{14}C
S.C.	Control (Alcoholic)	31.36	27.79	20.42	18.66

Patient	Case	12-24hr ^{3}H	12-24hr ^{14}C	0-24hr ^{3}H	0-24hr ^{14}C
S.C.	Control (Alcoholic)	4.25	3.94	56.03	50.3

Urinary recovery of radioactivity after an oral dose of ^{3}H + ^{14}C folic acid (5 mg) to control patient (alcoholism). The results are expressed as the percentage of the dose recovered during the time periods (0-6hr), (6-12hr) and (12-24hr).

Table 7.7.2

Patient	Case	Sample	Folic acid ^{3}H	Folic acid ^{14}C
S.C.	Control (Alcoholic)	0-6hr	23.09	19.97
		6-12hr	14.92	13.38
		12-24hr	2.03	1.83

Patient	Case	Sample	5-CH_3THF ^{3}H	5-CH_3THF ^{14}C	p-AcABG ^{3}H	p-AcABA ^{3}H
S.C.	Control (Alcoholic)	0-6hr	5.25	4.68	2.88	0.0
		6-12hr	3.03	2.82	2.03	0.78
		12-24hr	1.16	1.07	0.54	0.05

The relative distribution of the major labelled metabolites appearing in the various of urine samples in a control patient (alcoholism) after an oral dose of a mixture of ^{3}H + ^{14}C folic acid (5 mg). The results are expressed as the percentage of the dose.

Table 7.8.1

Patient	Case	Dose	0-6hr		6-12hr	
			^{3}H	^{14}C	^{3}H	^{14}C
J.B.	Control	50µg	1.75	0.18	0.52	0.07
D.C.	Control	57µg	0.13	0.13	0.94	1.06

Patient	Case	Dose	12-24hr		0-24hr	
			^{3}H	^{14}C	^{3}H	^{14}C
J.B.	Control	50µg	2.46	0.17	4.73	0.42
D.C.	Control	57µg	0.24	0.12	1.31	1.31

Urinary recovery of radioactivity after an oral administration of a mixture of ^{3}H + ^{14}C folic acid plus low dose of unlabelled 10-CHOFA (50µg, 57µg) to control patients. The results are expressed as the percentage of the dose recovered during (0-6hr), (6-12hr) and (12-24hr).

Table 7.8.2

Patient	Case	Dose	Sample	Folic acid ^{3}H	Folic acid ^{14}C
S.C.	Control	50µg	0-6hr	-	N.d.
			6-12hr		N.d.
			12-24hr	0.10	0.08
D.C.	Control	57µg	0-6hr		N.d.
			6-12hr	0.06	0.06
			12-24hr		N.d.

Patient	Case	Dose	Sample	5-CH_3THF ^{3}H	5-CH_3THF ^{14}C	p-AcABG ^{3}H	p-AcABA ^{3}H
S.C.	Control	50µg	0-6hr	N.d.		0.79	0.0
			6-12hr	N.d.		N.d.	
			12-24hr	0.62	0.04	N.d.	
D.C.	Control	57µg	0-6hr	N.d.		N.d.	
			6-12hr	0.14	0.19	0.30	0.0
			12-24hr	N.d.		N.d.	

The relative distribution of the major labelled metabolites appearing in the various of urine samples in control patient receiving an oral dose of a mixture of ^{3}H + ^{14}C folic acid plus low dose of unlabelled 10-CHOFA (50µg, 57µg). The results are expressed as the percentage of the dose.

N.d. = Not detected

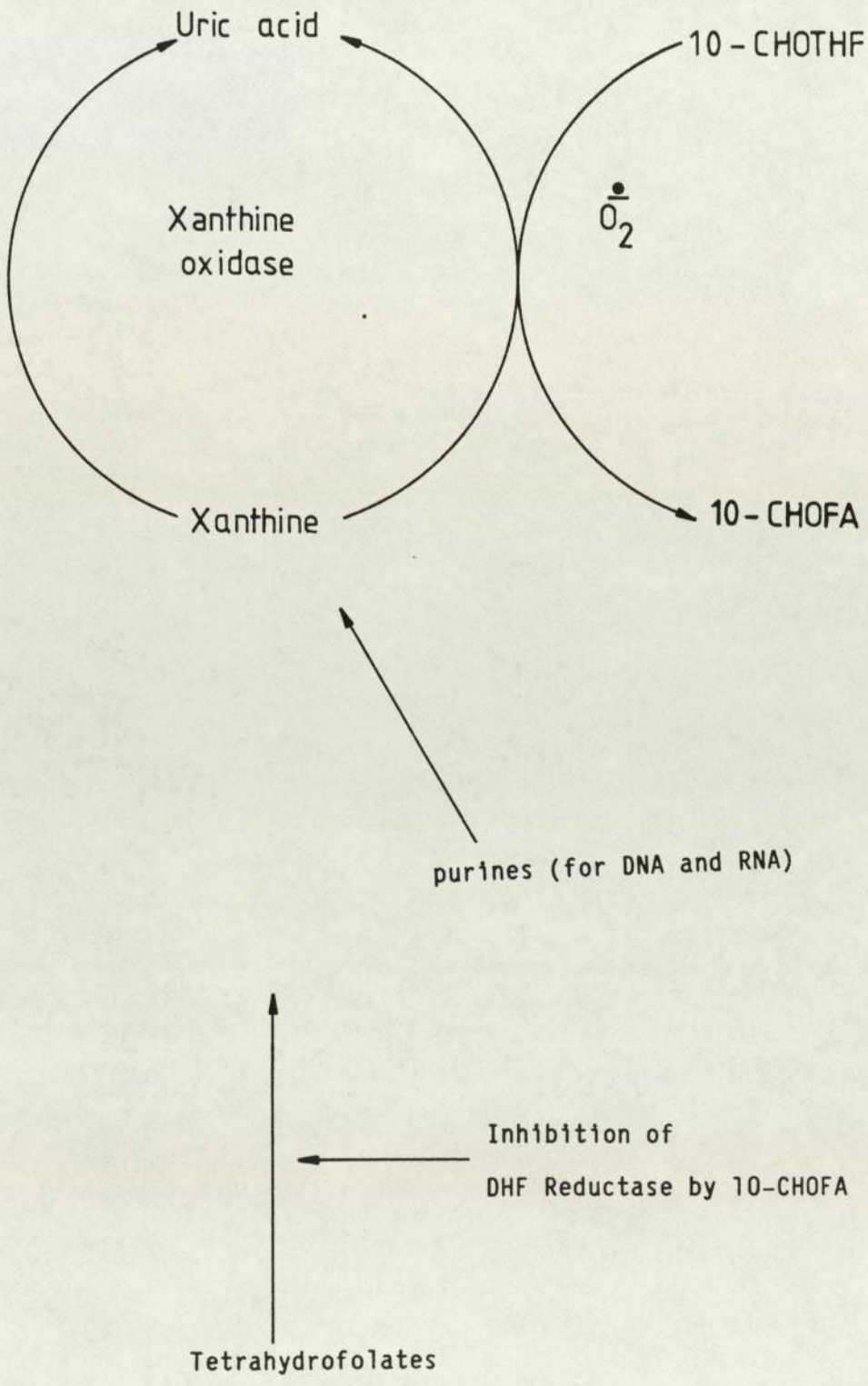


Figure 7-8-1 : Interaction of THF <u>in vivo</u> in the presence of 10-formylfolate

Suggestion for further work

A considerable catabolism has been shown to occur during the folate metabolism in man and rat (Blakley, 1969; Rowe, 1978; Pheasant, et al, 1981). The results presented in this thesis show that folate catabolism in vivo is a normal phenomenum in both normal hamster and in man. However the results of the breakdown of tissue folates following a dose of labelled folic acid to hamsters pre-treated with different agents also need to be further investigated because of the high percentage of liver folate breakdown that appears to take place. IO-CHOFA has also been shown to be extensively metabolised in the rat (Pheasant, et al, 1981) but it is only poorly utilised by man (Ratanasthien, et al, 1974; Saleh, 1981; Chapter 6 in this thesis). However, IO-CHOFA is metabolised to give largely scission product in the guinea pig and extensive folate breakdown has been demonstrated in the liver (Choolun, 1982). Thus the study of the IO-CHOFA in hamster might reveal its role in regulating DHF reductase activity in the normal cell.

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