STUDIES IN THE FOLATE METABOLISM OF RATS

A thesis
submitted for the degree of Doctor
of Philosophy

by

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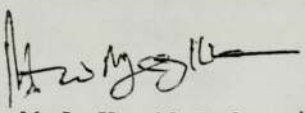
DECLARATION

This work was carried out between October 1974 and December 1977, in the Department of Chemistry, University of Aston in Birmingham.

This work was done independently and has not been submitted for any other degree.



M.A.K. Malghani

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SUMMARY

The metabolism of 2-^{14}C folic acid was studied in three weeks old weanling Wistar rats. Distribution of radioactivity after an oral dose of 2-^{14}C folic acid was determined in urine, faeces, liver and gut of the animals. Some of the urinary metabolites were characterized by column chromatography. The effects of an oral dose of methotrexate on 2-^{14}C folic acid metabolism in adult male Wistar rats were studied. An oral dose of 10 mg/kg body weight methotrexate was administered 24 hours before 2-^{14}C folic acid (76 microgram/kg body weight) dosing. The urinary metabolites characterized by column chromatography were compared and evaluated, quantitatively and qualitatively, with those present in the urine of control animals. Similarly metabolism of 3'-5'-9 ^{3}H folic acid was studied in the presence of methotrexate (10 mg/kg and 100 mg/kg) in rat. Large amount of unmetabolized folic acid was found in the urine of methotrexate treated animals. 10-Formyltetrahydrofolate and p-aminobenzoyl-L-glutamate were the other major metabolites present in the urine. The major metabolite in the urine of control rats was characterized as 5-methyltetrahydrofolate.

The metabolism of 5-^{14}C methyltetrahydrofolate was also studied. Since commercial 5-methyltetrahydrofolate was labelled with ^{14}C at labile methyl group, the metabolism was also studied using 2-^{14}C 5-methyltetrahydrofolate, isolated and purified from the urine of rats dosed with 2-^{14}C folic acid. The effect of methotrexate on 2-^{14}C folic acid and

3'-5'-9 ^{3}H folic acid metabolism was studied in the rats transplanted with 256 Walker carcinosarcoma.

It was concluded that methotrexate inhibits purine biosynthesis by interfering the steps involving formylfolate. This inhibitory action of methotrexate is in addition to the inhibition of dihydrofolate reductase. This in view, the effects of 6-mercaptopurine, a classical purine inhibitor, on folate metabolism was studied.

In addition to characterization of urinary metabolites an effort was made to identify the nature of metabolites present in the liver throughout the experiments. The main introduction describes historical background of folates, methotrexate and 6-mercaptopurine.

CONTENTS

CHAPTER 1

INTRODUCTION

Part A

The term folate is used to describe a widely distributed family of natural compounds, which are important for the growth and life of biological systems. Folates take their name from folic acid. Folates are indispensible for mammals and their deprivation, either caused by failure in absorption and uptake due to certain abnormalities in gastro-intestinal system or their unavailability in the diet ultimately leads to death. Mammals cannot synthesize their own folates, hence their only source is dietary folates. Most bacteria and pathogenic micro-organisms, on the other hand synthesize their own folates. Biosynthesis of required folates is therefore brought about by absorbing the necessary precursors from the environment they live in.

This chapter is devoted to some related aspects of literature on folates. It is difficult to document such a very vast and sometimes controversial literature in limited time and space, therefore no attempt is made to cover the whole literature; only some important features have been briefly reviewed.

Folates are pteridine derivatives and F.Gowland-Hopkins, demonstrator in physiology and chemistry at Guys Hospital, London, was the first man, who in 1889, drew the attention of scientists to the ornamental pigments of butter-flies which he thought were urates (1). In 1925-26 Wieland and Schopf (2,3) isolated white and yellow pigments from the wings of cabbage and brimstone butterflies in purer state and called these substances xanthopterin and leucopterin depending upon the colour (GK xanthos,yellow; leucos,white).

Purrman in 1940 established the structures of xanthopterin (4), leucopterin and isoxanthopterin (5). The structures of some pterins are shown in figure 1.

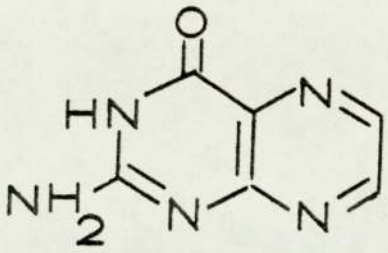
The difficulties in finding out the structures of pterins were due mainly to two reasons. Firstly the presence of impurities as pterins are poorly soluble in water and common organic solvents, hence resist purification, secondly pterins were confused with purines as they gave similar degradation products.

Natural pteridines are derivatives of 2-amino-4-hydroxypteridine and in general are named as pterins. Chemically pteridines are pyrimidopyrazines.

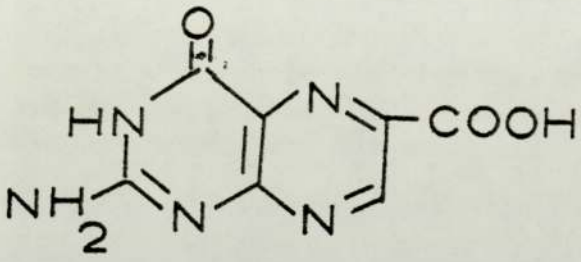
The ornamental natural pteridines of insects are merely waste products of certain biologically active pteridine derivatives, but a series of unrelated nutritional studies on monkeys, man and bacteria revealed the presence of biologically active pteridine derivatives in foods, specially in green leaves, yeast and liver, which in deprived subjects caused anaemia and related blood complications.

Wills in 1931 (6) reported that a number of patients with macrocytic anaemia, from different social groups in South India, were cured with autolyzed liver extracts.

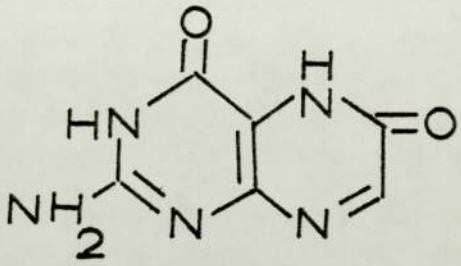
Experiments on monkeys later in 1938 (7), demonstrated the lack of certain essential factor in diet developed blood conditions characterized by anaemia, leukopenia, ulceration of gums, loss of weight and finally death. But if these animals were given yeast and liver extract preparations the blood picture remained normal. Similarly



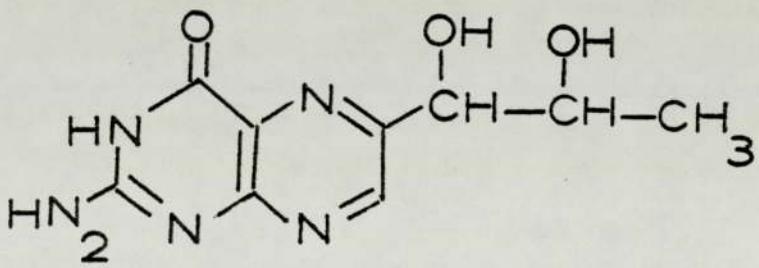
Pterin



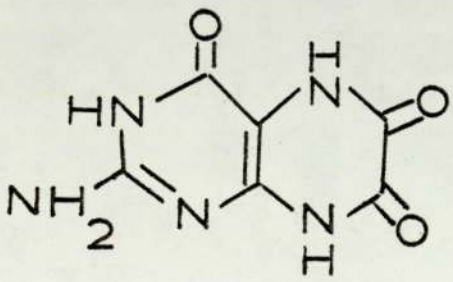
Pterin-6-carboxylic acid



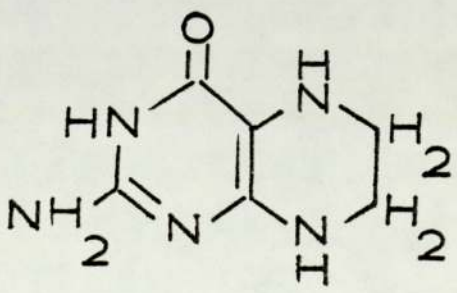
Xanthopterin



Biopterin



Leucopterin



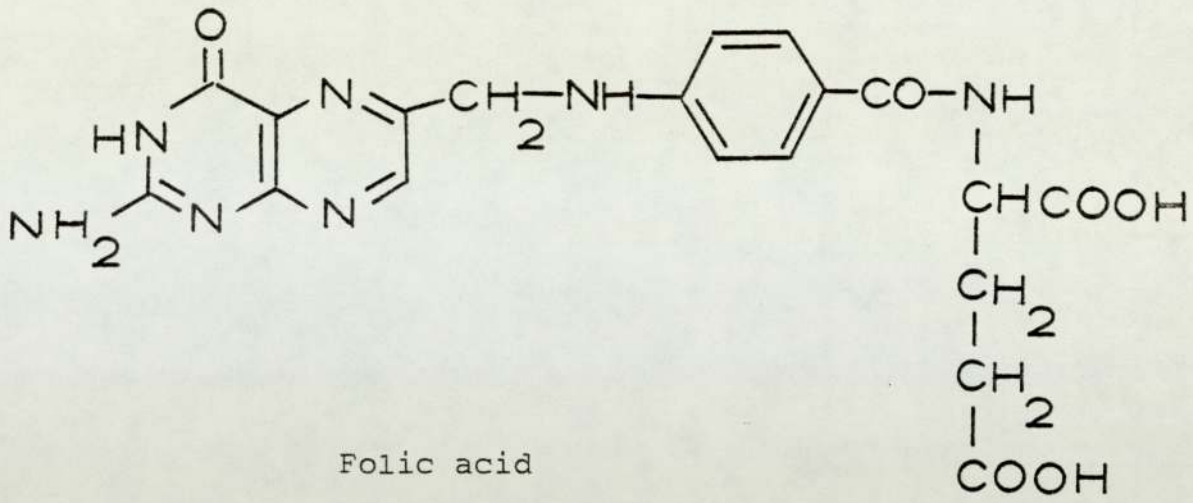
5,6,7,8-Tetrahydropterin

Figure 1

Stokstad et.al (8) reported that a factor is present in alfa alfa, wheat bran and yeast, which promoted the growth of chicks maintained on polished rice. It was also discovered that liver extracts prevented anaemia in chicks (9).

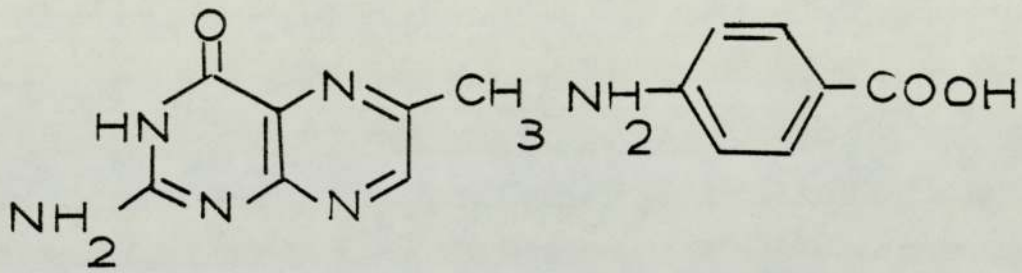
Mitchel et.al (10) extracted spinach and using streptococci as a test organism found high degree of activity in stimulating the growth of the bacteria. Since this growth promoting factor was abundant in leaves and grass they suggested the name "folic acid" (Latin-folium,leaf). It was realised that this growth promoting factor is a pteridine derivative, but the proof of its structure was given in 1946 by a group of workers by degradation and synthesis (11). Pteroyl glutamic acid (figure 2) is the alternative name for folic acid as suggested by these workers.

Folic acid molecule consists of three structural parts articulated into one. These parts are pteridine ring, para-aminobenzoic acid and L-glutamic acid (figure 2). When the first two units, that is pteridine ring and para-aminobenzoic acid, are joined together, a compound pteroic acid results and therefore its salts and radicals are called pteroates and pteroyl respectively. Hence when L-glutamic acid is joined with pteroic acid, pteroyl glutamic acid is formed. Further linkage of glutamic acid residues to the gamma-carboxyl group of pteroyl glutamic acid would give rise to pteroyl di-glutamic acid and so on. In general a folate with more than one glutamic acid part is called pteroylpolyglutamate.



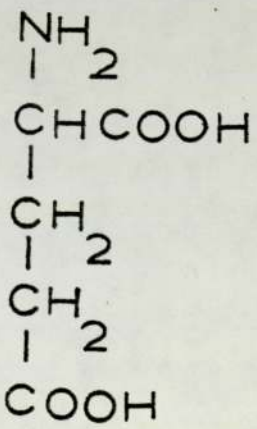
Folic acid

(Pteroylglutamic acid)



6-Methylpterin

p-Aminobenzoic acid



Glutamic acid

Figure 2

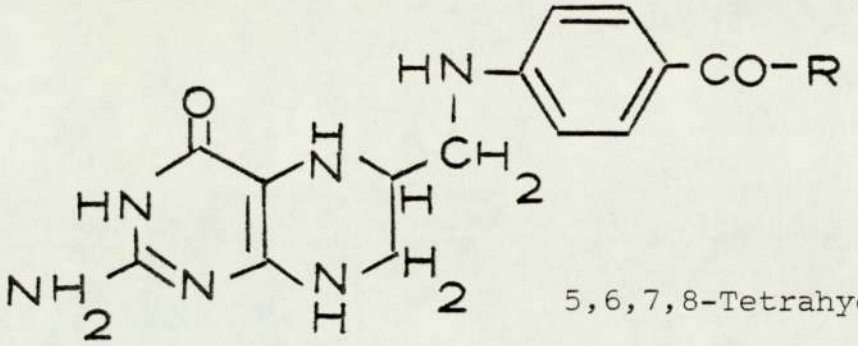
Biological Role of Folates

Folic acid co-enzymes function as a carrier of C-1 units required in the various metabolic steps, and in the synthesis of biologically important nucleic acid and cell building materials such as purines, methionine, and thymine. Folic acid as such does not take part in any of the above mentioned transfer of C-1 units, but is reduced to the folate co-enzyme 5,6,7,8 tetrahydrofolate (figure 3), before it serves this mediatory role. The enzyme responsible for the reduction of folic acid and di-hydrofolate to tetrahydrofolate in biological systems will be discussed later in this chapter.

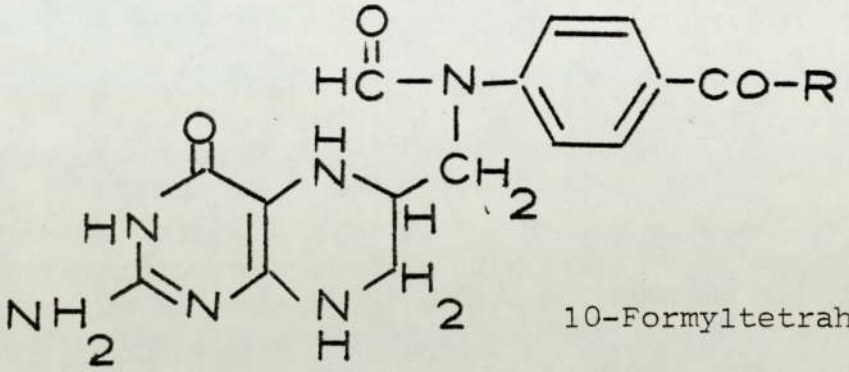
The co-enzyme tetrahydrofolate offers sites for one carbon groups at N5 and N10 positions via co-valent linkages and therefore this structural advantage makes it possible to accept and donate the one carbon groups in metabolic processes at different oxidation states like formate, formaldehyde and methanol (figure 3)

Biosynthesis of functional forms and the inter-relation of various steps involving tetrahydrofolate co-enzymes in the transfer of one carbon groups are summarized in figure 4.

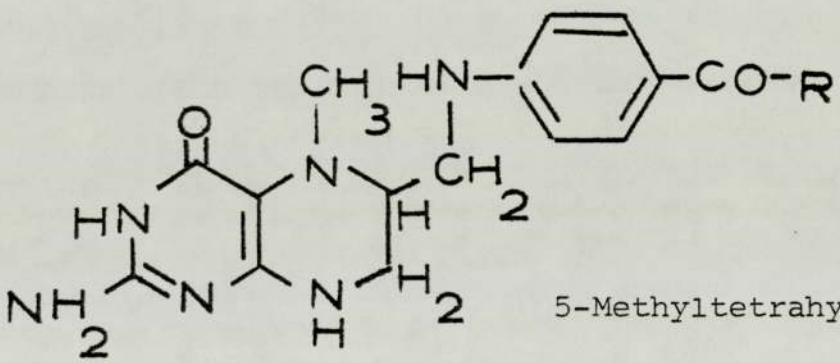
Welch et.al (12) in 1952 proposed the hypothesis that tetrahydrofolate was the co-enzyme involved in the activation of formate and formaldehyde. The active formate forms of tetrahydrofolate that is 5,10-methenyltetrahydro-folate and 10-formyltetrahydrofolate (13) are the donors of one carbon groups in the two steps of de-novo purine bio-synthesis (14,15,16,17,18,19).



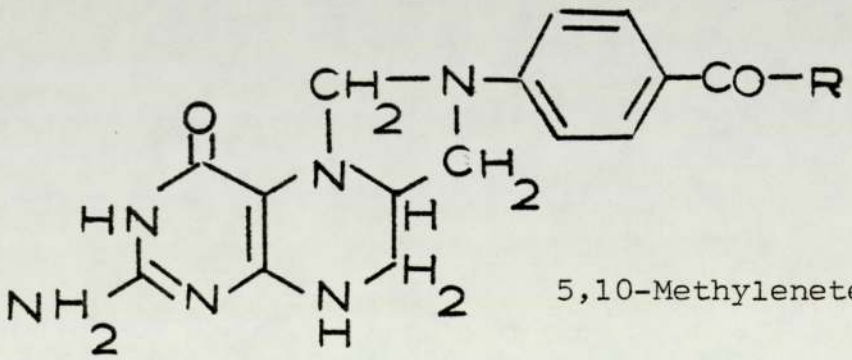
5,6,7,8-Tetrahydrofolate



10-Formyltetrahydrofolate



5-Methyltetrahydrofolate



5,10-Methylenetetrahydrofolate

R = Glutamyl part

Figure 3

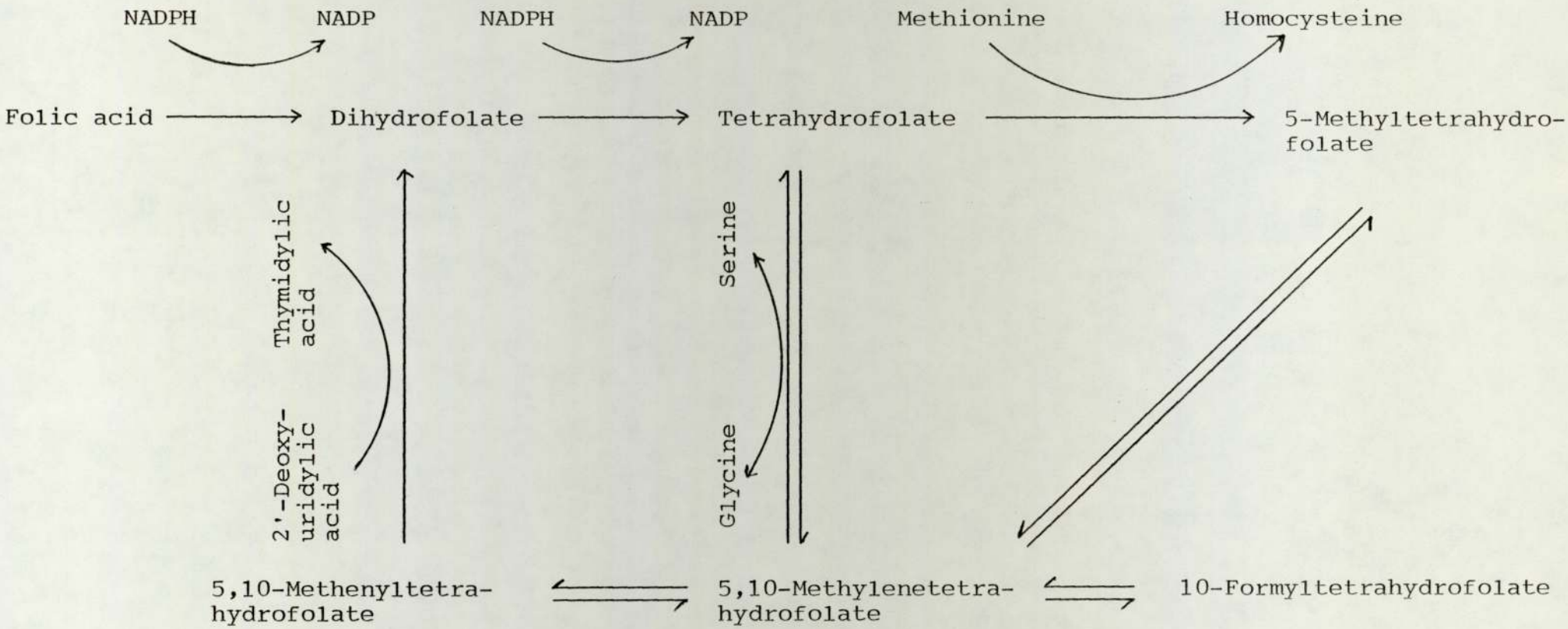


Figure 4 Metabolic interconversions of folate co-enzymes.

Formation of 10-formyltetrahydrofolate and 5,10-methenyl-tetrahydrofolate (formate levels)

Experiments on liver extracts showed that the ATP dependent 10-formyltetrahydrofolate synthetase or formate "activating" enzyme catalysed the formation of 10-formyl-tetrahydrofolate from formate and tetrahydrofolate interaction as follows (20,21):

$$HCOOH + ATP + \text{tetrahydrofolate} \xrightarrow{Mg^{++} \quad K^{+}} \text{10-formyltetrahydrofolate} + ADP$$

The enzyme 10-formyltetrahydrofolate synthetase is widely distributed in nature. It has been obtained from liver of chicks, pigeons, sheep and human erythrocytes (22).

The conversion of 10-formyltetrahydrofolate to 5,10-methenyltetrahydrofolate is catalysed by the enzyme 5,10-methenyltetrahydrofolate 5-hydrolase or "cyclohydrolase" found in pig kidney (23), rabbit liver (24) and beef liver (25). This interconversion also proceeds readily in the absence of enzyme in acidic medium.

$$\text{10-formyltetrahydrofolate} + H^{+} \rightleftharpoons (\text{5,10-methenyltetrahydrofolate})^{+} + H_2O$$

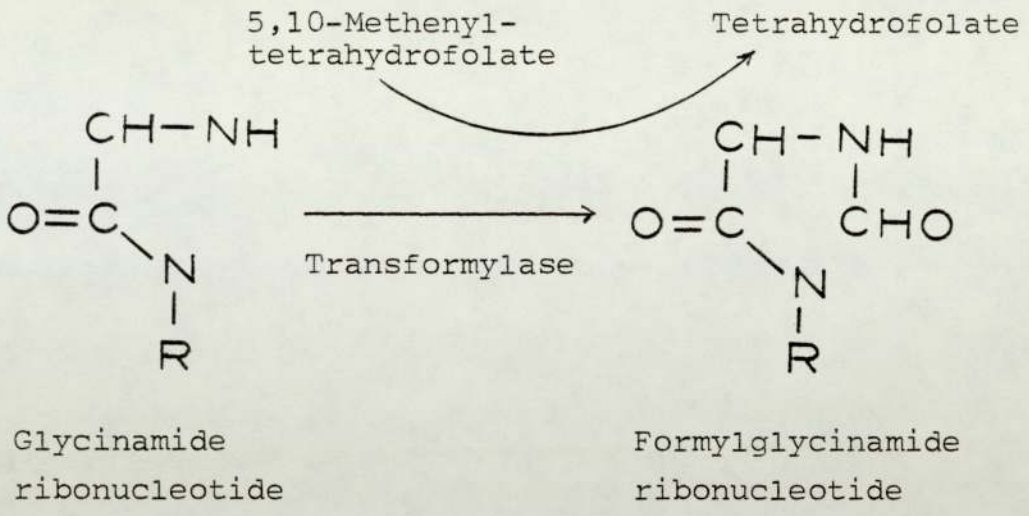
Steps in the de-novo purine biosynthesis utilizing 10-formyl-tetrahydrofolate and 5,10-methenyltetrahydrofolate

The folate co-enzymes 10-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate are involved in the two steps of the purine biosynthesis. The two steps are formation of C-2 and C-8 of the purine ring. The C-2 of the purine ring is formed as a result of 10-formyltetrahydrofolate interaction

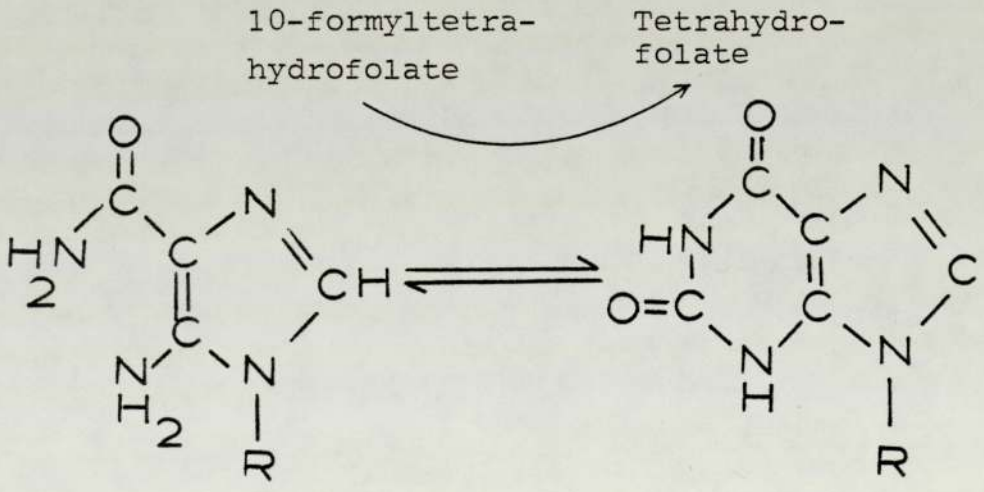
with 5-amino-4-imidazole carboxamide ribonucleotide. The product 5-formimido-4-imidazole carboxamide ribonucleotide finally is converted enzymatically to hypoxanthine ribonucleotide (inosinic acid) via ring closure. The first step, that is the formation of 5-amino-4-imidazole carboxamide ribonucleotide via 10-formyltetrahydrofolate is catalyzed by the enzyme 5-amino-4-imidazole carboxamide ribonucleotide transformylase. There are still doubts as to whether the two steps are catalyzed by the same enzyme or they proceed independently utilising different enzymes. Figure 5 shows the step of purine biosynthesis mediated by 10-formyltetrahydrofolate. This enzyme has been purified from the chicken liver (19).

One of the historical turning points towards recognition of the folate co-enzymes as a mediator of C-1 unit transfer was appreciated, when sulphonamide drugs were found to possess bacteriostatic activity due to the interference with the synthesis of folates in the bacteria. In the folate structure, para-aminobenzoic acid is one part and sulphonamides inhibit the condensation of this part with the pteridine portion in the synthesis of folates. As a result of inhibition of sulphonamides in E-coli, 5-amino-4-imidazole carboxamide ribonucleotide accumulates (26) and this is the step of purine biosynthesis mediated by 10-formyltetrahydrofolate mentioned above.

The possible effects of some folate antagonists on purine biosynthesis and related C-1 unit metabolism will be discussed later in this chapter.



Introduction of carbon-8



R = Ribose

Figure 5 Steps in purine biosynthesis mediated by folates.

The step of purine biosynthesis said to be mediated by 5,10-methenyltetrahydrofolate results in the formation of C-8 of the purine ring. Figure 5 shows this reaction. The 5'-phosphoribosyl glycinamide ribonucleotide receives one carbon unit from 5,10-methenyltetrahydrofolate at the terminal amino group. The enzyme glycinamide ribonucleotide transformylase mediates this probably irreversible reaction. The product of this reaction is 5'-phosphoribosyl-N-formylglycinamide ribonucleotide. Studies on the enzyme transformylase, using pigeon liver extracts, showed that tetrahydrofolate, ATP and formate were required for the formylation of glycinamide ribonucleotide (15,27).

The above conclusion might have been made wrongly, as the conditions for the formation of 10-formyltetrahydrofolate from 5,10-methenyltetrahydrofolate and vice versa described in this chapter previously resemble the above mentioned conditions of experiment. Therefore, the possibility that 10-formyltetrahydrofolate and not 5,10-methenyltetrahydrofolate mediates in the above mentioned step of purine biosynthesis cannot be ignored. However, Warren and Buchanan (17) claimed that 5,10-methenyltetrahydrofolate was responsible for the transformylation. In their experiment, they used chicken liver extract which also contains cyclohydrolase, the enzyme responsible for interconversion of 10-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate. Their result was based on the assumption that the enzyme was inactivated after prolonged storing, which they did before the actual reaction. Since the conversion of 5,10-methenyltetrahydrofolate

to 10-formyltetrahydrofolate and vice versa readily proceeds without any enzyme, the workers used maleate buffer to decrease the possibility of non-enzymic reaction.

In addition to the formylation reactions discussed above, 10-formyltetrahydrofolate is utilised in the formylation of methionine-transfer-ribonucleic acids, and when the formylated product formyl-methionine-t-ribonucleic acid initiates protein synthesis, methionine is incorporated only into the N-terminal positions of the protein. There are two types of methionine accepting transfer RNA in E-coli, only one of which is formylated. The enzyme which catalyses this formylation reaction is 10-formyltetrahydrofolate:methionyl-$tRNA_F^{met}$

$$\text{10-formyltetrahydrofolate} + \text{methionyl-}tRNA_F^{met} \longrightarrow$$

$$\text{N-formylmethionyl-}tRNA_F^{met} + \text{tetrahydrofolate}$$

The above kind of formyl transfer reaction occurs in mitochondria of micro-organisms only. A fuller discussion is given elsewhere (28,29,30,31).

<u>5-10-Methylenetetrahydrofolate, 5-methyltetrahydrofolate methionine and thymidylate synthesis</u>

5-10-Methylenetetrahydrofolate can be formed by the transfer of hydroxymethyl group from serine to tetrahydrofolate (32)

$$\text{tetrahydrofolate} + CH_2OH\text{-}CH(NH_2)\text{-}COOH \rightleftharpoons 5,10\text{-}CH_2\text{-}H_4 \text{ folate} + CH_2(NH_2)\text{-}COOH$$

The enzyme in this reaction is hydroxymethyltransferase.

The non-enzymic production of 5-10-methylenetetrahydrofolate requires formaldehyde and tetrahydrofolate (33).

5-10-methylenetetrahydrofolate is also precursor of methyl group of thymine.

5-10-methylenetetrahydrofolate + deoxyuridylate (dUMP) ⟶

dihydrofolate + thymidylate

De-oxythymidylic acid (dTMP) is formed as result of methylation of deoxyuridylic acid by 5-10-methylenetetrahydrofolate catalyzed by thymidylate synthetase. The reduction of C-1 unit to methyl group is brought at the expense of tetrahydrofolate which is converted to dihydrofolate such that the above reaction is simultaneously transfer and reduction of C-1 group. Dihydrofolate produced is reconverted to tetrahydrofolate by the enzyme dihydrofolate reductase. Pastore and Friedkin (34) using tritiated tetrahydrofolate, at 6 and 7 positions found that the tritium was incorporated into the methyl group of thymidylate. It was shown later (35) that thymidylate contained hydrogen from C-6 of tetrahydrofolate specifically.

The enzyme thymidylate synthetase is found in liver, tumor cells, thymus, bone marrow of man and most of the tissues in small amounts. This enzyme is susceptible to the action of antifolates such as methotrexate. Cells resistant to methotrexate accumulate thymidylate synthetase (36), as well as dihydrofolate reductase. The mechanism of inhibition of these enzymes by folate analogs is not fully understood.

Methionine Biosynthesis

The folate co-enzyme 5-methyltetrahydrofolate (see chapter 6) is the precursor of the methyl group of methionine (37,38). The earlier studies on chicks (39), demonstrated that vitamin B_{12} was important in the methionine biosynthesis from homocysteine. It was reported that the growth of vitamin B_{12} deficient chicks was increased by feeding homocysteine and vitamin B_{12} but not by the homocysteine alone. DuVigneaud et.al (40) demonstrated that homocysteine and choline together can replace methionine, suggesting that methyl group from choline is transfered in toto to homocysteine to form methionine. However, earlier studies show that D_2O feeding to rats (41) resulted in the deuterium labelled methyl group of choline and creatine, giving clue of de novo synthesis of methyl group. Such synthesis of methyl group, on the other hand, does not meet the total requirement of the rats and therefore exogenous supply of methionine remains essential for the normal health.

A large number of methyl group transfers in the biological systems (42) involve the formation of S-adenosyl-methionine called "active methionine".

In the micro-organism Escherichia coli, two alternative pathways for methionine synthesis are known. One depends on vitamin B_{12} (43) and the other does not depend on vitamin B_{12} (44). In case of the vitamin B_{12} dependent pathway, homocysteine is converted to methionine in the presence of 5-methyltetrahydrofolate which donates methyl group to homocysteine only when the enzyme methyltetrahydro-

folate-homocysteine transmethylase containing cobalt-methylcorrinoid as prosthetic group is present. In mammals the vitamin B_{12} dependent pathway is of crucial physiological importance in regulation and control of methionine metabolism (45).

In biological studies, synthetic dl-5-methyltetrahydrofolate (46) used is 50-50 mixture of two diastereoisomers which contain an asymmetric centre at C-6 of the pteridine ring. This asymmetric centre is introduced when 5-methyltetrahydrofolate is synthesized from folic acid (46). The enzyme vitamin B_{12} transferases can distinguish between the two diastereoisomers and are therefore stereo-specific in choosing the l-5-methyltetrahydrofolate for the transmethylation reaction (47,48). This does not necessarily mean that 5-methyltetrahydrofolate derived enzymically from tetrahydrofolate in the biological system also be levorotatory, but is designated so only because tetrahydrofolate which produces it is levorotatory.

5-Methyltetrahydrofolate "Trap Hypothesis"

The biosynthesis of methionine as discussed above, the vitamin B_{12} dependent pathway, requires two essential components, 5-methyltetrahydrofolate and methylcobalamine (vitamin B_{12} derivative) for the conversion of homocysteine to methionine. The vitamin B_{12} co-factor is required only in catalytic amounts, while 5-methyltetrahydrofolate in stoichiometric amounts. The relationship of vitamin B_{12} and folate can be explained by the 5-methyltetrahydrofolate "trap hypothesis".

The 5-methyltetrahydrofolate "trap hypothesis" was proposed by Noronha et.al (49) and Herbert Zalusky et.al (50). It was suggested that in vitamin B_{12} deficient subjects, 5-methyl-tetrahydrofolate accumulates or "piles up" in the folate pools due to diminished activity of methyltransferases. As a result of this metabolic trap folates other than 5-methyl-tetrahydrofolate are not available in adequate amounts to carry out C-1 unit transfers. As a consequence, purine and thymine biosynthesis is suppressed, which in turn impairs the DNA biosynthesis and "megaloblastosis" is caused.

The findings that administration of methionine to vitamin B_{12} deficient rats (51) and chicks (52) decreases the excretion of formiminoglutamic acid have been explained by the "methyl trap hypothesis" (50). Herbert Zalusky et.al (50), in their explanation, suggested that methionine in such subjects changes the folate metabolism of the liver from 5-methyltetrahydrofolate to formyltetrahydrofolate, since, methionine administration increases the amount of 10-formyl-tetrahydrofolate found in the liver.

Glutamic acid is formed as a result of L-histidine degradation, the last step involves tetrahydrofolate which acquires formimino group from formiminoglutamic acid and is converted to formiminotetrahydrofolate while glutamic acid is set free. The histidine degradation takes place in the following order:

L-histidine $\xleftarrow{\text{histidase}}$ Urocanic acid $\xleftarrow{\text{urocanase}}$ Imidazolone-propionic acid $\rightleftharpoons$ Formiminoglutamic acid $\xleftarrow{\text{formiminotransferase}}$ Glutamic acid

The enzyme formiminoglutamate formiminotransferase in the last reaction which involves tetrahydrofolate has been found in a variety of animal and human tissue (24).

It has been observed that an enzyme is present in pig liver (53) which splits N-formylglutamate and catalyzes the formation of 5-formyltetrahydrofolate, this enzyme may be the same as the enzyme responsible for the formation of formiminotetrahydrofolate from tetrahydrofolate (54) and formiminoglutamic acid.

Methotrexate administration results in excessive excretion of formiminoglutamic acid (55) in the urine. It also increased in megaloblastic anaemia due to folate deficiency.

Folinic acid (5-formyltetrahydrofolate) "Citrovorum factor"

The growth promoting effect of folates on certain bacteria has been extensively exploited for the identification and purification of folates from natural sources. Sauberlich et.al (56) in 1948 found that a factor in urine, yeast and liver is required by Leuconostic citrovorum (Pediococcus cervisiae) for their growth. This factor was identified as 5-formyltetrahydrofolate by a number of workers (57,58,59). Folinic acid is the only stable reduced folate, but it probably does not occur in mammalian tissue. If it does, it is derived from other reduced unstable folates. 5-formyl-tetrahydrofolate (folinic acid) activity for P.cerevisiae is not exclusive, since other tetrahydrofolates can also be utilised readily. The failure or less activity of these tetrahydro-folates for the growth of Pediococcus cerevisiae is due to

the fact that these tetrahydrofolates are unstable and undergo oxidation under the conditions of culture, while 5-formyltetrahydrofolate is stable (60). Nevertheless, micro-biological assay techniques used for the identification and estimation of folates are helpful but not very conclusive, since they are based mainly on differences like one mentioned above. However, use of micro-organisms for qualitative and quantitative evaluation of folates in biological samples, in presence of drugs which might interfere with the metabolism of bacteria itself, will undoubtedly lead to erroneous conclusions.

Three major strains of folate requiring bacteria used for the micro-biological assay of folates in biological samples are Lactobacillus casei ATCC No:7469, Streptococcus faecalis ATCC No:8043 and Pediococcus cerevisiae ATCC No:8081. It may be noted that folates containing more than two glutamate residues are not detected by any of the above mentioned bacteria.

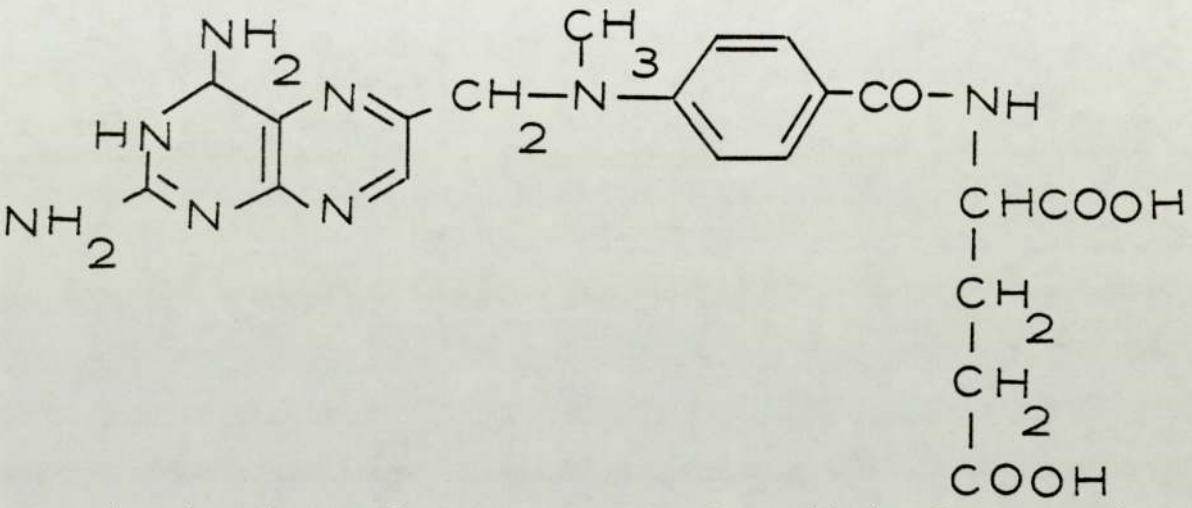
Role of folic acid in biological studies

Folic acid is much used for the metabolic studies of folates. It is converted to dihydrofolate and finally to tetrahydrofolate by the enzyme dihydrofolate reductase before it can carry out the C-1 unit transfers in the biological systems. Till recently use of natural folates in such studies was difficult and limited due to complex methods of synthesis and extraction from natural sources. In addition, natural folates are found in very minute quantities. Generally, folates are bound to proteins (61,62) and their liberation

from this may change their chemical nature.

The use of radioactive folic acid is discussed in the introductory pages of chapters dealing with folate metabolism in rat.

Cancer chemotherapy - Methotrexate and folate metabolism



4-amino-N_{10}-methylpteroylglutamic acid (methotrexate)

Methotrexate differs from folic acid only in two functions, the hydroxyl group at 4-position is replaced by an amino group and the hydrogen at N-10 position by a methyl group.

Methotrexate is one of the extensively used chemotherapeutic agents in human cancer. It is clear that methotrexate activity is due to the interference with the normal activities of folates, which play vital role in the biosynthesis of nucleic acids. Despite the huge amount of documentation, which sometimes complicates more than explains, on methotrexate activity, many questions are still unanswered e.g. toxicity, differential sensitivity, resistance.
In order to understand all complexities of methotrexate behaviour, a fuller knowledge of folate and methotrexate

metabolism in living mammalian system is essential.

As chemotherapeutic agent, methotrexate has been used with success in a number of neoplasms including leukaemia, and solid tumors like Burkitt's lymphoma.

The clinical use of folate antagonists was suggested by Lewisohn et.al (63), in 1946, for acute leukaemia of children. The most important observation of Farber et.al (106), in 1948, that aminopterin would produce temporary remission in children with acute leukaemia, led to the wide acceptance of folate antagonists for the treatment of various cancers. The best response with methotrexate has been found in rapidly growing tumors like choriocarcinoma (107) and acute lymphocytic leukaemia (108).

Absorption and distribution studies of methotrexate in man (64) demonstrated that methotrexate was absorbed quickly and was detectable in the serum of fasting subjects only after fifteen minutes of ingestion. The level of serum methotrexate reached a maximum within one hour. In cases with normal kidney function, excretion of methotrexate in urine was very fast and no methotrexate was detectable in the serum after eight hours. Henderson et.al claimed that in man (65) and animals like mice, dog and monkey (66), the drug was excreted almost completely in urine and faeces within 24 hours of administration. They suggested that the drug was poorly absorbed from gastro-intestinal tract and was mainly stored in the liver. However, an earlier report (67) showed that in man, no detectable store was found in liver, kidney, spleen, erythrocytes and leucocytes after small

doses of methotrexate between 18 and 120 days. Johns et.al (68) reported that tritiated methotrexate entered into cells at a slower rate than that found for similar doses of folate in man, 40% of dosed methotrexate (2.5 microgram/kg) was excreted in the urine in the first 48 hours and the remaining was excreted slowly over the next several weeks, mainly in the form of N-methyl-p-aminobenzoylglutamate and p-aminobenzoylglutamate. It is therefore right to assume that for all practical purposes methotrexate is not metabolised in man and the minor metabolites might have been derived (cf.,p29) through microbial metabolism in the gut or an extremely slow metabolism in man (69). This is in contrast to folic acid, which is rapidly converted to tetrahydrofolate metabolically (70).

The studies on uptake of folates across the small intestine of rat (71) have shown that folates are converted to neutral forms at acidic pH in order to achieve efficient transportation. Similar phenomena for methotrexate absorption and uptake has not yet been observed.

The studies on uptake of folic acid and its inhibitory analogs in Ehrlich ascites cells (72) was shown to be by a very slow process of diffusion. Similar slow diffusion of methotrexate was observed in human leucocytes (73). Reduced uptake of methotrexate in leukaemic cells in vitro (74) suggests that failure of response to the drug might be due to the impaired uptake. However, there is no conclusive evidence in the literature that drug resistance is function of impaired uptake only. On the contrary

there is equally strong evidence that methotrexate is absorbed by active transport mechanism in contrast to passive diffusion in the cell. Sirotnak et.al (75,76) proposed that uptake of tritiated methotrexate by L1210 leukaemic cells mediated via an active transport mechanism, since it required a source of energy. Some recent studies on uptake, metabolism and biliary excretion of methotrexate are discussed later in this chapter.

In conclusion, it can safely be assumed that methotrexate is absorbed quickly from the gastro-intestinal tract and large amounts are excreted within 48 hours of administration. As far as cellular uptake is concerned, to date there is no conclusive evidence in favour of any single mechanism of uptake, therefore, it is presumed that both passive and active means of uptake are possible within certain limits of extra-cellular drug concentration.

Dihydrofolate reductase and methotrexate action

The enzyme dihydrofolate reductase has two biological functions. It reduces folic acid to tetrahydrofolate via dihydrofolate and also reduces dihydrofolate formed as a result of thymidylate synthesis to tetrahydrofolate. Thymidylate synthesis from deoxyuridylate is mediated by tetrahydrofolate which in the course of reaction is oxidised to dihydrofolate.

$$\text{Folic acid} + \text{NADPH} + H^+ \xrightleftharpoons{\text{folate reductase}} \underset{\text{7,8, dihydrofolate}}{} \text{NADP}^+ +$$

$$\text{7,8, dihydrofolate} + \text{NADPH} + H^+ \xrightleftharpoons{\text{DHFR}} \underset{\text{5,6,7,8, tetrahydrofolate}}{} \text{NADP}^+ +$$

The above equations illustrate the two functions of the enzyme folate reductase.

The earlier studies on the enzyme were carried out with crude preparations from microbial, avian and mammalian sources. In man the enzyme has been found in liver, kidney, skin, bone marrow and white and red cells of peripheral blood (97,113,114,115,116,117). A significant activity of the enzyme in various organs, except for brain and skeletal muscle, of guinea pig has been observed; highest levels were found in liver and kidney (111). In general, distribution of the enzyme in other mammals like rats, mice, sheep and rabbit (112) show similar pattern as that of guinea pig (111).

The studies of Schulz (77) on dihydrofolate activity in the new born and pregnant rats showed that dihydrofolate reductase in the tissue of pregnant rats was 30% higher than in normal adults. On the other hand, liver and kidney of foetal rats had less activity in converting dihydrofolate to tetrahydrofolate, i.e. had lesser amounts of dihydrofolate reductase as compared to pregnant or normal adult rats. This activity rose considerably in new born rats in first ten days becoming normal in the fourth week of life.

Structural studies of dihydrofolate reductase have been carried out by a number of workers (78,79,80,81). Hunnekens et.al in 1970-71, working on chicken liver dihydrofolate reductase found that the enzyme preparation had molecular weight of 22000 and appeared to be a single polypeptide chain with two cysteine and four tryptophan amino acid residues in each molecule. The enzyme did not

contain any metal in the protein structure. The enzyme activity had two pH optima 7.5 and 4.5. At acidic value activity was two fold that of alkaline. At the acid pH optimum dihydrofolate was reacted three times faster than folic acid.

The earlier observations of Nichol and Welch (82) demonstrated that folic acid was converted to dihydrofolate and tetrahydrofolate in the sliced liver of rat and that the folate antagonist aminopterin (4-aminopteroylglutamic acid) blocked this reduction. This led to similar studies on methotrexate activity. Thus Osborn et.al (83) and Peters and Greenberg (84) in 1958 found that partially purified dihydrofolate reductase from chicken liver was inhibited both by methotrexate and aminopterin. The inhibition was complete with 5×10^{-7} M concentration of drug when substrate was dihydrofolate at a concentration of 5.3×10^{-4} M. Their initial conclusion was that inhibitors were competing with dihydrofolate for binding to the enzyme. They suggested that affinity of the enzyme for both dihydrofolate and inhibitor was high. Later studies showed that dihydrofolate reductase from chicken liver, sheep liver, calf thymus and Ehrlich ascites cells were inhibited by 10^{-8} M concentration of methotrexate. When equimolar amounts were used methotrexate inhibited the enzyme more at pH 6 than at 7.6 and 8.5, which indicated that at this pH the inhibitor was more tightly bound to the enzyme (85,86,87,88). It has been suggested that the enzyme activity is stoichiometrically inhibited if folic acid is used as substrate but on the other hand, methotrexate inhibits dihydrofolate reductase less effectively

if dihydrofolate was substrate (89).

It can be concluded from the above mentioned studies, that though methotrexate most probably inhibits the enzyme dihydrofolate reductase stoichiometrically under the conditions of experiments, that is mildly acidic medium (pH 6) and presence of limited competitive substrate. But in cells of living organism it is difficult to find the exactly similar conditions i.e. a pH value of 6 and also presence of limited competitive substrate. As dihydrofolate reductase is found in excess in most of the cells and only a very small fraction is required to maintain the intracellular reduced folate pool (90,91), therefore, much larger amounts of methotrexate are required for complete inhibition. Nixon et.al (92) demonstrated that in the presence of methotrexate, dihydrofolate derivatives accumulate in the murine lymphoma cells. Therefore, to bring about any effective blockade and to maintain this block, methotrexate in molar excess to the target enzyme is required (93).

The major problem in the treatment of neoplastic disease with methotrexate, is lack of drug response following treatment. The mechanism of resistance or the lack of response is still poorly understood; whether tumor cells acquire resistance or it is natural is not known.

Since dihydrofolate reductase is the major target enzyme of methotrexate activity, drug resistance might have partly to do with the enzyme and drug interaction and its repercussions. Thus, increased dihydrofolate reductase activity accompanied by drug resistance has been observed

in a number of animal tumor models (94,95). Bertino et.al (96,97) have observed similar increases of dihydrofolate reductase level in the leukocytes and erythrocytes after methotrexate treatment of leukaemia patients. Altered dihydrofolate reductase due to presence of methotrexate might be another reason of acquired resistance as observed in tumor models (98,99). Whether or not this occurs in humans is not known.

Protection of normal tissue from methotrexate toxicity

Use of methotrexate in the treatment of cancer poses another danger, that of toxicity to normal host tissue. Methotrexate by its inhibitory action on dihydrofolate reductase, indirectly depletes the folate co-enzymes. Presumably the actively dividing cells, whether neoplastic or normal, would contain higher levels of dihydrofolate reductase due to the excessive demand for DNA synthesis. Hence they will equally be effected by the methotrexate action. Tissues like bone marrow and gastro-intestinal epithelium of the host would therefore be more susceptible to drug toxicity.

To protect the normal tissue from the toxic effects of methotrexate, leucovorin (5-formyltetrahydrofolate, 100) has been extensively used as a rescue therapy. It is assumed that 5-formyltetrahydrofolate by-passes the methotrexate block and hence prevents the toxic effect of methotrexate on host organs (101). In addition 5-methyltetrahydrofolate may also rescue the normal cells as Halpern et.al (102) demonstrated that murine malignant cells in tissue culture

cannot convert 5-methyltetrahydrofolate to other forms of reduced folates like 5-10-methylenetetrahydrofolate, 10-formyltetrahydrofolate and 5-10-methenyltetrahydrofolate, which are required for purine and pyrimidine biosynthesis. However, rescue of normal cells is only possible when these cells in contrast to malignant cells can convert 5-methyl-tetrahydrofolate to the above mentioned co-enzymes.

Apart from 5-formyltetrahydrofolate, purine and thymidine also have been found to protect cells from the toxicity of methotrexate. Thus either adenosine or hypoxanthine or inosine together with thymidine were found necessary to protect mouse bone marrow in vitro (103). This suggests that methotrexate has some inhibitory effects on purine and pyrimidine biosynthesis apart from dihydrofolate reductase. Hryniuk (104) demonstrated that cytotoxic effects of methotrexate on the L5178Y murine leukaemia cell line could be partially prevented by a purine source alone, on the other hand, if thymidine and a purine were given together protection was complete.

Toxicity does not only result due to dihydrofolate reductase inhibition, it may also arise by the delayed drug excretion. Thus renal dysfunction is usually associated with high dose drug infusion (121). In addition a metabolite of methotrexate, 7-hydroxymethotrexate has also been identified in the urine of monkey and man (105) after high dose infusion. Another metabolite, methotrexate-diglutamate (4-amino-4-deoxy-N_{10}-methylpteroylglutamyl- -glutamate) has also been found in the liver of rat (109) and man which is

retained in the liver several months after methotrexate administration (110). The methotrexate-diglutamate has been reported to possess methotrexate like behaviour in inhibiting dihydrofolate reductase (118).

Enterohepatic recirculation of methotrexate has also been reported which might also contribute to the toxicity of methotrexate (119).

Shin et.al (120) have reported that presence of methotrexate in the rat liver decreases the uptake of tritiated folic acid in addition to decreased conversion of folate to polyglutamate. Similarly, Buehring et.al (123) demonstrated that administration of methotrexate, aminopterin or tetrahydromethotrexate to rats, two hours before injection of tritiated folic acid decreased the incorporation of radioactivity by the liver. In addition, the intra-cellular proportion of polyglutamates relative to monoglutamates was decreased. They also reported that in presence of methotrexate more than 90% of dosed tritiated folic acid was excreted as such. The authors claimed that when methotrexate was administered 24 hours prior to tritiated folic acid, the decrease of uptake in the liver was less than the decrease of uptake of folic acid observed when methotrexate was administered 2 hours before folic acid. These observations indicate change of metabolism in the liver after methotrexate administration.

Slavikova and Slavik (122) demonstrated that the toxicity as a result of reduced folate antagonist administration to mice could not be explained by reductase

inhibition as they found that tetrahydroderivatives of methotrexate and aminopterin were several hundred times less effective in inhibiting the enzyme dihydrofolate reductase. They suggested that antileukaemic activity of these reduced derivatives of methotrexate and aminopterin depended upon interference with folate co-enzymes and not exclusively upon inhibition of dihydrofolate reductase. The authors also suggested that the inhibition of two enzymatic steps in the de novo synthesis of purine ring and of thymidylate synthetase, contribute significantly towards the resulting toxicity. The two steps in the de novo synthesis of purine ring mentioned above include the formylation of ribose-glycineamide and amino-imidazolecarboxamide.

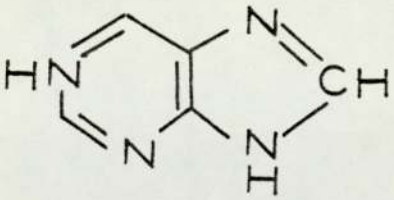
Purine Antagonist - 6-Mercaptopurine

The requirement of the folate co-enzyme 10-formyl-tetrahydrofolate for the biosynthesis of C-2 of purine ring was discussed earlier in this chapter. It was stated that 10-formyltetrahydrofolate reacts with 5-amino-4-imidazole-carboxamide ribonucleotide to produce 5-formimido-4-imidazole-carboxamide ribonucleotide in the presence of the enzyme 5-amino-4-imidazolecarboxamide ribonucleotide transformylase. The product of the above reaction is then converted enzymatically to hypoxanthine ribonucleotide (inosinic acid).

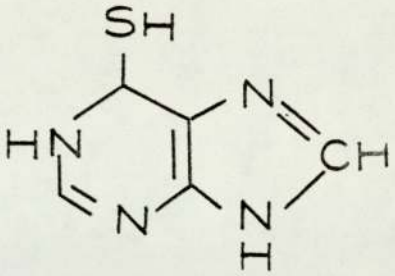
It was observed in the course of experiments in this thesis, that 10-formyltetrahydrofolate levels in the urine of rats dosed with methotrexate 24 hours prior to folic acid, compared to other reduced folates were increased. The requirement of 10-formyltetrahydrofolate for the de novo purine biosynthesis involving the formation of C-2, therefore, suggests that methotrexate is somehow interfering with this step of purine biosynthesis directly or indirectly. It seems logical, therefore, to find out metabolism of folates in mammals in the presence of purine analogs which might interfere at the same site of folate metabolism. Hence, 6-mercapto-purine was used in one of the experiments documented in chapter 8 of this thesis. In the following pages a brief review of the literature on the metabolism and the possible sites of action of 6-mercaptopurine is given.

Metabolism and sites of action

6-Mercaptopurine is an analog of adenine in which amino group is replaced by SH group. It was synthesized by



Adenine



6-mercaptopurine

Elion et.al (124) in 1952. It was first tested as an anti-leukaemic agent by Burchenal et.al (125).

Skipper (126) in 1954 reported that 6-mercapto-purine inhibited the purine biosynthesis by inhibiting glycine-^{14}C or formate-^{14}C incorporation into the nucleic acid purines in mouse intestine, Adenocarcinoma 755 and Sarcoma 180. Skipper (126) also claimed that 6-mercaptopurine does not significantly inhibit adenine incorporation into nucleic acids but it inhibits hypoxanthine incorporation into polynucleotides.

Formate incorporation into purines was inhibited by 6-mercaptopurine in rat spleen (127), Ehrlich ascites carcinoma (128), leukaemia L1210 cells (129) and also in micro-organisms L.casei (130), S.faecalis (131) and E.coli (132).

Bennett et.al (129) demonstrated that 6-mercapto-purine inhibited the incorporation of formate in L1210 cells in mice at the lowest dose of 1mg/kg. The authors also reported inhibition of de novo purine synthesis in Sarcoma 180. They stated that 6-mercaptopurine inhibits the de novo synthesis of purine in DNA and RNA equally.

Ramey (136) claimed that 6-mercaptopurine and its ribonucleotide are acceptors of methyl group from S-adenosylmethionine in the presence of S-adenosylmethionine-specific transmethylase in the preparations from the mouse liver. The suggested pathway for the formation of 6-methyl-mercaptopurine ribonucleotide is as follows.

6-mercaptopurine ⟶ 6-mercaptopurine ribonucleotide ⟶ 6-methylmercaptopurine ribonucleotide.

However, Bennett and Allan (137) demonstrated that 6-mercaptopurine was methylated by a non-enzymatic transfer of methyl group from S-adenosylmethionine in vitro. They suggested that though 6-mercaptopurine is a good acceptor of methyl group in vitro and some methylation might be expected in intact cells, the conversion of 6-mercaptopurine - ribonucleoside to methyl mercaptopurine ribonucleoside does not occur in intact cells to significant extent to be of significance in the metabolism of 6-mercaptopurine.

Hakala and Nichol (138), Bennett and Adamson (139) reported that the inhibition caused by 6-mercaptopurine or 6-methylmercaptopurine ribonucleoside is prevented or reversed by 4-amino-5-imidazolecarboxamide or hypoxanthine in cultured cells. Hakala and Nichol (138) reported that the major effect of ribonucleotide of 6-mercaptopurine was exerted at an early step in the biosynthesis of purines and in cultures in which the de novo pathway of purine synthesis is blocked by methotrexate and growth of the cells was totally dependent on a supplement of purine added to the medium. The growth inhibition was reversed by 4-amino-

Salser et.al (133) studied formate incorporation 1 and 8 days after 6-mercaptopurine administration. They concluded that the effect of 6-mercaptopurine may differ from tissue to tissue and that kidney and liver synthesized more soluble nucleotide when the animals received 6-mercaptopurine 24 hours or 8 days prior to incorporation studies.

Studies with radioactive 6-mercaptopurine demonstrated that it is rapidly metabolised in the mouse with the excretion of 6-thiouric acid as a major product (134). Elion et.al (134) used two types of labelled 6-mercaptopurine, with ^{14}C at 8-position of purine ring and ^{35}S in mercapto group, for studying the incorporation into nucleic acids and metabolism in mice. When 1mg of ^{35}S-mercaptopurine was given by intraparitoneal injection, 43.5% radioactivity was excreted in first four hours and rest after two days. The same amount of excretion was observed after using ^{14}C-mercaptopurine, the only difference was that in latter observation some $^{14}CO_2$ was detectable in the expired air. However, the authors did not mention any quantitative relation of $^{14}CO_2$ compared to the dosage. They stated that concentration of radioactivity during the first few hours after intraparitoneal injection was highest in the gut and very low in the brain. Again the authors did not mention the quantitative proportions to the dosage with time.

Similar studies in man (135) demonstrated comparable results to that in mice. 6-mercaptopurine labelled with ^{35}S at 6-position was metabolised rapidly as in mice except that more sulphate was excreted in the urine.

5-imidazolecarboxamide when the cells were grown under conditions in which purines are synthesized entirely de novo. They concluded that 6-mercaptopurine can inhibit at least two steps in the pathway of purine biosynthesis, one prior to the formation of 4-amino-5-imidazolecarboxamide ribonucleotide and second conversion of inosinate to adenylate.

Tidd and Paterson (140) in 1974 reported that delayed cytotoxic effects of 6-mercaptopurine in mouse leukaemic cells in culture were due to incorporation of 6-mercaptopurine into cellular DNA. When 6-mercaptopurine was isolated from DNA it was found to be present not as such but was converted to thioguanine deoxynucleotide. This suggests that conversion of 6-mercaptopurine to 6-thioguanine was involved. However, there was direct correlation between the incorporation of 6-mercaptopurine into DNA and cytotoxicity or cell lethality. On the other hand, Scannel and Hitchings (143) reported a very contradictory finding that a mouse ascites tumor line Ad755, sensitive to 6-mercaptopurine did not incorporate as much 6-mercaptopurine into DNA as a 6-mercaptopurine resistant cell line. In addition the resistant cell line was found to have 6-thioguanine present in DNA in deoxynucleotide linkage.

The anabolic conversion of 6-mercaptopurine to ribonucleotide occurs when it reacts with phosphoribosyl-pyrophosphate in the presence of enzyme hypoxanthine-guanine-phosphoribosyltransferase, thus Brockman (141,142) showed that bacterial and mammalian cells resistant to 6-mercapto-purine had lost the above mentioned enzyme.

CHAPTER 1 (Part B)

A CRITICAL ANALYSIS OF METHODS USED IN FOLATE METABOLISM STUDIES: GENERAL OBJECTIVES OF THIS THESIS

Following the dicovery of folic acid and elucidation of its structure a number of its derivatives were isolated, purified and identified in natural sources such as food and mammalian tissues. Clinical and biochemical studies followed, of their absorption, excretion, retention and utilisation in man and animals. As knowledge of folates progressed it was realised that the problems were much more complex than had been thought. Major difficulties in this field were caused by the unstable chemical nature of these compounds, the minute amounts in which they were present and the lack of any precise chemical means of characterising and estimating the folates.

Much work has been done using microbiological assay techniques. In this method three types of micro-organisms Lactobacillus casei, Streptococcus faecalis and Pediococcus cervisiae are used for the measurement and identification of the folates. As yet no single micro-organism has been discovered which requires only one folate monoglutamate. Thus of the folate monoglutamates P.cervisiae requires all the tetrahydromonoglutamate derivatives except 5-methyltetrahydrofolate. S.faecalis is active for the same group as well as dihydrofolate, folic acid and 10-formylfolic acid and L.casei utilises all of these and 5-methyltetra-hydrofolate. The only common folate for which a specific microbiological assay can be used for identification and assay is 5-methyltetrahydrofolate and this can be done using the difference between L.casei and S.faecalis assay. In addition to this lack of specificity these micro-organisms

are inactive for folates containing more than three glutamate residues and the various break down products of folates. Sterilization of the assay material against oxidation is essential and this is usually done by addition of large amounts of ascorbate. As polyglutamates containing more than three glutamic acid residues are not available to these organisms these compounds have to be broken down by prior incubation with crude extracts of conjugases to the monoglutamates or diglutamates. Such procedures may destroy or alter the folate moiety by chemical or biochemical means. Thus liver folates are not active for L.casei until treated with conjugases. Drugs such as methotrexate, if present in the tissue will also interfere with the microbiological assay.

More recently tritium labelled folic acid particularly 3'-5'-9 ^{3}H folic acid has been used to follow the metabolism of folates. While this technique permits the use of small amounts,"physiological,doses" of folic acid, easy tracing, quantitative measurement and identification it may give rise to errors because of tritium exchange in the animal or because of scission of the folate molecule by chemical or biological means.

Gel permeation chromatography on Sephadex G15 of folates offers separation of various monoglutamate fractions between themselves and from polyglutamates. The folate polyglutamates appear close to the void volume and the monoglutamates appear later. However, gel permeation chromatography cannot be used satisfactorily as a single

technique for the identification of folates, for example 5-methyltetrahydrofolate is not separable from folic acid.

Ion-exchange column chromatography using DE52 and steadily increasing ionic strength gradient is more specific in separating the folate species. However, although 5-methyltetrahydrofolate and folic acid are clearly separable by this technique 10-formylfolate, 10-formyl-tetrahydrofolate and 5-formyltetrahydrofolate elute very close together.

The common practice of identifying folates by their behaviour on pre-calibrated column chromatographs both gel permeation and ion-exchange is likely to lead to errors in identification because the precise position of elution will depend on sample size, column packing, flow rate, temperature and minor variations in column packing and composition. The possibility of errors is greatest when compounds elute in similar positions, eg. 5-methyl-tetrahydrofolate and folic acid in G15 gel permeation chromatography and least when they elute in widely different positions, eg. the same two compounds on DE52 ion-exchange chromatography.

Thin layer chromatography of monoglutamates followed by their detection on the layer by microbiological assay using L.casei, S.faecalis and P.cervisiae (bio-autography) has been used in a few studies. Because of the specific microbiological assay and the high resolution capacity of thin layer chromatography this technique is capable of a very high degree of specificity.

To identify the various folate species and other metabolites described in this thesis, both DE52 ion-exchange and gel permeation G15 Sephadex were used. In addition to characterising the different metabolites by pre-calibration of the columns used, co-chromatography with authentic standards on both types of columns was widely used. In appropriate cases identification was assisted by comparison of UV spectra and appropriate colour tests. 2-^{14}C and 5-^{14}C labelled folates were used in some studies while others were performed with a mixture of 2-^{14}C and 3'-5'-9' ^{3}H folic acid. This latter technique permitted the identification of scission products in urine.

In a previous study (ref?) it was observed that rats with Walker 256 carcinosarcoma excreted relatively more 10-formyltetrahydrofolate in the urine in comparison to normal animals when dosed with 2-^{14}C folic acid. Similar experiments were done on young Wistar rats to see if the same relative increase in 10-formyltetrahydrofolate was found in normal young animals with increased cellular proliferation.

These experiments were then followed by a study of effects of methotrexate on folate metabolism using 2-^{14}C folic acid, a mixture of ^{3}H and ^{14}C labelled folic acid and 5-methyltetrahydrofolate labelled with ^{14}C in the pterin ring. A study then followed of the effects of methotrexate on the metabolism of the methyl group from 5-^{14}C methyltetrahydrofolate. In a final series of experiments the effects of 6-mercaptopurine on folate metabolism and the

combined effects of implanted Walker 256 tumour and methotrexate were studied.

This thesis seeks to extend the hypothesis previously advanced (Blair,1975)[144] that in cellular proliferation the amount of 10-formyltetrahydrofolate relative to 5-methyltetrahydrofolate is increased, to explore the transport and metabolism of 5-methyltetrahydrofolate and to extend in some depth our knowledge of the effect of well established anti-tumour drugs on folate metabolism.

CHAPTER 2

MATERIALS AND METHODS

Each chapter dealing with experiments include a Materials and Methods section, however, general description of methods and materials used in the experiments is summarised in this chapter.

Wistar rats were orally dosed with radioactive folates with or without prior oral administration of drugs (methotrexate or 6-mercaptopurine). The radioactive folates include 2-^{14}C folic acid, 3'-5'-9 ^{3}H folic acid, a mixture of 2-^{14}C folic acid and 3'-5'-9 ^{3}H folic acid , 5-^{14}C methyl-tetrahydrofolate and 2-^{14}C-5-methyltetrahydrofolate. All radioactive chemicals were purchased from Radio-chemical Centre, Amersham, except 2-^{14}C-5-methyltetrahydrofolate, which was isolated and purified from the urine of Wistar rats orally dosed with 2-^{14}C folic acid.

Administration of radioactive folates and drugs

The chemicals were administered by stomach intubation under mild ether anaesthesia. A 1 ml glass syringe with a especially designed curved metalic tube was used to administer required amount of particular chemical in distill water (a solution or suspension). A plastic tube was used instead of metalic tube for dosing the young rats. The rats were killed by placing them on cotton wool soaked in ether in a desicator.

Housing of animals and collection of biological samples

The animals were housed in metabolic cages designed for separate collection of urine and faeces (Metabowl, Jencons Ltd; Hemel Hempstead, Herts; U.K.). Urine and faeces were

collected in round bottomed flasks attached at the lower ends of the separator, leading from the main chamber of the metabowl residing the rat. Occasional blocking of urinary tube of separator by faeces was prevented by removing the blocked faeces with help of a glass rod. The faeces was freed from the occasionally droped food pellets into the faeces, before or after freeze drying.

The urine samples were collected in flasks containing 5 mls of 0.05 molar phosphate buffer, pH 7.0, containing anti-oxidants (i.e. dithiothreitol, sodium ascorbate). The separator of the metabowl and the flasks were wrapped with tin foils to protect the contents from direct light.

The animals were killed 24 hours or 48 hours after radioactive folate administration. The gut and liver of each animal was removed immediately after killing. The extraction of liver for the identification of folate metabolites was carried out by methods described later in this chapter.

The gut was washed thoroughly with water. Faeces, some of the livers and gut were freeze dried and powdered for the measurement of radioactivity using Biological Material Oxidiser.

Measurement of radioactivity in urine, faeces, liver and gut

Radioactivity of a fraction of urine sample (10 microlitre, 50 microlitre or 1 ml) was counted in a Nuclear Enterprise liquid scintillation counter type NE 8310 using 10 mls of a cocktail of toluene (500 mls), Fisons emulsifier E (250mls) and 2,5-diphenyloxazole (2.5 gm). Standards and blanks were

counted in a similar fashion until constant count rates were achieved. Corrections for quenching and background were made whenever necessary.

Gut, faeces and liver as mentioned earlier were freeze dried and powdered. A known quantity of each sample was oxidised in a Beckman Biological Material Oxidise (BMO) at 900 ^{o}C in a stream of oxygen, which finally pushes the gaseous combustion products (including CO_2 and water) through combustion tube and catalyst bed. The gaseous product bubble through one or two traps (attached to the other end of the combustion tube) containing trapping counting solutions. Only one trap was used for ^{14}C labelled materials, the trap contained 15 mls of Fisons absorber P (Fisons, Loughborough, Leics; U.K.), a scintillation cocktail designed for the collection of $^{14}CO_2$. Duplicate combustion of each sample was carried out. The standards and blank combustion were carried out in the same way. The dually labelled materials containing both ^{14}C and ^{3}H were oxidised, the end products (containing $^{14}CO_2$ and $^{3}H_2O$) were first passed through a cold trap (placed in a methanol and dry ice bath) where tritiated water condensed and $^{14}CO_2$ was trapped into another trap attached to the first one. The second trap contained 15 mls of Fisons absorber P. 15 mls of Fisons Dioxane D was poured into the first trap containing $^{3}H_2O$, allowing the ice to melt and mix. The contents were poured into a counting vial and counted. The standards and blanks were oxidised and counted similarly. In addition standards and blanks were counted directly in the counter which gave the gross CPM of

standard and calibration blank. Thus the latter observation provided with correction factor (correction factor = DPM added/ DPM recovered), which if multiplied with specific counts of unknown sample would give correct values (for details see Beckman instructions by Michael A. Kravetz).

Extraction of livers

(i) Boiled liver extract

The freshly removed liver were cut into small pieces and plunged into a solution of boiling 0.05 molar phosphate buffer, pH 7.0, containing 2-5% (w/v) sodium ascorbate and 5 mg/100 mls dithiothreitol. The contents were left boiling for about 5 minutes, cooled to room temperature and centrifuged to remove precipitated protein and stored at -15°C until column chromatography. Details of this extraction procedure is given elsewhere (171).

(ii) Cold liver extract

The freshly removed liver was homogenised at room temperature in 0.05 molar phosphate buffer, pH 7.0, and solid material was removed by centrifugation and filtration.

(iii) Trichloroacetic acid (TCA) liver extract

The freshly removed liver was homogenised at room temperature with 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/ 100 mls dithiothreitol. To resultant homogenate 10% trichloroacetic acid was added dropwise till the pH of the contents changed to pH 1. The contents were centrifuged and filtered. The pH of the extract was brought back to pH 7 by adding 1 N sodium hydroxide solution. The extract was stored at -15°C until required.

(iv) Freeze dried extract

The liver was completely freeze dried and homogenised with 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/100 mls dithiothreitol, centrifuged and filtered. The extract was stored at -15°C until required.

In most of the experiments procedure (i) has been used for the preparation of liver extracts. This procedure ensures inactivation of conjugases and thus prevent the breakdown of polyglutamates (172).

Column chromatography

(i) DEAE ion-exchange column chromatography

Diethylaminoethyl cellulose (Whatman DE52) was packed into columns (2 sq.cm. x 50 cm) after standard procedures involving degassing and equilibration with buffer solution. The ion-exchange columns used throughout these experiments were equilibrated with 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/100 mls dithiothreitol and eluted with a gradient of 0-1 molar sodium chloride in 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/100 mls dithiothreitol.

The DE52 ion-exchange columns were calibrated with most of the unlabelled folate monoglutamates, which were detected by using an LKB Uvicord 11 and a chart recorder (LKB Instruments, Croydon, Surrey, U.K.). In addition the columns were calibrated with radioactive folates used in the experiments i.e. 2-^{14}C folic acid, 3'-5'-9 ^{3}H-folic acid, 2-^{14}C-5-methyltetrahydrofolate and 5-^{14}C methyl-tetrahydrofolate.

(ii) G15 Sephadex column chromatography

Sephadex G15 columns were equilibrated and packed using standard methods, into (2 sq.cm x 60 cm) columns. The columns were eluted with 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/100 mls dithiothreitol and calibrated with authentic folate monoglutamates either labelled or unlabelled.

Identification of metabolites

Liver extracts and pooled urine samples were applied to both DE52 ion-exchange and G15 Sephadex columns. A sample of 10 mls was applied to DE52 column and 5 mls sample was applied to G15 Sephadex column (unless otherwise mentioned). The radioactive fractions of peaks after urine chromatography were rechromatographed with authentic materials both on DE52 ion-exchange and G15 Sephadex columns and final identification was mostly based on co-chromatography (match) of authentic materials with radioactivity on both columns. The unlabelled materials were identified either by UV absorption or chemically (colour reactions). The radioactive fractions (under a peak for rechromatography and identification) were chosen, which stood well clear of adjacent peaks (middle fractions between two peaks were rejected). The pooled fractions were reduced to required volumes by partial lyophilization.

CHAPTER 3

METABOLISM OF 2-^{14}C FOLIC ACID IN YOUNG RAT.

Introduction

This chapter reports some aspects of metabolism of 2-^{14}C folic acid in three weeks old weanling Wistar rats. Some of the observations in this experiment have been compared with the previous reports on the metabolism of 2-^{14}C folic acid in adult male Wistar rats (145).

The experiments documented here were intended to find out the effects of age on the metabolism of oral folic acid, since little information could be found in the literature on this aspect.

Barford and Blair (145) reported presence of four metabolites in the urine of male adult Wistar rats, namely folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and a newly identified metabolite 4a-hydroxy-5-methyltetrahydrofolate after oral administration of 2-^{14}C folic acid. The authors could not produce any conclusive evidence for polyglutamate formation in the liver after 2-^{14}C folic acid administration.

Vidal and Stokstad (146) reported presence of 5-formyltetrahydrofolate in addition to 5-methyltetrahydrofolate and 10-formyltetrahydrofolate in the urine of rats receiving intraperitoneal injections of ^{3}H folic acid.

It has been observed (77) that dihydrofolate reductase in the tissues of pregnant rats was 30% higher than in normal adults. It was also reported that liver and kidney of foetal rats had less activity in converting dihydrofolate to tetrahydrofolate and that the activity rose considerably in new born rats in first ten days becoming normal in the

fourth week of life. This observation suggests that liver and kidney of foetal rats contains less dihydrofolate reductase.

The formation of reduced folates like 5-methyl-tetrahydrofolate and 10-formyltetrahydrofolate have been detailed in chapter 1 of this thesis. 5-Methyltetrahydrofolate is formed from 5,10-methylenetetrahydrofolate enzymically. The formation of 5-methyltetrahydrofolate has been reported to be irreversible under physiological conditions (147). 5-Methyltetrahydrofolate was observed to be one of the major urinary reduced folate after oral administration of 2-^{14}C folic acid (145).

Liver of the rats, however, has been found to contain polyglutamates (148), derivatives of reduced folates, mostly 5-methyltetrahydrofolate and formyltetrahydrofolate polyglutamates (149).

This chapter details distribution of 2-^{14}C folic acid in urine, faeces, liver and gut of three weeks old weanling rats. DE52 ion-exchange and G15 sephadex column chromatograms of 0-6, 6-24 and 24-48 hours urine samples and liver extracts have been presented with reference to similar distribution and urinary analysis in adult rats.

Materials and Methods

Three week old male weaning Wistar rats were purchased from Messrs Bantam and King. 2-^{14}C Folic acid (specific activity 58.2 uCi/mmol) was obtained from Radiochemical Centre, Amersham. The chemicals used for making buffer solutions were of purest grade.

The animals were orally dosed with 76 microgram/kg body weight 2-^{14}C folic acid in water. Each animal was housed in a separate metabowl (Jencons metabowls) designed for the separate collection of urine and faeces. The rats were fed and watered ad libitum and were killed 48 hours after 2-^{14}C folic acid dosing.

Collection of biological samples and measurement of radioactivity

The urine samples were collected between 0-6, 6-24 and 24-48 hours in flasks containing 5 mls of 0.05 molar phosphate buffer, pH 7.0, containing 2 gm/100 mls of sodium ascorbate and 2 mg/100 mls dithiothreitol.

Faeces were collected in empty flasks. The estimation of radioactivity was carried out by oxidising known amounts of freeze dried and powdered samples in Beckman biological material oxidiser. The $^{14}CO_2$ was collected into 15 mls of Fisons absorber P (Fisons, Loughborough, Leics., U.K). Duplicate burning of each sample was carried out together with controls. The radioactivity was counted in a Nuclear Enterprises liquid scintillation counter type NE8310. Appropriate corrections were made for quenching and background.

The liver and gut of the animals were taken out immediately after killing. Gut and some of the livers were freeze dried, powdered and oxidised for the determination of radioactivity.

Amount of radioactivity in urine samples were measured by counting 50 microlitres fractions in liquid scintillation counter, by using 10 mls of a cocktail of toluene (500 mls), Fisons emulsifier E (250 mls) and 2,5-diphenyloxazole (2.5 gms) in a vial. The standards and blanks were counted in a similar fashion and corrections for quenching and background were made.

Column chromatography of urine samples and liver extracts

The 0-6, 6-24 and 24-48 hours urine samples were applied to DEAE-cellulose (Whatman DE52) columns (2 sq.cm. x 50 cm). The columns were equilibrated with 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/100 mls dithiothreitol before applying the sample. The columns were eluted with a linear gradient of 0-1 molar sodium chloride in 0.05 molar phosphate, pH 7.0, containing 5 mg/100 mls dithiothreitol. 5 mls eluent fractions (samples) were collected by LKB automatic fraction collector. The conductivity of every tenth fraction was determined by Mullard conductivity cell.

The urine samples were also applied to G15 sephadex columns (2 sq.cm. x 60 cm). The columns were eluted with 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/100 mls dithiothreitol.

Liver extracts were chromatographed on DE52 ion-exchange and G15 sephadex columns. Four different extraction procedures were used. These are boiled extraction, cold extraction, trichloroacetic acid extraction and freeze dried extraction. The extraction procedures have been described in chapter 2 of this thesis.

RESULTS

Recovery of radioactivity

Table 1 shows the distribution of radioactivity in urine, faeces, liver and gut of three week old Wistar rats receiving 2-^{14}C folic acid (76 microgram/kg) orally. The distribution is expressed as a percentage of the dosed 2-^{14}C folic acid radioactivity. The total radioactivity excreted in urine and retained in liver and gut on average accounts for about 65% of the total dosed 2-^{14}C folic acid radioactivity. Thus this experiment shows that about 35% of the dosed 2-^{14}C folic acid radioactivity is retained by the animal after 48 hours in the animal system in addition to the radioactivity recovered in liver and gut. The liver and gut retained about 12% and 5% of dosed radioactivity respectively after 48 hours. Table 2 has been taken from Barford and Blair (145) which shows distribution of radioactivity in urine, faeces and liver of male adult Wistar rats following 2-^{14}C folic acid dosing (78 microgram/kg).

Column chromatography of urine samples

Figures 3.1, 3.2 and 3.3 show DE52 ion-exchange column chromatograms of 0-6, 6-24 and 24-48 hours urine samples respectively.

The 0-6 hours urine chromatogram (figure 3.1) shows four peaks which appear at 0.9, 0.6, 0.4 and 0.1 molar sodium chloride concentration on the gradient. The peak appearing at 0.9 molar sodium chloride concentration was identified as folic acid by its elution position and co-chromatography with authentic folic acid on G15 sephadex

column. Other peaks appearing at 0.6 and 0.4 molar sodium chloride concentration on the gradient were similarly identified as 5-methyltetrahydrofolate and 10-formyltetrahydrofolate respectively. The fourth peak which appears at the start of the gradient was not identified, however, it is not 4a-hydroxy-5-methyltetrahydrofolate, xanthopterin and 2-amino-4-hydroxypteridine.

The 6-24 hours urine chromatogram (figure 3.2) shows five peaks. The peaks appearing at 0.9, 0.6 and 0.4 molar sodium chloride concentration were identified as folic acid, 5-methyltetrahydrofolate and 10-formyltetrahydrofolate, while the other two peaks could not be identified.

Figure 3.3, which is DE52 ion-exchange column chromatogram of 24-48 hours urine sample, shows two peaks. The major peak appearing at 0.6 molar sodium chloride concentration on the gradient was identified as 5-methyltetrahydrofolate on the basis of its elution position and co-chromatography with authentic 5-methyltetrahydrofolate on G15 sephadex column.

Figures 3.4, 3.5 and 3.6 are G15 sephadex column chromatograms of 0-6, 6-24 and 24-48 hours urine samples respectively. These chromatograms show that 5-methyltetrahydrofolate and folic acid have not appeared separately.

Column chromatography of liver extracts

Figures 3.7, 3.8, 3.9 and 3.14 are G15 sephadex column chromatograms of liver extracts prepared by four extraction methods.

Figure 3.7 is a G15 sephadex column chromatogram of 'cold' liver extract prepared by homogenising the freshly taken out liver in 0.05 molar phosphate buffer, pH 7.0, and removing some of the solid material by centrifugation and filtration. Figure 3.7 shows two major peaks, smaller peak appearing just after void volume. This peak may be some polyglutamate form of folate. The second major peak appears where 5-methyltetrahydrofolate appears on this column. Figure 3.11 is a DE52 ion-exchange column chromatogram of the same 'cold' liver extract.

Figure 3.8 shows a G15 sephadex column chromatogram of liver extract prepared by dropping chopped liver into 20 mls of boiling 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/100 mls dithiothreitol. The contents were left boiling for five minutes, centrifuged at room temperature and filtered. The extract was stored at -15^{o}C. The chromatogram shows one major peak at the void volume of G15 sephadex column, which is characteristic of polyglutamates. DE52 ion-exchange column chromatogram of the same 'boiled' liver extract (figure 3.12) shows only one peak appearing at 0.6 molar sodium chloride concentration on the gradient.

Figure 3.9 is a G15 sephadex column chromatogram of liver extract prepared by using 10% trichloroacetic acid for the precipitation of solid material. The freshly taken out liver was homogenised at room temperature with 10 mls of 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/100 mls dithiothreitol. To the resultant homogenate 10% trichloroacetic acid was added until the pH of the contents became pH 1.

The precipitated material was homogenised and solid material was removed by filtration and decantation. The pH of the extract was brought back to pH 7 by adding sufficient 1.0 N sodium hydroxide solution. Figure 3.9 shows two major peaks. One peak appearing after 35th fraction appears to be folic acid on this column. Since there is no peak of folic acid on DE52 ion-exchange column chromatogram of the same extract (figure 3.10), the above mentioned peak is not folic acid. The second peak which appears after fraction 50 on G15 sephadex column (figure 3.9) did not co-chromatograph with 10-formyltetrahydrofolate, 5-formyltetrahydrofolate, folic acid, xanthopterine and pteroic acid.

Figure 3.14 is G15 sephadex column chromatogram of liver extract prepared after homogenising the freeze dried liver in 15 mls of 0.05 molar phosphate buffer, pH 7, containing 5 mg/100 mls dithiothreitol. The resulting homogenate was centrifuged and filtered. The extract was stored at -15^{o}C until chromatographed. Figure 3.14 shows two peaks none of which appear at the expected site of polyglutamate. However, DE52 ion-exchange column chromatogram of the same extract (figure 3.13) shows three peaks appearing at about 0.3, 0.4 and 0.6 molar sodium chloride concentration on the gradient which are the calibrated sites of 4a-hydroxy-5-methyltetrahydrofolate, 10-formyltetrahydrofolate and 5-methyltetrahydrofolate respectively.

Rat No	Urine			Liver	Gut	Faeces	Total
	0-6 hrs	6-24 hrs	24-48 hrs				
1	5.8	7.1	1.8	20.0	6.3	40.3	81.3
2	8.6	2.3	2.3	17.0	8.1	40.0	78.3
3	7.3	8.1	5.8	10.7	2.1	35.8	69.8
4	9.1	7.3	3.5	3.1	9.2	38.9	71.1
5	8.2	5.3	3.0	10.7	4.9	40.9	73.0
6	11.2	6.6	1.7	15.5	7.4	30.0	72.4
7	8.9	4.1	2.3	6.3	6.4	41.0	69.0
8	19.9	4.6	4.5	-	4.8	27.1	60.9
9	2.5	4.4	3.7	-	4.2	35.8	50.6
10	18.8	2.5	1.3	-	2.7	24.6	49.9
11	10.0	10.4	2.5	-	3.0	28.3	54.2
12	6.2	1.0	8.1	-	3.1	35.5	53.9
13	4.1	6.1	4.8	-	5.1	44.5	64.6
14	6.1	3.3	3.6	-	5.9	32.5	51.4
15	3.6	7.8	6.2	-	2.6	60.8	81.0
Average	8.7	5.4	3.7	11.9*	5.0	37.1	65.4

Table 1 Distribution of radioactivity in urine, liver, gut and faeces (expressed as a percentage of 2-^{14}C folic acid dosed) of rats killed 48 hours after 2-^{14}C folic acid administration.

* Average recovery in seven rats.

Rat No	Urine			Liver	Faeces	Total
	0-6 hrs	6-24 hrs	24-48 hrs			
1	2.9	4.4	4.5	12.3	45.4	69.5
2	4.6	7.2	7.9	12.5	44.2	76.4
3	7.7	13.0	5.9	11.6	52.4	90.6
4	5.1	4.7	1.9	20.1	42.8	74.6
5	4.2	2.4	1.5	18.7	39.8	66.6
6	19.7	1.3	0.9	17.6	24.1	63.6
7	1.2	0.8	2.2	17.1	57.1	78.4
8	5.3	4.4	2.2	12.5	53.0	77.4
Average	6.3	4.8	3.4	15.3	44.8	74.6

Table 2 Distribution of radioactivity in urine, faeces and liver (expressed a percentage of 2-^{14}C folic acid dosed) of adult male Wistar rats following an oral dose (78 microgram/kg) of 2-^{14}C folic acid.

Note: The above table has been taken from Barford and Blair (145).

Chromatogram	Metabolites			
	1	2	3	4
Figure 3.1	33.6	30.0	25.4	9.0
Figure 3.2*	9.5	30.0	11.5	11.2
Figure 3.3	-	62.6	17.9**	-

Table 3 Distribution of radioactivity in various urinary metabolites as resolved by DE52 ion-exchange column, expressed as a percentage of total radio-chromatographed on each column.

Metabolites 1, 2 and 3 appear at the calibrated sites of folic acid, 5-methyltetrahydrofolate and 10-formyltetra-hydrofolate respectively on DE52 ion-exchange column. Metabolite 4 has not been identified and appears at the start of the gradient.

* DE52 ion-exchange column chromatogram of 6-24 hrs urine (figure 3.2) shows a peak just before 10-formyltetra-hydrofolate which could not be identified and has not been included in this table.

** The figure may not represent 10-formyltetrahydrofolate as the peak does not exactly coincide with the calibrated site of 10-formyltetrahydrofolate.

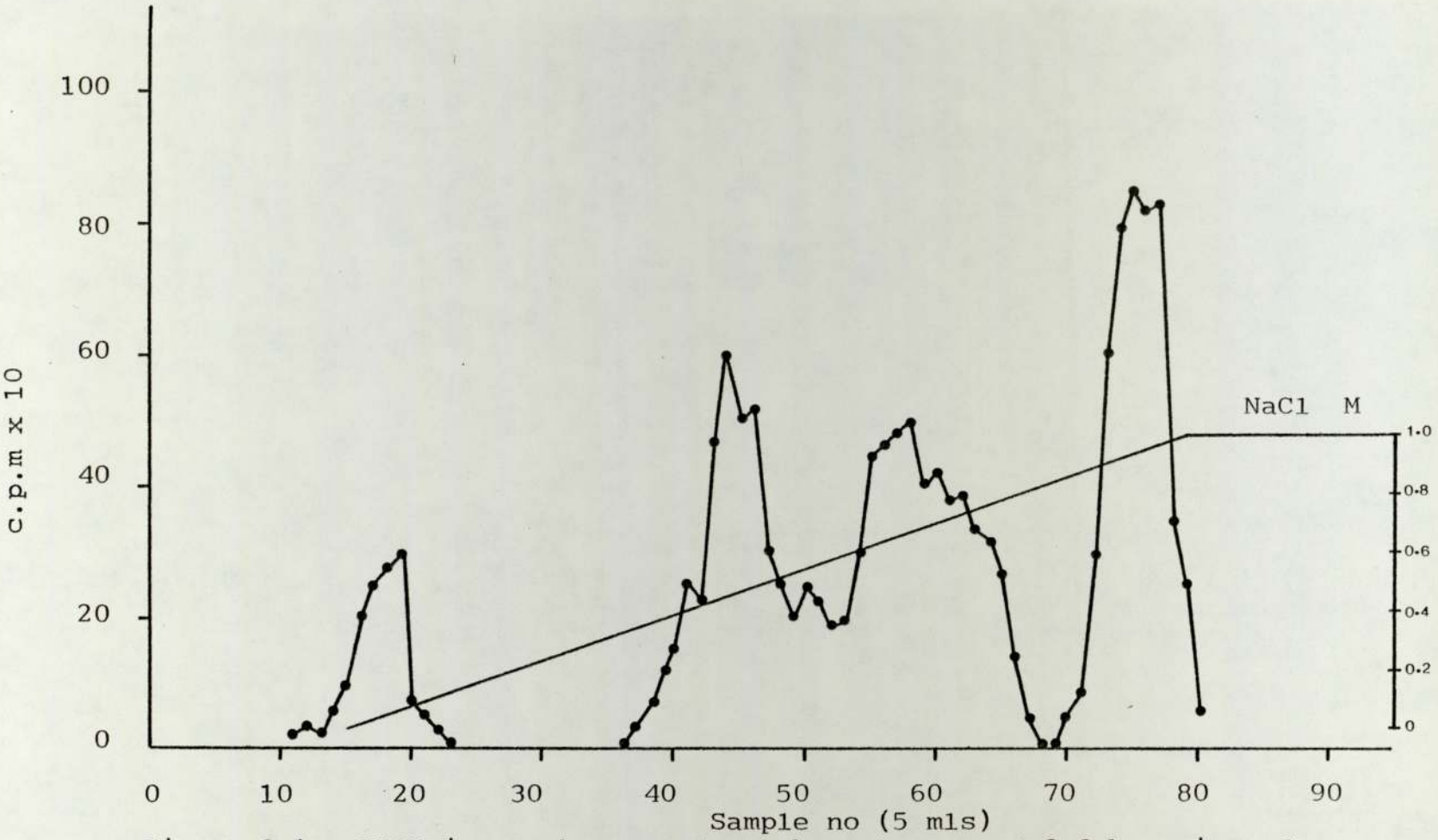


Figure 3.1 DEAE ion-exchange column chromatogram of 0-6 hr urine of young rats receiving an oral dose of 76 microgram/kg carbon-14 labelled folic acid.

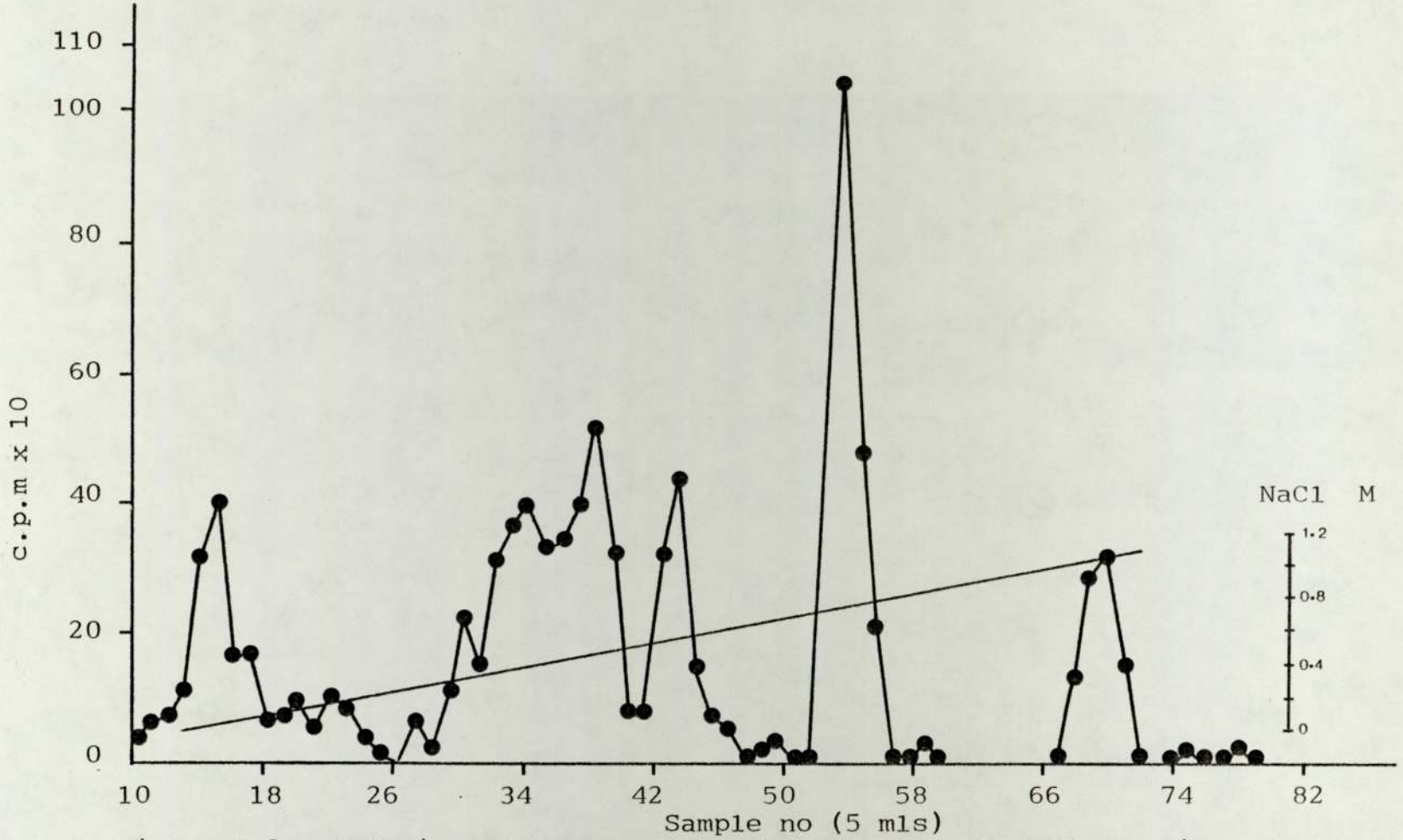


Figure 3.2 DEAE ion-exchange column chromatogram of 6-24 hr urine of young rats receiving an oral dose of 76 microgram/kg carbon-14 labelled folic acid.

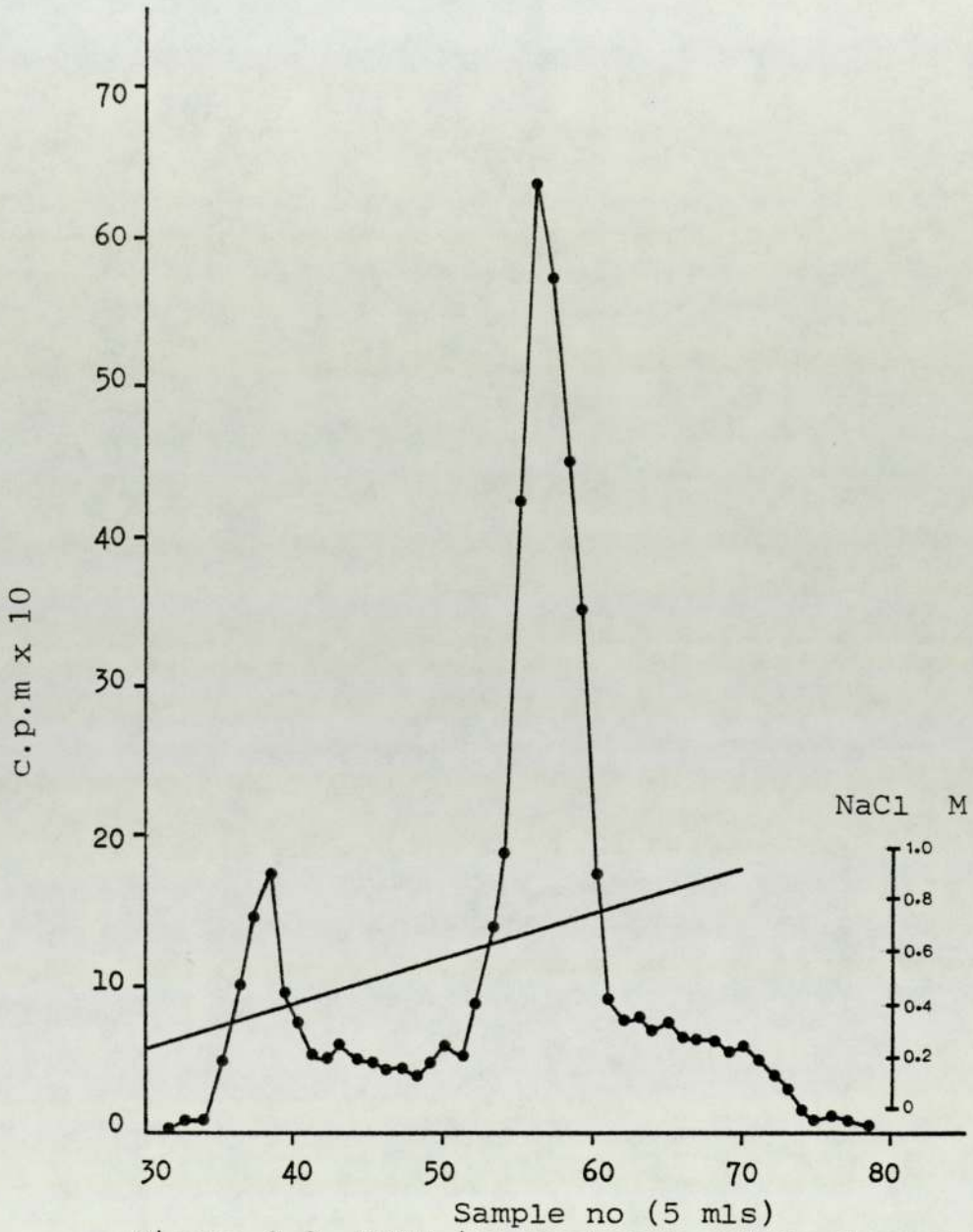


Figure 3.3 DEAE ion-exchange column chromatogram of 24-48 hr urine of young rats receiving an oral dose of 76 microgram/kg carbon-14 labelled folic acid

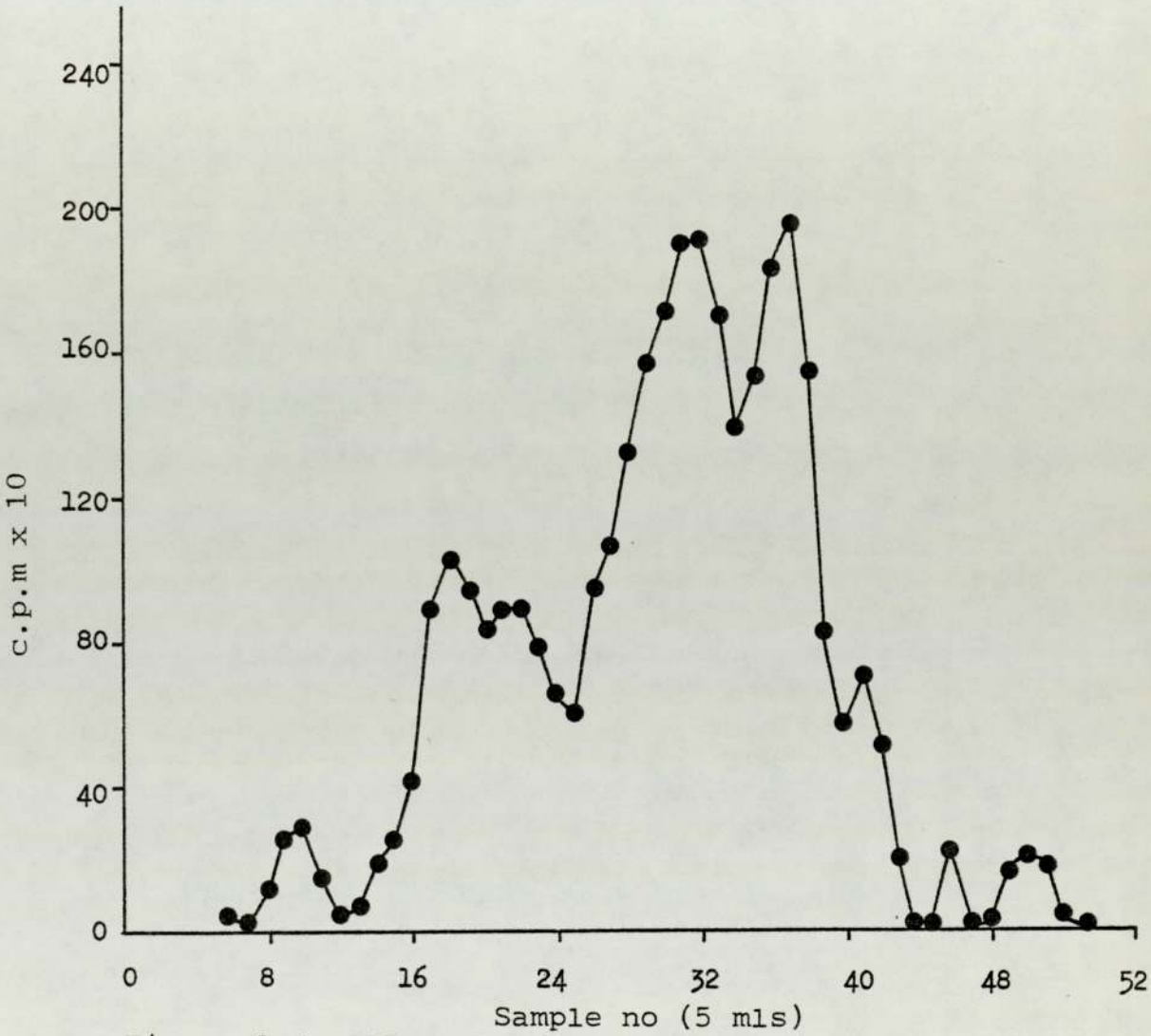


Figure 3.4 G15 sephadex column chromatogram of 0-6 hr urine of young rats receiving an oral dose of 76 microgram/kg carbon-14 labelled folic acid.

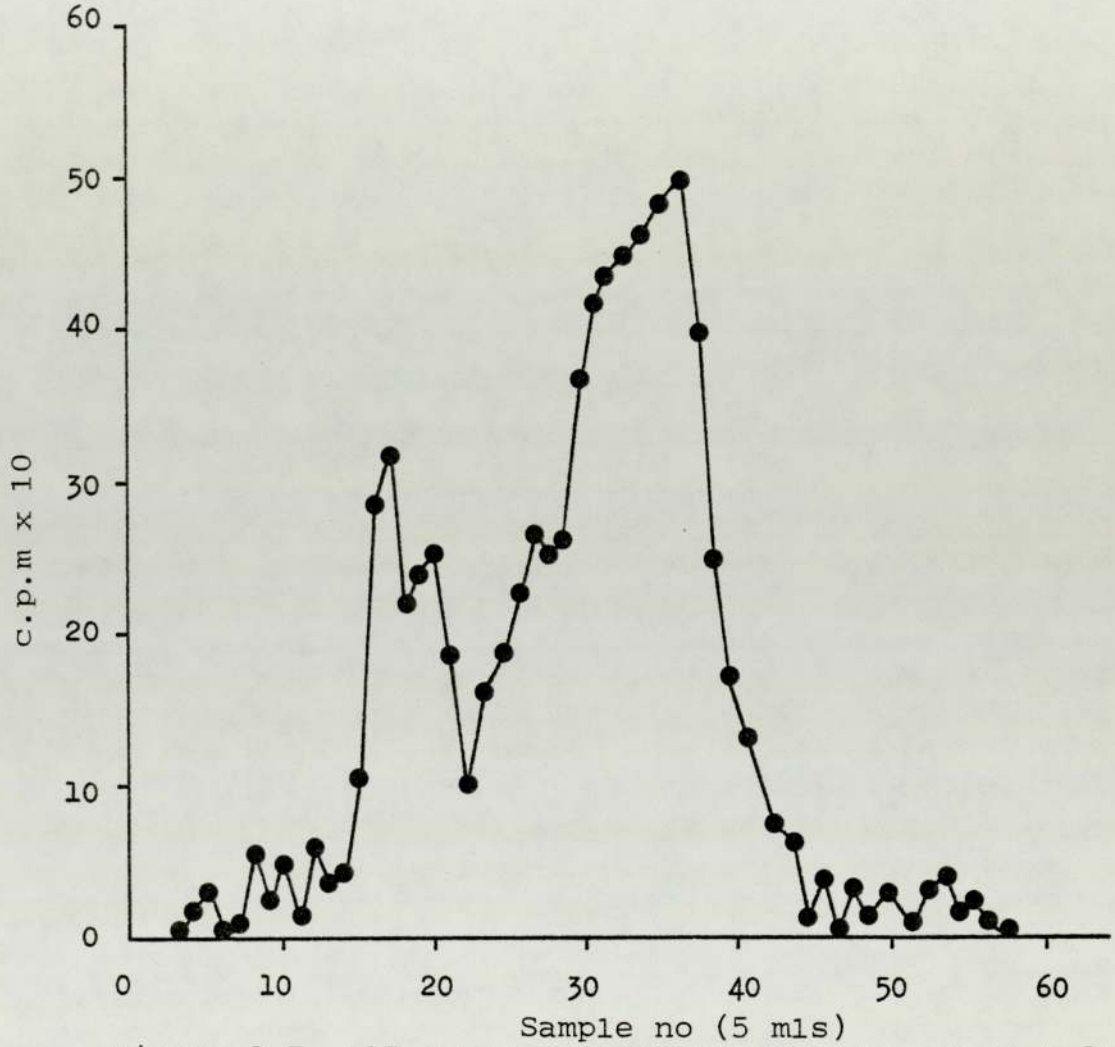


Figure 3.5 G15 sephadex column chromatogram of 6-24 hr urine of young rats receiving an oral dose of 76 microgram/kg carbon-14 labelled folic acid.

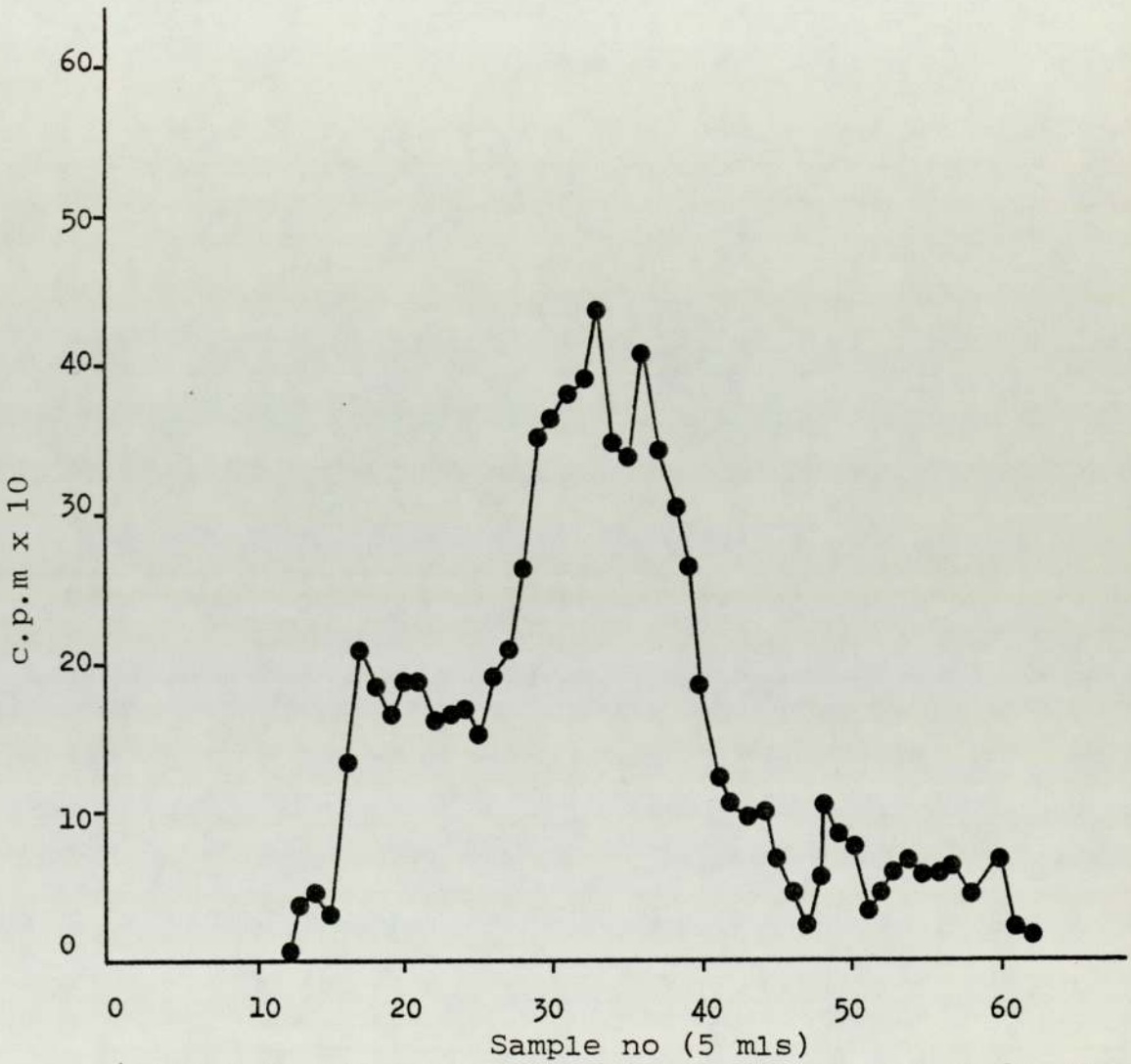


Figure 3.6 G15 sephadex column chromatogram of 24-48 hr urine of young rats receiving an oral dose of 76 microgram/kg carbon-14 labelled folic acid.

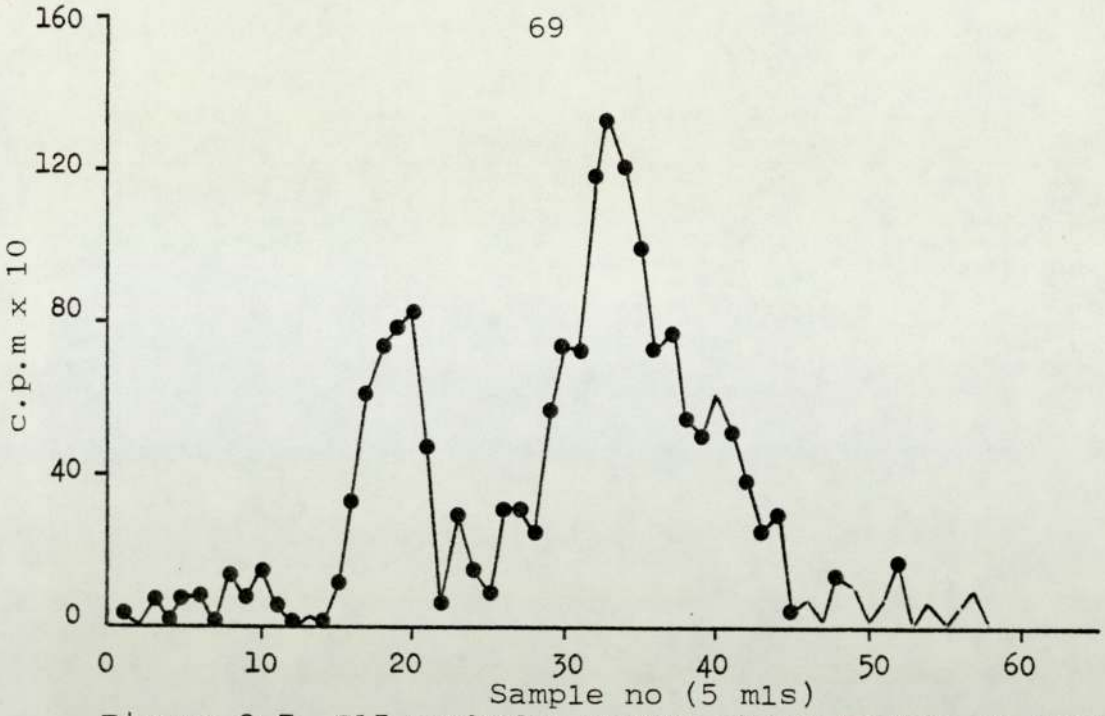


Figure 3.7 G15 sephadex column chromatogram of 'cold' liver extract of young rats receiving carbon-14 labelled folic acid, killed 48 hours after folic acid administration.

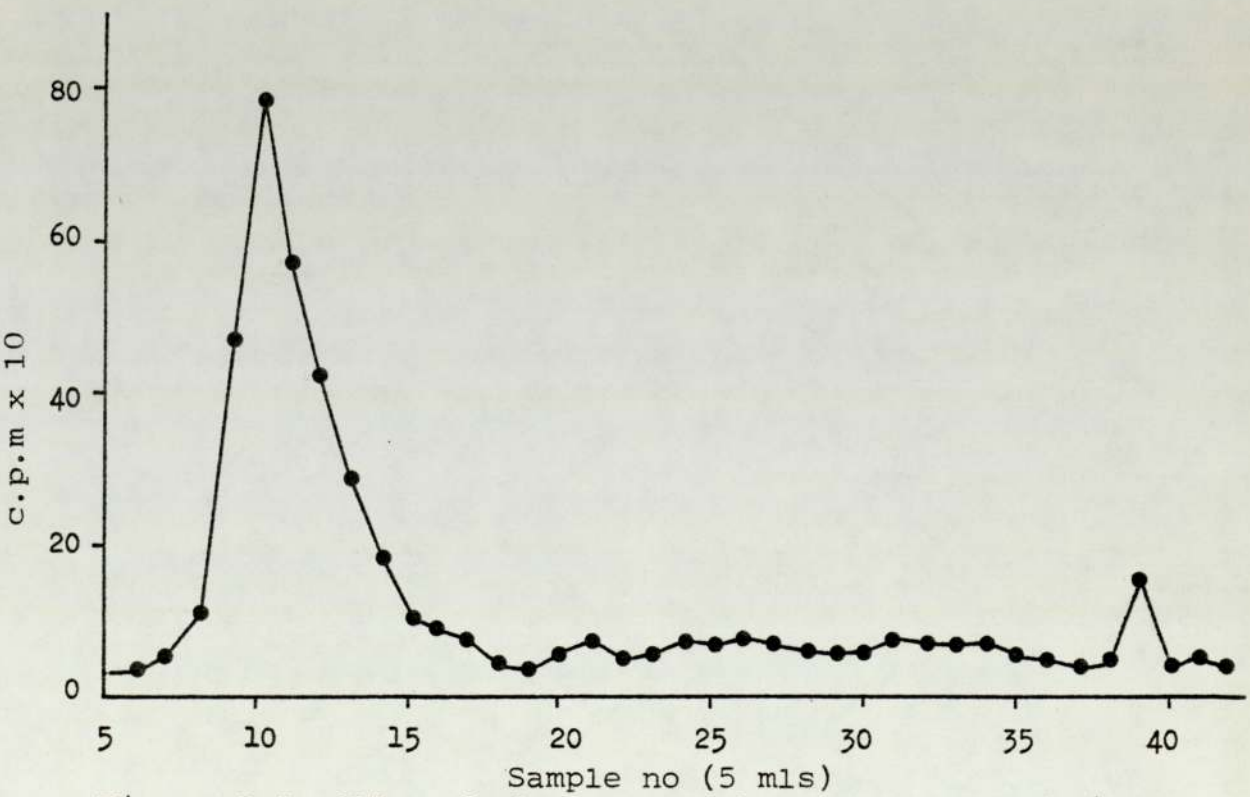


Figure 3.8 G15 sephadex column chromatogram of 'boiled' liver extract of young rats receiving carbon-14 labelled folic acid, killed 48 hours after folic acid administration.

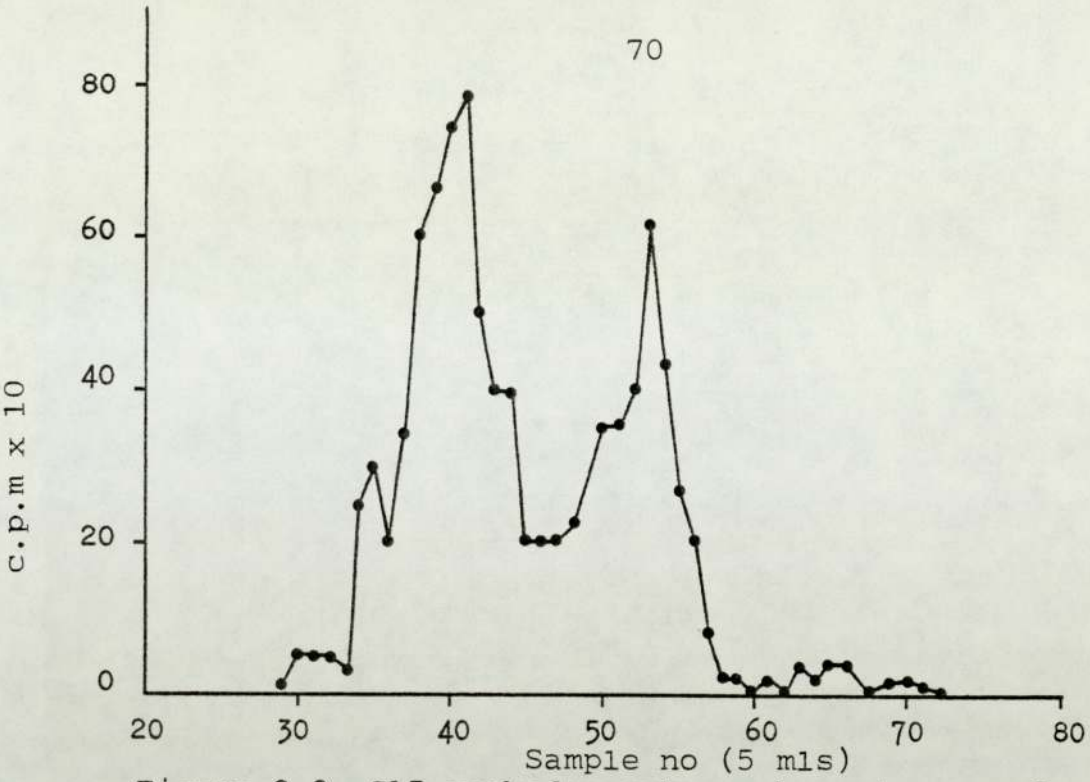
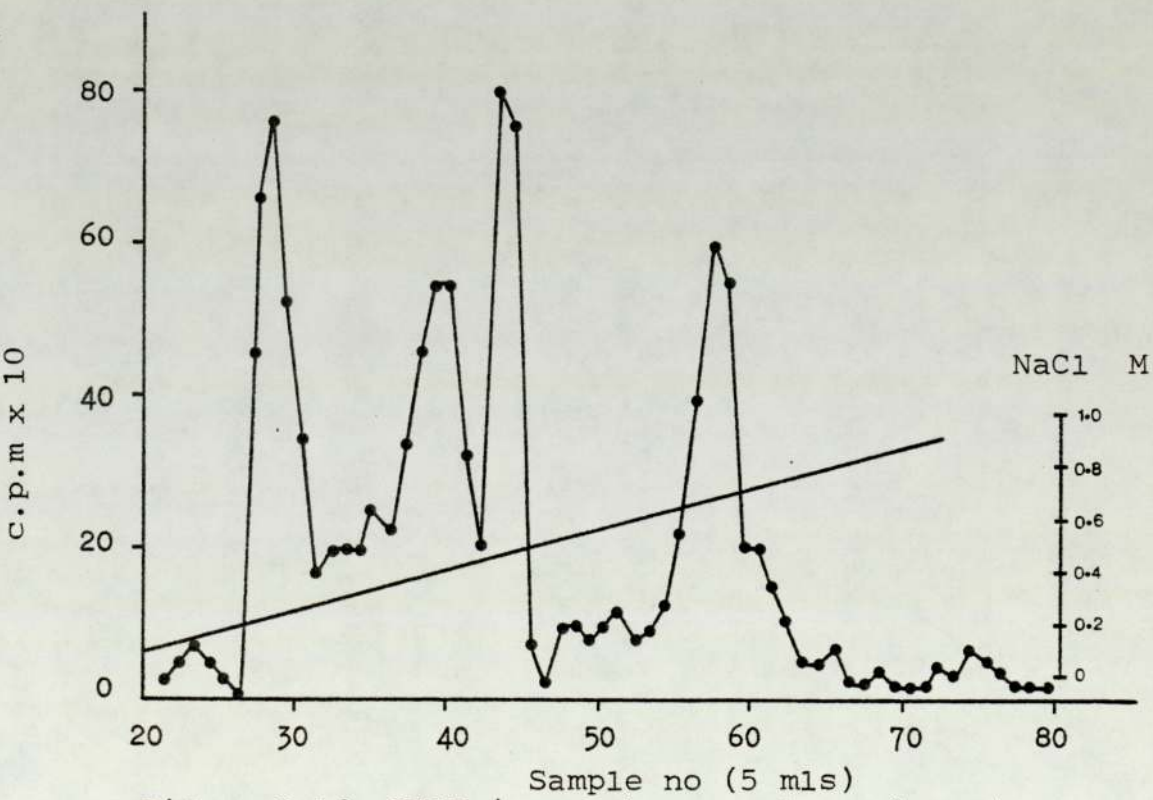


Figure 3.9 G15 sephadex column chromatogram of 'trichloroacetic acid' (TCA) liver extract of young rats receiving carbon-14 labelled folic acid, killed 48 hours after folic acid administration.



Fifure 3.10 DEAE ion-exchange column chromatogram of 'trichloroacetic acid' (TCA) liver extract of young rats receiving carbon-14 labelled folic acid, killed 48 hours after folic acid administration.

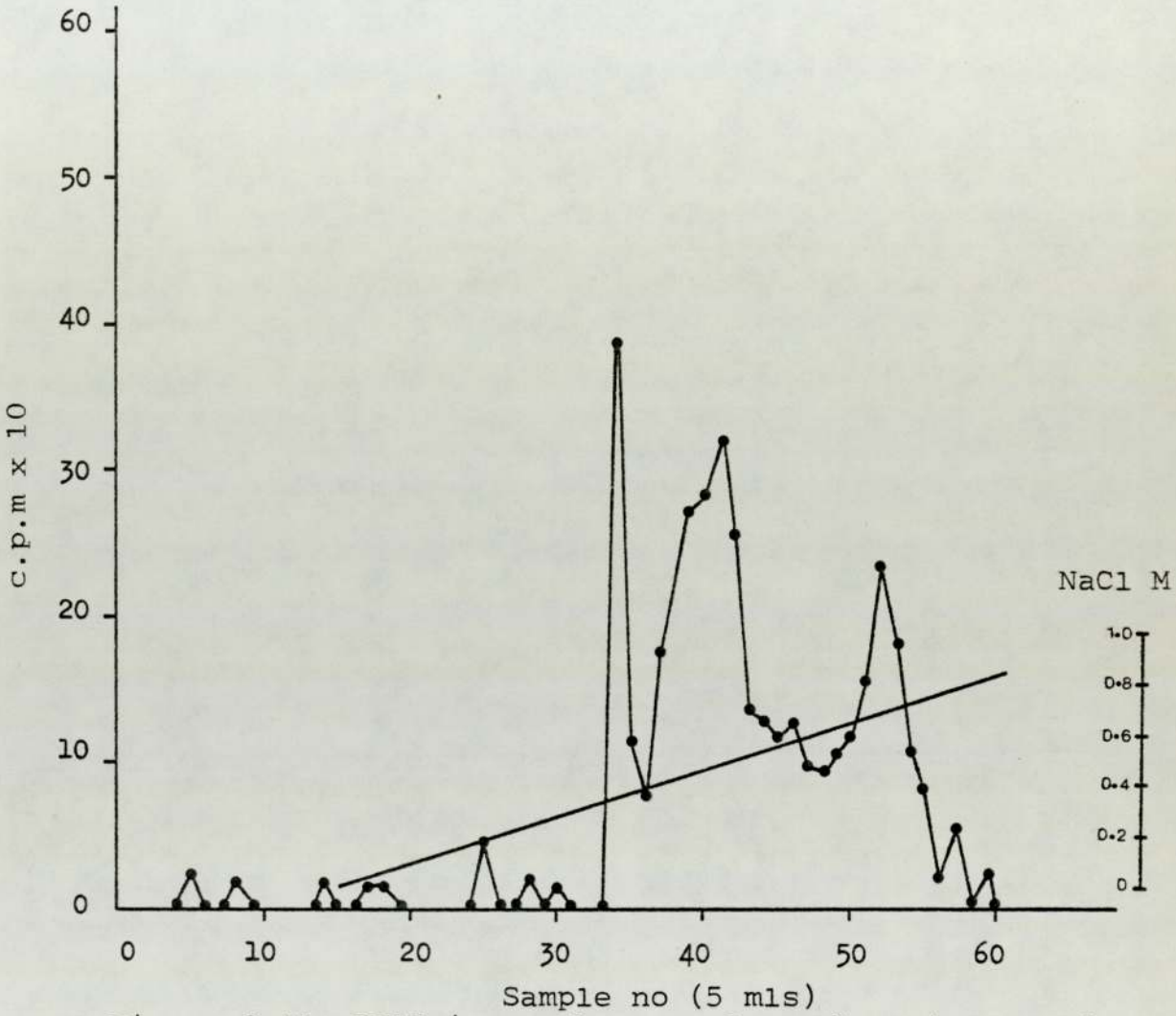


Figure 3.11 DEAE ion-exchange column chromatogram of 'cold' liver extract of young rats receiving carbon-14 labelled folic acid, killed 48 hours after folic acid administration.

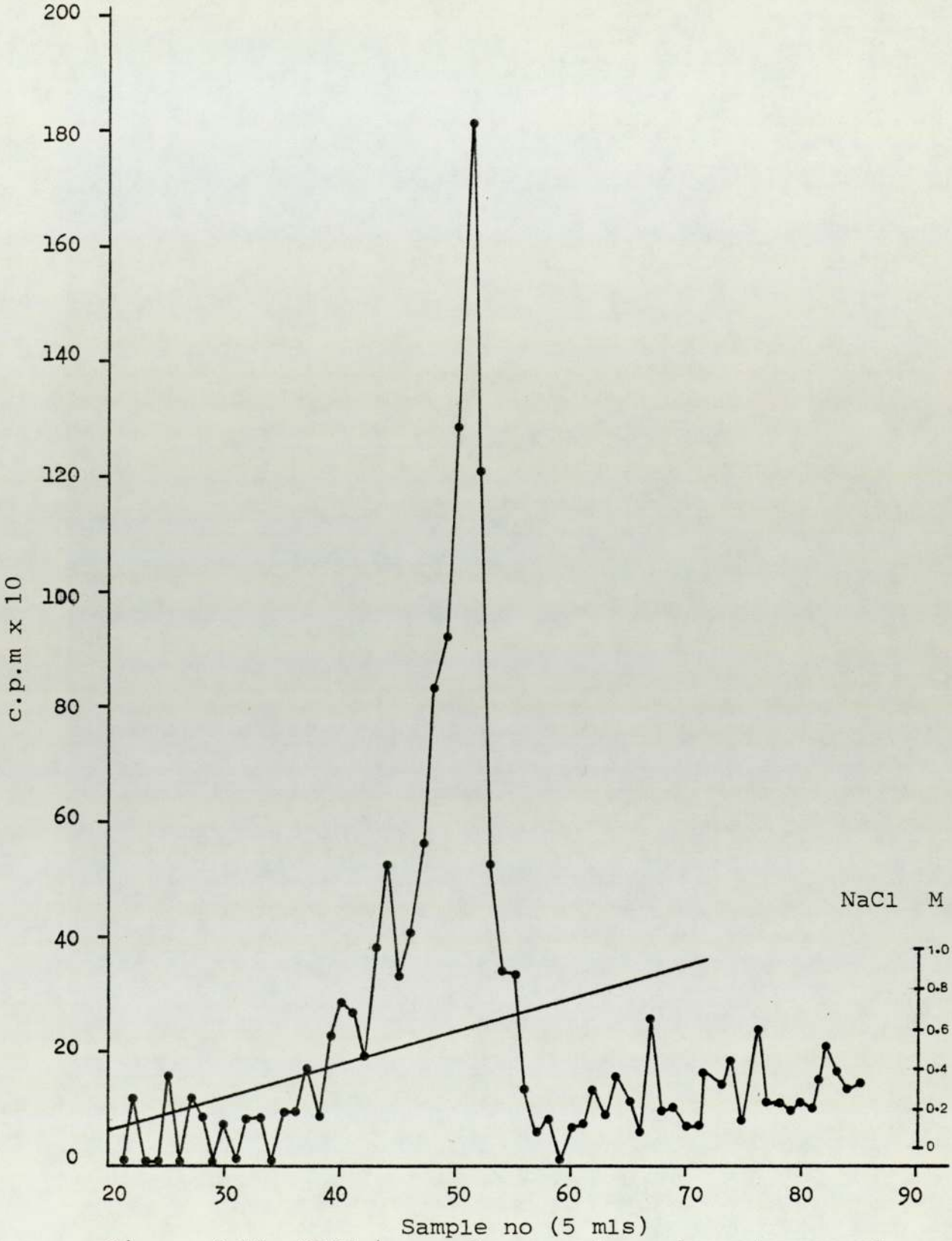


Figure 3.12 DEAE ion-exchange column chromatogram of 'boiled' liver extract of young rats receiving carbon-14 labelled folic acid, killed 48 hours after folic acid administration.

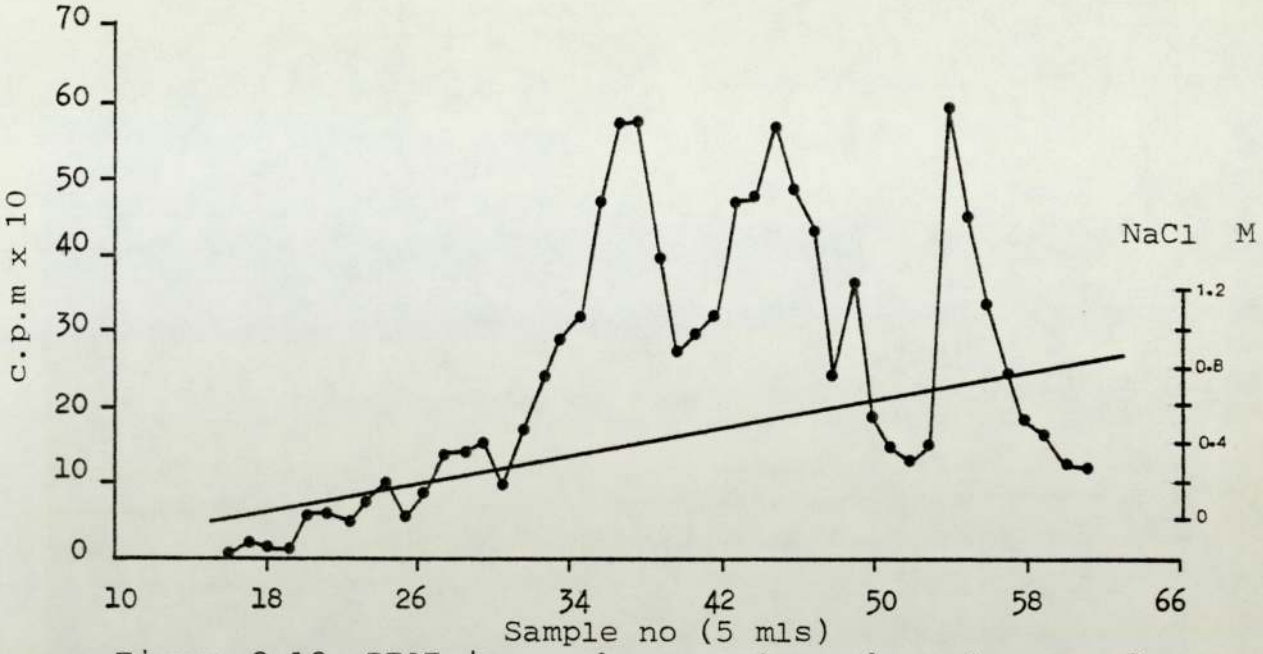


Figure 3.13 DEAE ion-exchange column chromatogram of 'freeze dried' liver extract of young rats receiving carbon-14 labelled folic acid, killed 48 hours after folic acid administration.

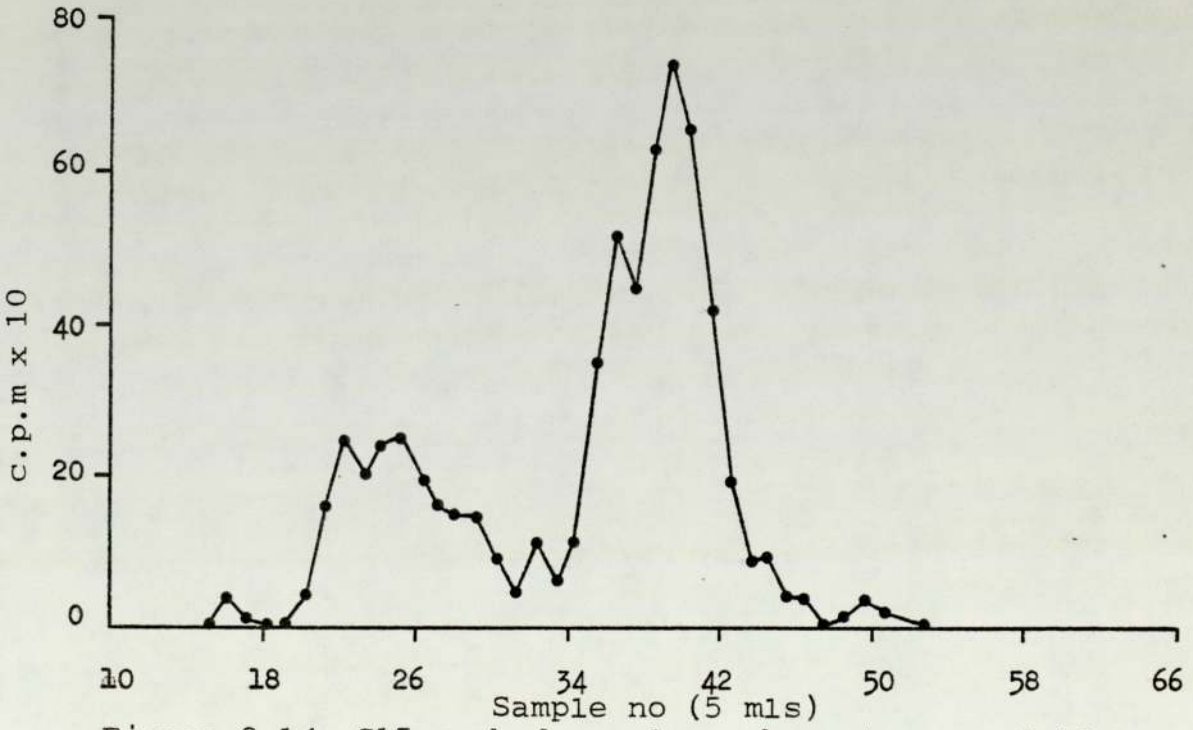


Figure 3.14 G15 sephadex column chromatogram of 'freeze dried' liver extract of young rats receiving carbon-14 labelled folic acid, killed 48 hours after folic acid administration.

Discussion

Experiments documented in this chapter indicate that pattern of metabolism of 2-^{14}C folic acid in three week old male Wistar rats is not very much different from the metabolism of 2-^{14}C folic acid in male adult Wistar rats reported by Barford and Blair (145).

The distribution of radioactivity of an oral dose of 2-^{14}C folic acid (76 microgram/kg) in urine, faeces, liver and gut is given in table 1. Similar distribution of orally dosed 2-^{14}C folic acid (78 microgram/kg) radioactivity in adult male Wistar rats after 48 hours is given in table 2. Comparison of tables 1 and 2 shows that young rats excrete more radioactivity in urine in 48 hours (17.8%) compared to adult rats (13.9%). The excess radioactivity is excreted in 0-6 hours urine sample by young animals (young 8.7%, adult 6.3%).

Radioactivity retained by the liver of young animals is less (11.9%) as compared to adult rats (15.3%). The faeces of young animals in 48 hours contained about 37% of the dosed radioactivity,while faeces of adult rats contained about 44% in the same time period after comparable amounts of 2-^{14}C folic acid administration. This indicates that uptake of 2-^{14}C folic acid in young animals (63%) is higher than in adults (56%). Not significant see page 265

Column chromatography of urine samples

Table 3 shows the distribution of radioactivity in the metabolites present in 0-6, 6-24 and 24-48 hours

urine samples as resolved by DE52 ion-exchange column chromatograms (figures 3.1, 3.2 and 3.3). The distribution expresses percent of the chromatographed radioactivity associated with each metabolite. Table 3 shows that amount of folic acid in urine decreases with time. The urinary radioactivity associated with folic acid in 0-6 hours urine sample is 33.6%, in 6-24 hours urine sample is 9.5% and there is no folic acid in the 24-48 hours urine sample. On the other hand, however, 5-methyltetrahydrofolate increases from 30% in 0-6 and 6-24 hours urine samples to 62.6% in 24-48 hours urine sample. Amount of 10-formyltetrahydrofolate decreases from 25.4% in 0-6 hours urine to 11.5% in 6-24 hours urine. The amount of 10-formyltetrahydrofolate in 24-48 hours urine could not be established.

Barford and Blair (145) observed that 5-methyltetrahydrofolate represents 20% of the urinary radioactivity after 2-^{14}C folic acid administration to adult rats, while it became 40% in 6-24 hours urine sample. They also reported that 10-formyltetrahydrofolate in urine decreases with time. However, the authors reported identification of a new metabolite 4a-hydroxy-5-methyltetrahydrofolate, which could not be identified in the urine of young rats in these experiments.

Column chromatography of liver extracts

G15 sephadex column and DE52 ion-exchange column chromatograms of the liver extracts prepared using different methods show altogether different pictures of the metabolites.

The presence of polyglutamates was observed only in the chromatograms of extracts prepared by 'boiling' method which ensures inactivation of conjugases. G15 sephadex column chromatogram of 'boiled' liver extract (figure 3.8) shows a major peak at the void volume of the column, which is characteristic of polyglutamates. The same peak appeared at higher concentrations of sodium chloride on the gradient on DE52 ion-exchange column (figure 3.12).

CHAPTER 4

METABOLISM OF 2-^{14}C FOLIC ACID IN RAT AFTER ORAL METHOTREXATE

Introduction

Folic acid is converted to dihydrofolate and tetrahydrofolate by the enzyme dihydrofolate reductase.

$$\text{Folic acid} + \text{NADPH} + H^+ \underset{\text{folate reductase}}{\rightleftharpoons} \text{NADP}^+ + \text{7,8, dihydrofolate}$$

$$\text{7,8, dihydrofolate} + \text{NADPH} + H^+ \underset{\text{DHFR}}{\rightleftharpoons} \text{NADP}^+ + \text{5,6,7,8, tetrahydrofolate}$$

The enzyme reduces folic acid to dihydrofolate at a much slower rate than it reduces dihydrofolate. Properties of the enzyme dihydrofolate reductase have been reviewed elsewhere (81,85,117,150,151).

The tetrahydrofolate produced is converted to the co-enzymes which act as a carrier of 'one carbon' units in various vital biosynthetic reactions (see chapter 1). Continuous production of tetrahydrofolate is therefore essential for 'one carbon' unit transfers. Most important of these reactions are those leading to biosynthesis of purines and thymidylate. The co-enzyme for the conversion of deoxyuridylic acid to thymidylic acid is 5-10-methylenetetrahydrofolate.

$$\text{dUMP} + \text{5,10-methylenetetrahydrofolate} \longrightarrow \text{TMP} + \text{dihydrofolate}$$

This reaction is catalyzed by the enzyme thymidylate synthetase which involves both a transfer and reduction of the methylene group (35). Friedkin (152) stated that thymidylate synthetase is an enzyme of central importance

in the metabolism of folates.

The co-enzyme required for purine synthesis is 10-formyltetrahydrofolate. It transfers the formyl group into the 2-position of the purine ring. 10-formyltetrahydrofolate reacts with 5-amino-4-imidazole carboxamide ribonucleotide to produce 5-formamido-4-imidazole carboxamide ribonucleotide.

5-amino-4-imidazole carboxamide ribonucleotide + 10-formyltetrahydrofolate $\rightleftharpoons$ 5-formamido-4-imidazole carboxamide ribonucleotide + tetrahydrofolate

The enzyme which catalyzes this reaction is 5-phosphoribosyl-5-formamido-4-imidazole carboxamide tetrahydrofolate 10-formyltransferase which utilises 10-formyltetrahydrofolate specifically as a formyl donor in the above reaction (13). The product 5-fomamido-4-imidazole carboxamide ribonucleotide is converted to inosinic acid via ring closure.

Methotrexate is a synthetic analog of folic acid and has been used in the treatment of various cancers (153). Although the mechanism of its action as antifolate in the isolated cellular system has been studied to a large extent, little work has been done on the physico-chemical interactions with the folate metabolism in the whole living mammalian system. The need for such 'in vivo' studies is inevetable, however, for the understanding of its selective toxicity for certain neoplasms over normal tissue.

Cell division is stopped by methotrexate (163).

It has been concluded that the enzyme dihydrofolate reductase is inhibited by methotrexate and hence prevents the reduction of folic acid to dihydrofolate and dihydrofolate to tetrahydrofolate by binding to the enzyme dihydrofolate reductase (154,155).

Administration of a single dose of methotrexate to rats has resulted in the retention of methotrexate in the tissue, perhaps bound to dihydrofolate reductase, in small amounts for many months (160,161,162).

Methotrexate toxicity is reversed by 'citrovorum factor' (5-formyltetrahydrofolate) and this rescue is effective given before or after methotrexate administration (100,156,157,158,159). Folic acid pretreatment is also able to protect against the toxicity of a single dose of methotrexate (164).

It was hoped that the experiments documented in this chapter would help in the better understanding of the folate metabolism in the presence of methotrexate in the rat and thereby enable us to investigate those sites of its action which are involved with the folate metabolism.

A single methotrexate oral dose (10mg/kg body weight) was given to the rats 24 hours prior to 2-^{14}C folic acid (70 microgram/kg body weight) oral dose. The control animals were orally dosed with the same quantity of 2-^{14}C folic acid only. Both groups of animals were kept under similar conditions. The urine samples and the liver extracts were chromatographed on DE52 ion-exchange and G15 sephadex columns for the identification of radioactive

metabolites. The amounts of dosed radioactivity distributed in urine, faeces, liver and gut was estimated.

Materials and Methods

2-^{14}C folic acid was purchased from the Amersham Radio Chemical Centre. Methotrexate was purchased from the Lederle Chemical Company. All the rest of the chemicals used in the making of buffer solutions, scintillation cocktails and antioxidants were of Analar grade.

A group of 18 male adult Wistar rats weighing 150-200 gms received methotrexate 10mg/kg body weight orally in water suspension. A group of 8 male adult Wistar rats of the same weight and age as the group of rats mentioned above received same quantity of water orally without methotrexate. Both groups of animals were then dosed orally with 2-^{14}C folic acid (76 microgram/kg body weight) 24 hours after methotrexate and water dosing. The animals were singlely housed in metabolic cages (Jencons metabowls), food and water was supplied ad libitum.

Six of the methotrexate dosed animals were killed 48 hours after 2-^{14}C folic acid administration and the rest of all the animals were killed after 24 hours of the same dose.

Collection of biological materials

Urine samples

The urine samples were collected in flasks containing 5 mls of 0.05 Molar phosphate buffer pH 7 containing 5 gms of ascorbate/100 mls and 5 mg/100 mls of dithiothreitol. The flasks were changed six hours after 2-^{14}C folic acid dose for the rats killed after

24 hours of the dose and were changed after 6 and 24 hours for the rats killed after 48 hours of the same dosing.

The total radioactivity in all the urine samples was determined by counting 50 micro-litres of each sample using 10 mls of standard cocktail composed of Fisons emulsifier E 250 mls, 2,5-diphenyloxazole 2.5 gms and toluene 500 mls.

Faeces

The faeces for all times between 2-^{14}C folic acid dose and killing the animals were collected in empty flasks and freeze dried. The total radioactivity in the faeces was determined by oxidising the known quantities of powdered freeze dried samples in a Beckman Biological Material Oxidiser. The $^{14}CO_2$ was collected in 15 mls of Fisons absorber. The same oxidation of "blanks" and standards was repeated. The samples were counted for the radioactivity in Nuclear Enterprise liquid-scintillation counter NE8310. Quenching errors were removed with the help of appropriate quenching curves.

Gut and liver

All the gut and livers were immidiately removed after killing the animals. The gut and some of the livers were subjected to the same type of burning and counting after freeze drying the samples for the estimation of radioactivity, as in the case of faeces. Some of the livers were extracted for the quantitative analysis of the radioactive substances by column chromatography. Three different methods of extraction were applied. "Hot" extracts

were prepared by putting the small divided pieces of livers into 15 mls of boiling 0.05 M phosphate buffer pH 7 containing 5 gms/100 mls ascorbate and 5 mg/100 mls dithiothreitol. The contents were left boiling for ten minutes. The solid protein part of liver was rejected after centrifugation. Cold extracts were made by homogenizing the fresh liver in 15 mls of 0.05 M phosphate buffer pH 7 containing 5 gms/100 mls ascorbate and 5 mg/100 mls dithiothreitol. The solid material was rejected after centrifugation. The third method applied was similar to the cold extract but the protein was removed by using 10% trichloroacetic acid, and the pH of the extract was brought back to pH 7 by adding sodium hydroxide solution.

Column chromatography of liver extracts and urine samples

The urine and liver extract samples were applied to DE52 ion-exchange and G15 sephadex columns. The 5 mls fractions were collected using automatic LKB fraction collector. The radioactivity in each fraction was determined, and the total recovered radioactivity in the fractions was usually close to the amount of radioactivity put on the column.

RESULTS

Recovery of radioactivity

Tables 1,2 and 3 show the percentage of the dosed radioactivity recovered in the urine, faeces, liver and gut of the animals. Table 1 gives the amounts of the total percent radioactivity recovered in the above mentioned samples of the control rats killed 24 hours after 2-^{14}C folic acid administration. Table 3 shows the same for methotrexate treated animals, while table 2 gives the amounts of percent radioactivity found in the same biological samples of the rats treated with methotrexate and killed 48 hours after 2-^{14}C folic acid oral dose.

The control animals on average excreted 8.6% of the dosed radioactivity in 0-6 hours and 8.6% again in 6-24 hours urine samples (table 1). Table 3 shows that the methotrexate treated animals on average excreted 13.6% and 8.2% of the dosed radioactivity in the urine samples between 0-6 hours and 6-24 hours respectively.

Comparing the above results it can be seen that the amount of radioactivity recovered in the 0-6 hours urine of methotrexate treated animals is 4.9% greater than the amount of radioactivity recovered in the urine of control animals collected during the same period. The urine of both control and methotrexate treated animals collected between 6-24 hours, however, show no significant difference in the radioactive content.

Table 2 shows the distribution of the dosed radioactivity in the biological samples of methotrexate treated animals killed 48 hours after 2-^{14}C folic acid

administration. The 0-6 hours urine of these animals on average contained 25.4% of the dosed radioactivity, which is 16.8% greater than the amount of radioactivity in the corresponding urine of control animals. 6.6% of the dosed radioactivity was excreted in the urine collected between 6-24 hours, while 3.4% was excreted in the 24-48 hours urine sample.

The radioactivity recovered in the 0-24 hours faeces of control animals on average was 36.3% of the dosed radioactivity, while the same for the methotrexate treated animals of table 3 was 42.5%. The two figures perhaps cannot be compared as four of the methotrexate treated animals did not have any faeces. On the other hand, the percent radioactivity recovered in the 0-48 hours faeces of methotrexate treated animals killed 48 hours after 2-^{14}C folic acid administration was only 22.3% (table 2).

The average radioactivity recovered from the livers of four control animals 13.1% (table 1). 3.8% and 5.3% of the dosed radioactivity was found in the liver of the animals treated with methotrexate and killed 48 hours and 24 hours after an oral dose of 2-^{14}C folic acid respectively. This indicates the decreased uptake of 2-^{14}C folic acid in presence of methotrexate in the liver. The percent recovery of radioactivity in the gut of control animals was 4.8% (table 1) and the same for the methotrexate treated animals was 2.8% (table 3).

Constipation was generally observed in the case of methotrexate treated animals in these experiments and

was remarkable in the animals of table 2, which perhaps explains the low amounts of radioactivity appearing in the faeces, as the dry weight of faeces was less than it was in the case of control animals. But on the other hand, comparison of tables 1 and 3 suggests that the passage of 2-^{14}C folic acid across the gastro-intestinal tract in methotrexate treated animals has been curtailed by the methotrexate already present in the gut. The excessive amounts of radioactivity appearing in 0-6 hours urine samples of methotrexate treated animals, might be due to the less uptake of 2-^{14}C folic acid in tissues of the animal.

Column chromatography of the urine samples

The 0-6 and 6-24 hours samples of the control and methotrexate treated animals after an oral dose of 2-^{14}C folic acid was chromatographed on DE52 ion-exchange (DEAE cellulose) and G15 sephadex columns.

Figure 4.1 shows a 0-6 hours urine sample DE52 chromatogram of control rats. The metabolites are labelled as 1,2,3,4 and 5. On the basis of elution position on the sodium chloride gradient and co-chromatography, metabolites 1 to 4 represent folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and 4a-hydroxy-5-methyltetra-hydrofolate respectively.

Figure 4.2 shows a DE52 chromatogram of 6-24 hours urine sample of the same rats. Table 4 shows the distribution of the radioactivity among the metabolites expressed as a percentage of the total radioactivity

chromatographed on each of the above mentioned columns. It shows that folic acid quantity decreases in the 6-24 hours urine sample, while 5-methyltetrahydrofolate increases with time. 10-formyltetrahydrofolate on the other hand decreases in quantity with time. 6-24 hours urine sample also show the increased amounts of 4a-hydroxy-5-methyltetrahydrofolate.

Figure 4.4 shows a DE52 chromatogram of 0-6 hours urine of methotrexate treated animals. Figure 4.3 is a DE52 chromatogram of 6-24 hours urine of the same rats. Table 4 gives the approximate distribution of the metabolites as a percentage of the chromatographed total activity on the columns. Metabolites are labelled as 1,2,3 and 4 in the chromatogram of 0-6 hours urine (figure 4.4) and 1,3 and 4 in the chromatogram of 6-24 hours urine (figure 4.3).

Studying the elution position of these metabolites in the light of the calibrations with authentic materials and co-chromatography, suggests that the metabolites 1 to 4 are folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and 4a-hydroxy-5-methyltetrahydrofolate respectively. Similarly the metabolites in the case of 6-24 hours urine are 10-formyltetrahydrofolate (peak 3) and most probably 4a-hydroxy-5-methyltetrahydrofolate (peak 4).

Comparing the two chromatograms (figures 4.3 and 4.4), it is apparent that 5-methyltetrahydrofolate has disappeared in the 6-24 hours urine and folic acid has decreased, while the amount of 10-formyltetrahydrofolate

has increased and it appears that it is the only major metabolite of 2-^{14}C folic acid in the urine of methotrexate treated animals after six hours.

Comparison of the 0-6 hours urine chromatograms of normal and methotrexate treated animals (figures 4.1 and 4.4), indictes crucial differences in the amounts of already mentioned metabolites. The amount of folic acid in the methotrexate treated animals is very high as compared to the control animals urine in the first six hours. All but metabolite number 5 appeared in the 0-6 hours urine of the methotrexate treated animals, though in very small quantities as compared to the 0-6 hours urine of control rats in the DE52 column chromatograms. Sephadex G15 chromatography of the urine samples was not very helpful in the identification, as the columns did not separate the metabolites. The G15 chromatograms are shown in figures 4.5 and 4.6

Column chromatography of liver extracts

The liver extracts of both control and methotrexate treated animals were chromatographed on DE52 and G15 sephadex columns. Most of the liver extracts from liver of methotrexate treated animals failed to give any prominent radioactive peaks, as the dilution is inevitable in the making of liver extracts. The small amount of radioactivity present in the liver of methotrexate treated animals is perhaps the main reason for not obtaining any detectable peaks in the chromatograms.

Figure 4.7 shows a DE52 ion-exchange column chromatogram of liver extract of control animals. The extract was prepared by hot extraction procedure (see chapter 2). Figure 4.8 is G15 sephadex column chromatogram of the same liver extract.

Figure 4.9 is DE52 ion-exchange column chromatogram of liver extract of control animals. Extract was prepared by hot extraction procedure, however, no anti-oxidants were used. Figure 4.10 is G15 sephadex column chromatogram of the same liver extract.

Figure 4.11 shows a G15 sephadex column chromatogram of 'boiled' liver extract of rats receiving methotrexate before 2-^{14}C folic acid administration. Figure 4.12 is DE52 ion-exchange column chromatogram of the same liver extract.

Rat No	Urine		Faeces	Liver	Gut	Total
	0-6 hrs	6-24 hrs				
1	8.2	2.3	30.7	15.6	3.2	60.0
2	4.9	5.9	29.0	8.2	6.3	54.3
3	8.3	30.1	24.3	11.2	5.8	79.7
4	11.1	5.2	47.3	17.6	4.3	85.5
5	7.2	4.2	37.2	-	2.0	-
6	16.1	2.9	39.9	-	3.4	-
7	3.6	13.9	40.1	-	5.9	-
8	9.3	4.5	42.2	-	7.5	-
Average	8.6	8.6	36.3	13.1	4.8	69.9

Table 1 Distribution of radioactivity in urine, faeces, liver and gut (expressed as percentage of 2-^{14}C folic acid dosed) of control animals killed 24 hours after 2-^{14}C folic acid administration.

Rat No	Urine			Faeces	Liver	Gut	Total
	0-6 hrs	6-24 hrs	24-48 hrs				
1	21.3	9.8	2.6	17.7	2.8	4.1	58.3
2	30.5	3.3	2.9	11.0	4.7	1.8	54.2
3	33.0	4.1	1.9	-	3.6	5.3	-
4	28.3	3.1	8.4	12.1	3.2	2.7	57.8
5	20.6	5.0	2.0	41.2	5.1	1.7	75.6
6	18.9	14.1	2.5	29.4	3.2	2.4	70.5
Average	25.4	6.6	3.4	22.3	3.8	3.0	63.3

Table 2 Distribution of radioactivity in urine, faeces, liver and gut (expressed as percentage of 2-^{14}C folic acid dosed) of methotrexate treated animals killed 48 hours after 2-^{14}C folic acid administration.

Rat No	Urine		Faeces	Liver	Gut	Total
	0-6 hrs	6-24 hrs				
1	30.7	21.7	-	-	3.0	-
2	17.4	7.1	42.3	-	1.6	-
3	18.0	18.9	-	-	2.9	-
4	14.1	2.5	50.2	6.2	1.0	74.0
5	14.2	2.5	38.7	6.4	2.0	63.8
6	16.7	6.6	49.3	4.9	2.8	80.3
7	3.7	4.9	60.9	3.9	1.4	74.8
8	9.6	8.2	-	-	7.9	-
9	23.0	9.2	-	-	-	-
10	5.6	4.5	33.8	-	-	-
11	6.2	6.5	19.2	-	-	-
12	3.3	5.5	45.6	-	-	-
Average	13.5	8.2	42.5	5.3	2.8	73.2

Table 3 Distribution of radioactivity in urine, faeces, liver and gut (expressed as percentage of 2-^{14}C folic acid dosed) of methotrexate treated animals killed 24 hours after 2-^{14}C folic acid administration.

Chromatograms	Metabolites				
	1	2	3	4	5
Fig 4.1	42.6	18.6	15.5	15.5	7.8
Fig 4.2	12.7	38.1	16.1	26.7	6.4
Fig 4.3	25.0	-	58.3	16.7	-
Fig 4.4	72.2	11.5	9.5	6.8	-

Table 4 Distribution of radioactivity in metabolites expressed as a percentage of the total radioactivity chromatographed.

The metabolites numbered as 1 to 4 in the above table appeare at authentic marker sites of folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and 4a-hydroxy-5-methyltetrahydrofolate respectively. The metabolite numbered as 5 has not been identified.

Chromatograms	Metabolites				
	1	2	3	4	5
Fig 4.1	3.7	1.6	1.3	1.3	0.7
Fig 4.2	1.1	3.3	1.4	2.3	0.6
Fig 4.3	2.0	-	4.8	1.4	-
Fig 4.4	9.7	1.6	1.3	1.0	-

Table 5 Distribution of radioactivity in metabolites expressed as a percentage of the total radioactivity dosed (i.e. as a percentage of the total 2-^{14}C folic acid dosed).

The metabolites numbered as 1 to 4 in the above table appear at authentic marker sites of folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and 4a-hydroxy-5-methyltetrahydrofolate respectively. The metabolite numbered as 5 has not been identified.

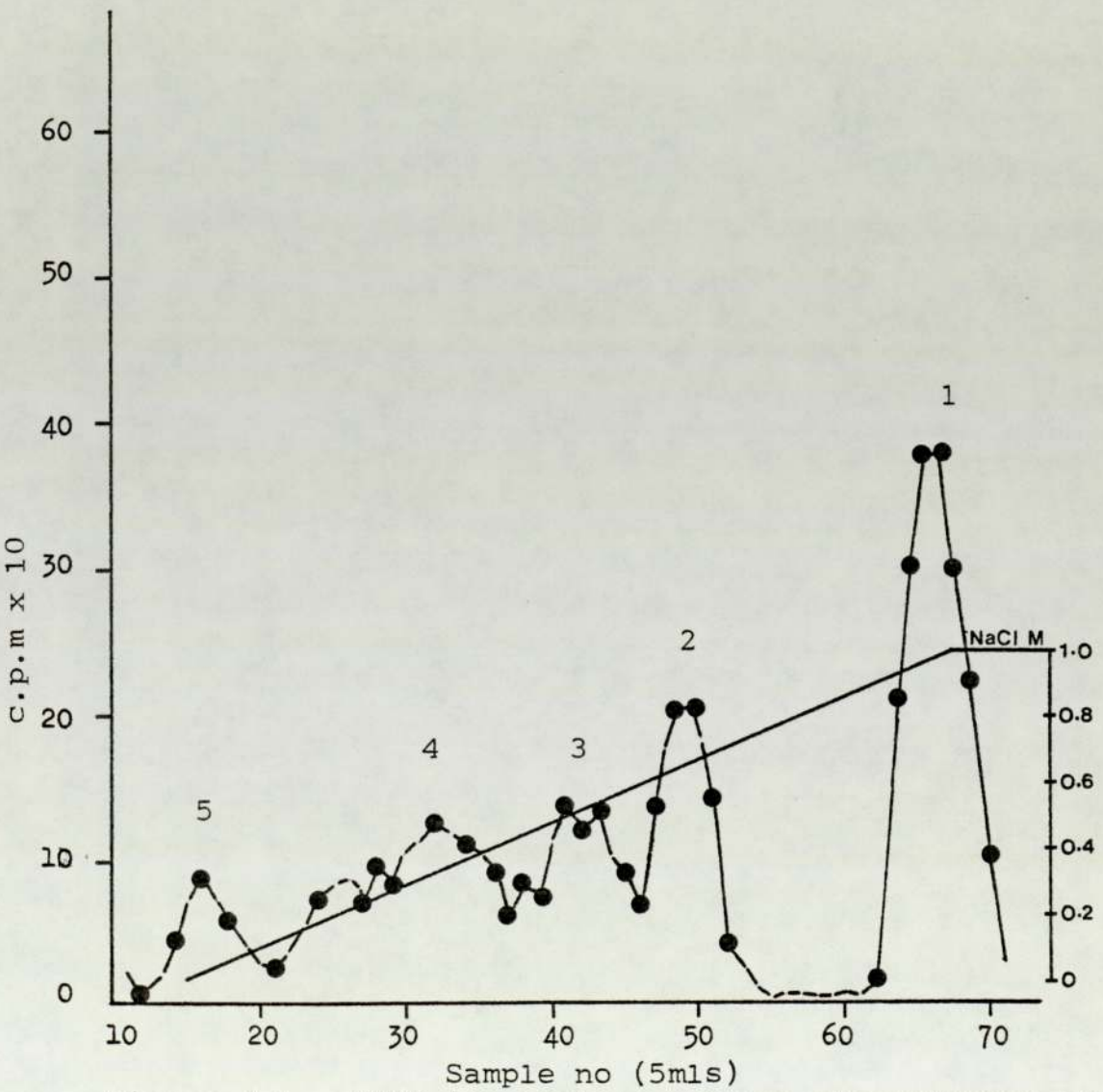


Figure 4.1 DEAE ion-exchange column chromatogram of 0-6 hr urine of rats receiving carbon-14 labelled folic acid.

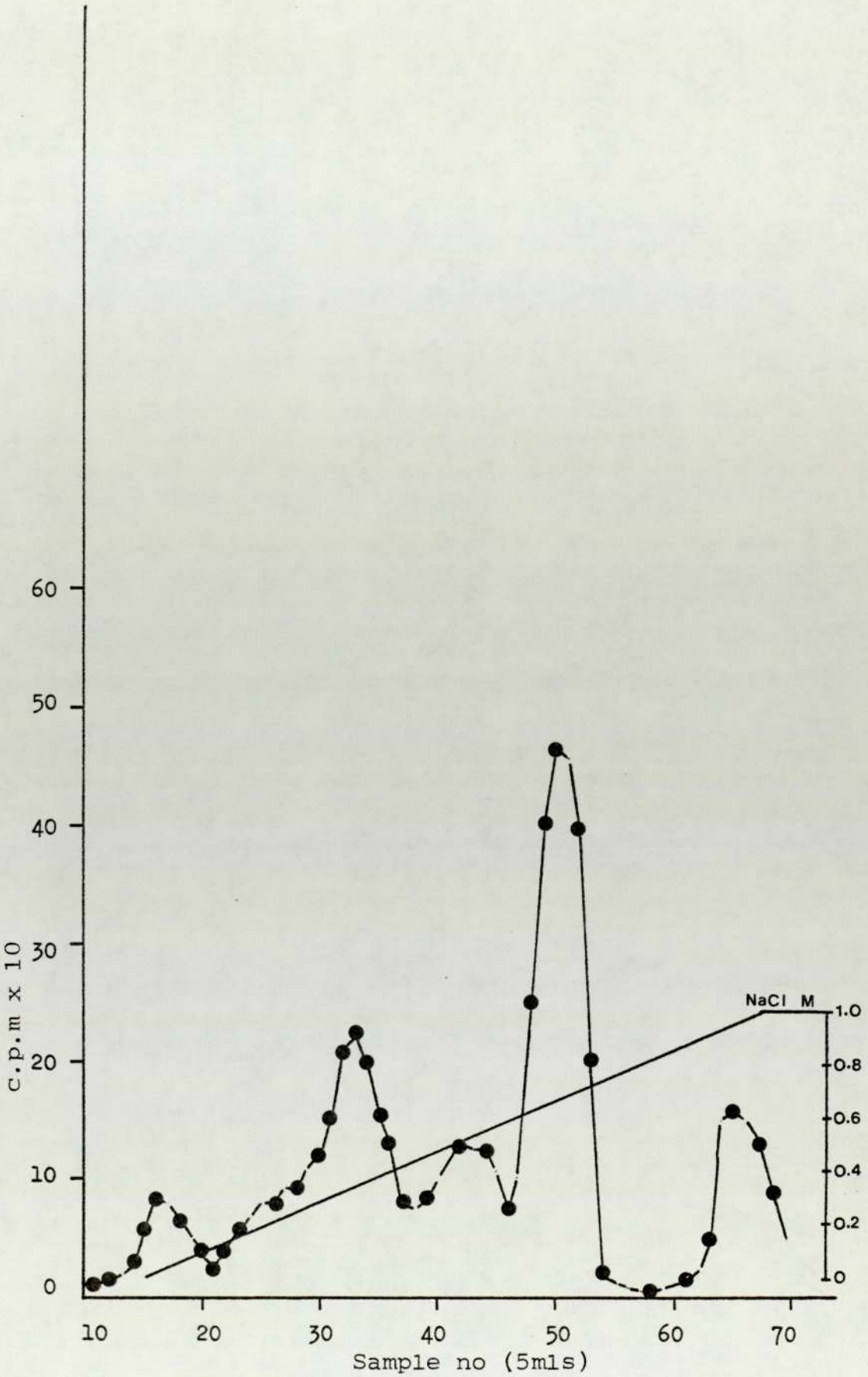


Figure 4.2 DEAE ion-exchange column chromatogram of 6-24 hr urine of rats receiving carbon-14 labelled folic acid.

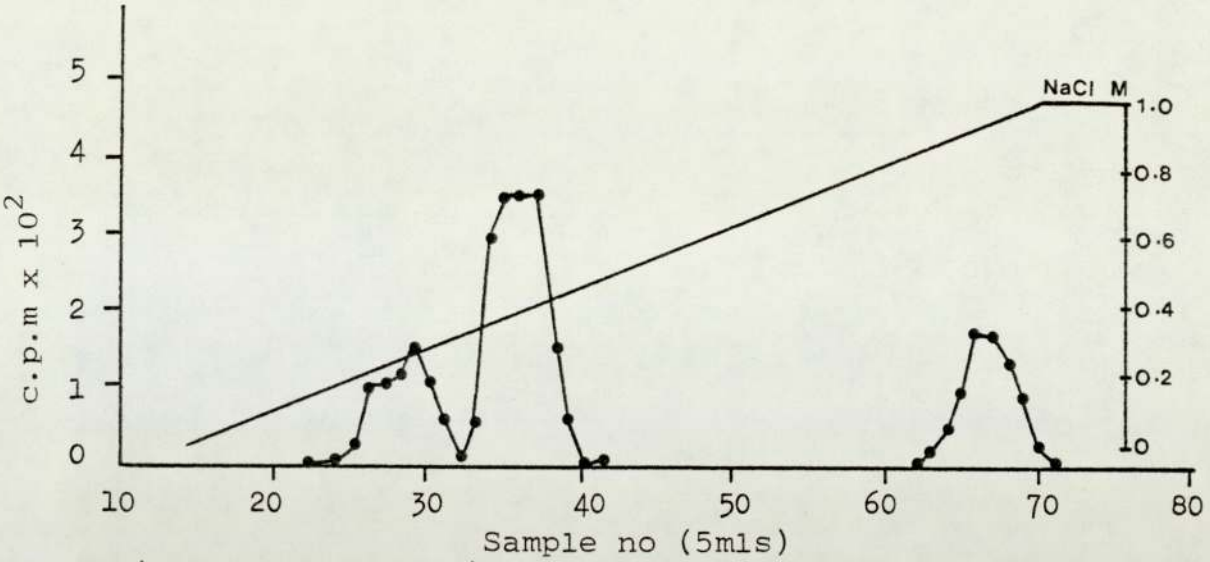


Figure 4.3 DEAE ion-exchange column chromatogram of 6-24 hr urine of rats receiving carbon-14 labelled folic acid 24 hours after methotrexate administration.

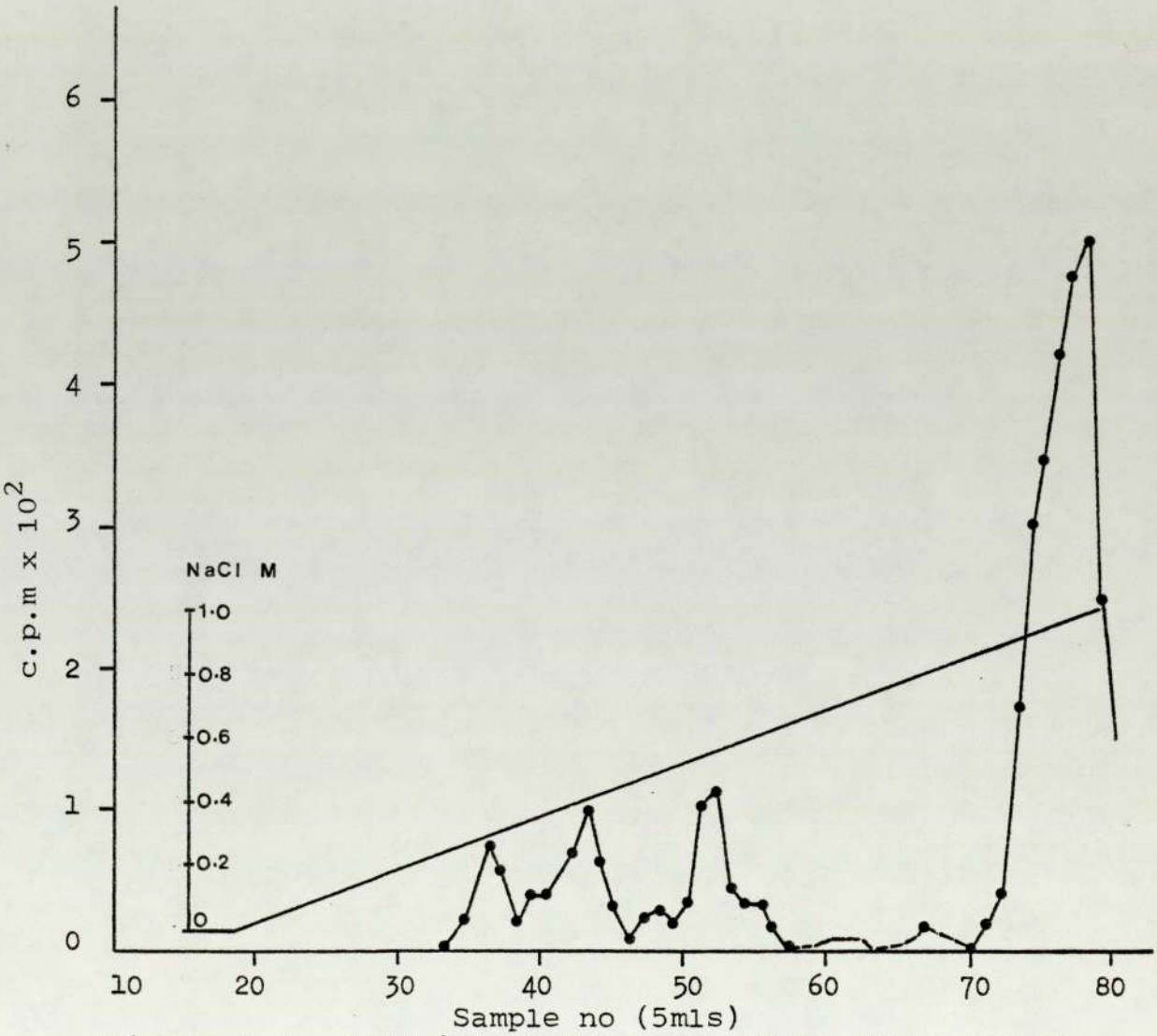


Figure 4.4 DEAE ion-exchange column chromatogram of 0-6 hr urine of rats receiving carbon-14 labelled folic acid 24 hours after methotrexate administration.

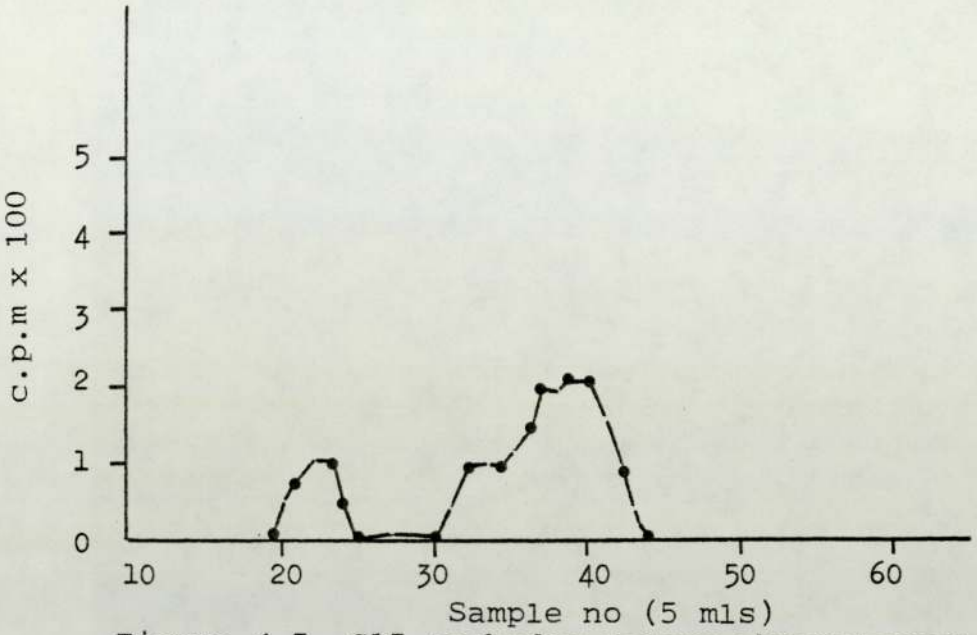


Figure 4.5 G15 sephadex column chromatogram of 6-24 hr urine of rats receiving carbon-14 labelled folic acid 24 hours after methotrexate administration.

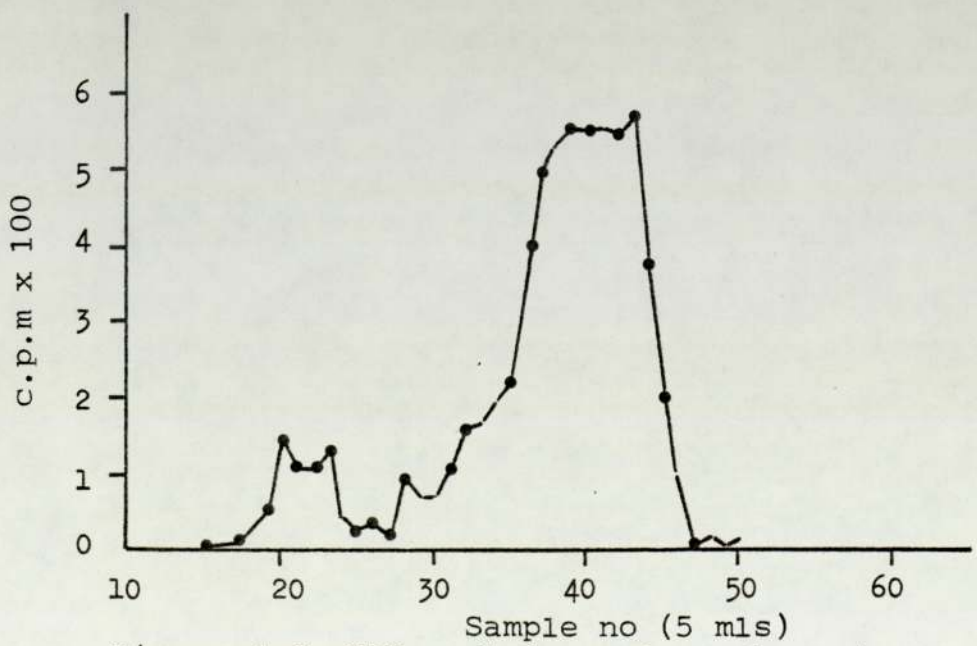


Figure 4.6 G15 sephadex column chromatogram of 0-6 hr urine of rats receiving carbon-14 labelled folic acid 24 hours after methotrexate administration.

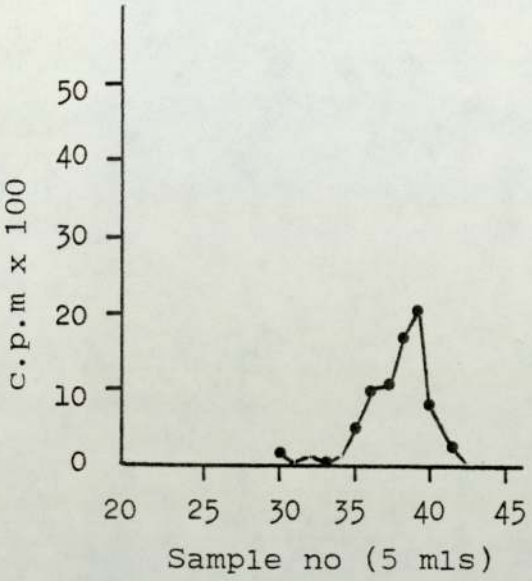


Figure 4.7

DEAE ion-exchange column chromatogram of 'boiled' liver extract of rats receiving carbon-14 labelled folic acid. Liver extract was prepared without using anti-oxidant.

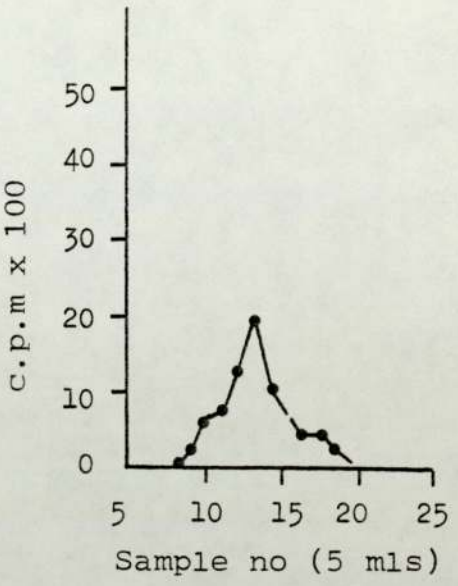
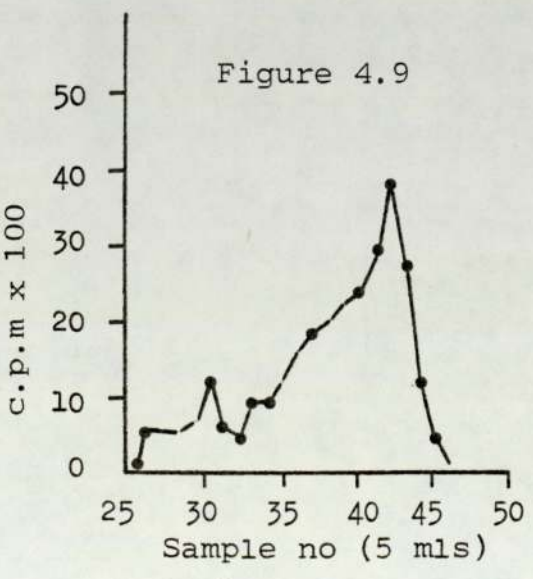


Figure 4.8

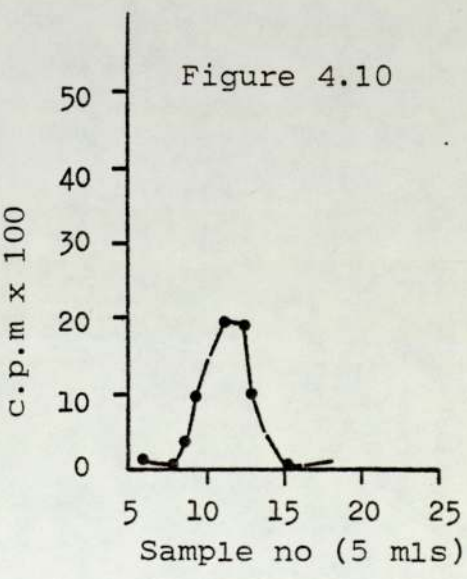
G15 sephadex column chromatogram of 'boiled' liver extract of rats receiving carbon-14 labelled folic acid. Liver extract was prepared without using anti-oxidant.

Figure 4.9



DEAE ion-exchange column chromatogram of 'boiled' liver extract of rats receiving carbon-14 labelled folic acid.

Figure 4.10



G15 sephadex column chromatogram of 'boiled' liver extract of rats receiving carbon-14 labelled folic acid.

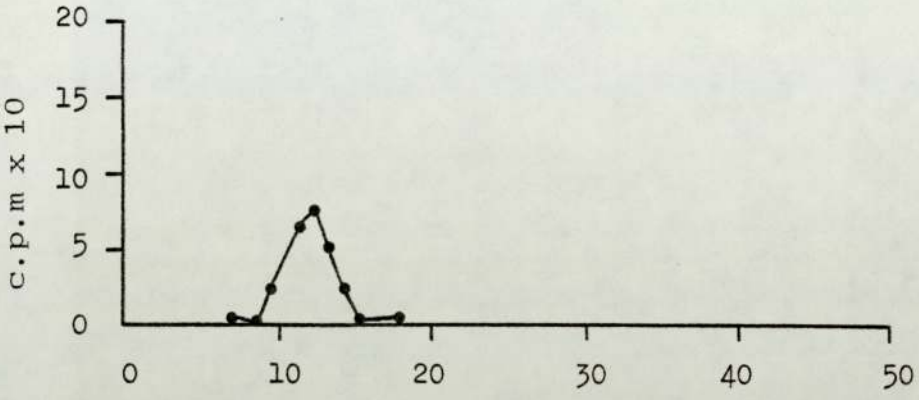


Figure 4.11 G15 sephadex column chromatogram of 'boiled' liver extract of rats receiving carbon-14 labelled folic acid, 24 hours after methotrexate administration.

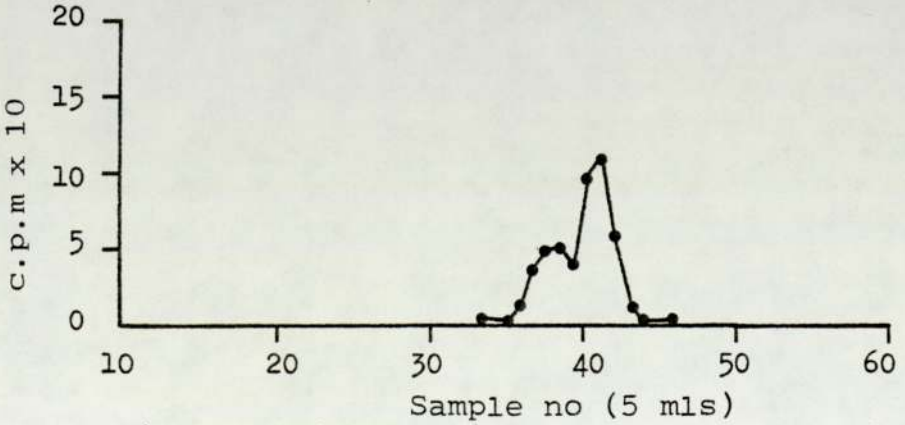


Figure 4.12 DEAE ion-exchange column chromatogram of 'boiled' liver extract of rats receiving carbon-14 labelled folic acid, 24 hours after methotrexate administration.

Discussion

Distribution of 2-^{14}C folic acid (76 microgram/kg body weight, dosed orally) radioactivity in biological samples of control and methotrexate treated rats show quantitative differences. Tables 1,2 and 3 give the amounts of radioactivity recovered in urine, faeces, liver and gut of control and methotrexate treated animals. The overall recovery of radioactivity varied between 54.2% and 85.5%.

Figure 4.1 shows a DE52 ion-exchange chromatogram of 0-6 hours urine of control rats. The identified metabolites numbered as 1 to 4 in figure 4.1 are 2-^{14}C folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and 4a-hydroxy-5-methyltetrahydrofolate respectively. Blair et.al and Barford et.al have also reported the same finding (144,145).

The amount of radioactivity recovered in the 0-6 hours urine sample of control rats was 8.6% (table 1) of the total radioactivity dosed, whereas, 42.6% of the chromatographed radioactivity in figure 4.1 appeared as folic acid (table 4). Therefore, on average 3.7% of the total dosed 2-^{14}C folic acid was excreted as unmetabolised 2-^{14}C folic acid. Similarly, 1.6%, 1.3%, 1.3% and 0.7% of the total dosed folic acid was excreted as 5-methyltetrahydrofolate, 10-formyltetrahydrofolate, 4a-hydroxy-5-methyltetrahydrofolate and unidentified metabolite respectively (table 5).

The 6-24 hours urine sample of the same control

rats contained 8.6% of the total dosed radioactivity (table 1). Table 4 shows the distribution of radioactivity in metabolites appearing when chromatographed on a DE52 ion-exchange colimn (figure 4.2). Converting the distribution given in table 4 into percent of the total dosed radioactivity, it appears that 6-24 hours urine of control animals contains 1.1%, 3.3%, 1.4%, 2.3% and 0.6% of the total dosed radioactivity as folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate, 4a-hydroxy-5-methyltetrahydrofolate and unidentified metabolite respectively (table 5).

Comparing the above results, it appears that the quantity of folic acid has decreased from 3.7% in 0-6 hours urine to 1.1% in 6-24 hours urine (table 5). This shows that after an oral dose of 2-^{14}C folic acid, 4.8% of the total dose was excreted as unmetabolised folic acid in the urine in 24 hours. On the other hand, 5-methyltetrahydrofolate excretion increased from 1.6% in 0-6 hours urine to 3.3% in 6-24 hours urine (table 5). Therefore, 4.9% of the dosed radioactivity was excreted as 5-methyltetrahydrofolate in the urine in 24 hours.

It has been shown that the enzyme dihydrofolate reductase is inhibited by very small amounts of methotrexate in vitro (83). The chromatography of urine samples (figures 4.3 and 4.4) of the rats dosed orally with a single dose of methotrexate (10mg/kg body weight) 24 hours before the folic acid dose indicate that, nevertheless, amounts of reduced folates is far less than the control animals, the

qualitative pattern of ^{14}C-folate metabolism has not significantly changed.

The 0-6 hours and 6-24 hours urine samples of methotrexate treated animals contained 13.5% and 8.2% of the dosed radioactivity respectively (table 3). Figure 4.4 shows a DE52 chromatogram of 0-6 hours urine sample of methotrexate treated rats. Distribution of various metabolites, expressed as a percentage of the total radioactivity chromatographed, is given in table 4. 72.2% of the chromatographed radioactivity in figure 4.4 represents unmetabolised folic acid, which is equivalent to 9.7% of the total dosed 2-^{14}C folic acid. Other metabolites like 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and 4a-hydroxy-5-methyltetrahydrofolate are also present in small amounts. In the 0-6 hours urine of control rats, however, only 3.7% of the dosed 2-^{14}C folic acid was excreted as unmetabolised folic acid.

The 6-24 hours urine of the same methotrexate treated rats contains only 2% of the dosed folic acid as unmetabolised folic acid. This shows that the unmetabolised folic acid in the urine is predominantly excreted within 6 hours of folic acid administration. The excessive excretion of unmetabolised folic acid in the 0-6 hours urine suggests that the enzyme dihydrofolate reductase is at least partially inhibited by methotrexate at an early stage.

The metabolite 5-methyltetrahydrofolate could not be found in the 6-24 hours urine of methotrexate treated

animals, whereas it was a major metabolite in the 6-24 hours urine of control animals. On the other hand, 10-formyl-tetrahydrofolate level was 4.8% as compared to 1.4% in the urine of control animals collected during the same period. The increased amounts of 10-formyltetrahydrofolate, suggests that administration of methotrexate has in some way interfered in the utilisation of this metabolite in the rat. This indicates that methotrexate is interfering in the biosynthesis of purine, suppressing the incorporation of formate -^{14}C into adenine and guanine, reducing the normal purine synthesis and in turn nucleic acid. However, the possible significance of finding excessive 10-formyl-tetrahydrofolate in the urine can only be evaluated after comparing the event with the folate metabolism in the presence of a genuine purine inhibitor in vivo. p213

The retained amount of radioactivity in the liver is proportional to the uptake of folic acid or its metabolites. Table 1 shows that on average 13.1% of the dosed radioactivity has been retained in the liver of control animals killed 24 hours after 2-^{14}C folic acid administration. On the other hand, the liver of methotrexate treated animals on average retained 5.3% of the dosed radioactivity (table 3). The animals receiving methotrexate and killed 48 hours after 2-^{14}C folic acid administration show that the radioactivity in the liver was only 3.8% (table 2).

The lesser amounts of radioactivity in the liver of methotrexate treated animals suggest that the higher

amounts of bound methotrexate is present in the site, and also that of dihydrofolate reductase. The 'prevention and rescue' of toxicity of methotrexate by citrovorum factor (5-formyltetrahydrofolate) before or after methotrexate treatment probably would suggest that at least excessive amounts of methotrexate are eliminated from the sites of its higher concentration such as liver. This also suggests that binding of methotrexate to dihydrofolate reductase at these sites is not completely irreversible as reported in literature (150).

The qualitative analysis of liver extracts of methotrexate treated animals has shown the presence of polyglutamates comparable to the qualitative analysis of normal rat liver extracts. A major peak appearing at the 0.4 M concentration of sodium chloride on the gradient, which also a marker site of appearance of 10-formyltetrahydrofolate on DE52 ion-exchange column, but behaviour of this peak on G15 sephadex column differs from that of 10-formyltetrahydrofolate as it appeared at void volume, a charecterstic of polyglutamates. No further investigation was pursued for the number of glutamate residues.

CHAPTER 5

METABOLISM OF 3'-5'-9 ^{3}H-$2^{14}C$-FOLIC ACID IN RAT AFTER LOW AND HIGH DOSES OF METHOTREXATE

Introduction

Chemically synthesized folic acid used for the biochemical studies contains three structural units (i) the pteridine "ring", (ii) para-aminobenzoic acid and (iii) glutamic acid. Most of the workers in the field of folate metabolism using radio-isotope tracer techniques for the "follow up" of the radioactive folic acid, in the intact animal system, either use folic acid labelled with ^{3}H at 3'-5'-9 positions of para-aminobenzoic acid part of the molecule, or ^{14}C labelled at the 2-position of pteridine part. The results of the two ways of approaching the folate metabolism problem very often end up with drastic controversial conclusions both qualitatively and quantitatively.

The complications may have arisen in the identification of the break-down products due to scission in the folate molecule at ^{9}C-^{10}C linkage, as a result of an artifact in the collection, extraction and storing procedures or presence of such a product or products as a proper long term metabolites of the animal system. In both the cases a product or products constituting other than the labelled part of the molecule would escape detection depending upon the kind of labelled folate used. In addition to the problem, the identifiable product sharing the labelled part of the molecule may be confused with a folate appearing at the same marker position of the column. The above mentioned possibilities of erroneous conclusions and controversial results after using the two

kinds of labelled folic acid are evident, especially when column chromatography is employed as the only means of the quantitative work.

In addition "^{3}H-exchange in the biologic environment might have contributed in the confusion of the results if ^{3}H folic acid is used in the experiments.

In the hope of avoiding some of the mentioned anomalies concerning either of the labelled folic acid used and the consequences thereby, mixture of the 3'-5'-9' ^{3}H folic acid and 2-^{14}C folic acid was administered to the animals in the course of the experiments stated in this chapter.

The results of the experiments in chapter 4 of this thesis demonstrated, that, though a single oral dose of 10 mg/kg body weight of methotrexate, twenty four hours before an oral dose of 2-^{14}C folic acid supressed the folate metabolism to large extent, it did not completely inhibit the formation of normal metabolites, which was otherwise expected of the drug, considering the ability of the drug to bind with the enzyme dihydrofolate reductase. It was, therefore, assumed that perhaps in our experiments there was not enough amount of methotrexate available, for the complete inhibition of the enzyme at various sites of its greater concentration, due to smaller dose of methotrexate. Much wanted basis for the better understanding of the whole picture of methotrexate-folate interaction in the mammalian system, was therefore to administer a higher dose of methotrexate and administer

duel labelled folic acid to overcome the probable possibility of confusing the break-down products with the folates, before reaching any optimistic or clear conclusion.

Rats were dosed with methotrexate in water suspension orally, some of them receiving 100 mg/kg body weight and some 10 mg/kg body weight. A group of rats were controlled, receiving distilled water only without methotrexate. All these animals received mixture of 2-^{14}C folic acid and ^{3}H folic acid (2-^{14}C folic acid 76 microgram/kg body weight and ^{3}H folic acid 8 microgram/kg body weight per animal). The urine samples were chromatographed on ion-exchange and gel permeation sephadex G15 columns. All the detectable radioactive peak fractions were re-chromatographed on both types of columns with the suspected analar grade cold materials for comparing the radioactive peaks with the U.V. peaks of the cold materials added. Isotope dilution test was applied to the major tritium peak appearing on ion-exchange column. Liver extracts were subjected to the similar chromatographic analysis techniques as urine samples. The distribution of the radioactivity was estimated by burning the freeze-dried samples of liver, gut and faeces in the biological material oxidiser. The amount of radioactivity in the urine samples were estimated by counting the fractions of urine samples directly in the counter.

Materials and Methods

Methotrexate was purchased from Lederle Chemical Company Limited, 3'-5'-9 ^{3}H folic acid with specific activity 500 micro Ci/mmol and 2-^{14}C folic acid with specific activity 58 micro Ci/mmol was purchased from the Radiochemical Centre, Amersham, U.K. 10-formylfolic acid, 5-formyltetrahydrofolic acid, para-aminobenzoic acid, xanthopterin, isoxanthopterin, 2-amino-4-hydroxypteridine were of the purest grade and were chromatographed in order to confirm the purity before use. All the chemicals used in the making of buffer solutions and as antioxidants were of analar grade. Standard column packing materials were used and repeated chromatography with same resins and celluloses were avoided wherever necessary.

10 adult male Wistar rats weighing 200-250 grams each, were dosed with methotrexate orally in water suspension, six of them received a dose of 100 mg/kg body weight and rest of the four with a dose of 10 mg/kg body weight of methotrexate. Another group of four male adult Wistar rats received same amount of distilled water only without methotrexate. All of the doses were given under mild ether anaesthesia. Twenty four hours later all the above mentioned groups of animals were dosed orally with a mixture of 3'-5'-9 ^{3}H folic acid and 2-^{14}C folic acid in water. Each animal receiving 8 microgram/kg body weight of 3'-5'-9 ^{3}H folic acid and 76 microgram/kg body weight of 2-^{14}C folic acid in a mixture of the labelled materials in water.

Animals were housed separately in Jencon's metabowls, fed and water ad libitum. The animals were killed after 24 hours of the folic acid mixture dosing. The urine samples and faeces were collected between the second dosing of folic acid mixture and killing the animals. The details are given below.

Collection of biological samples

The urine samples were collected between 0-6 hours and 6-24 hours in flasks containing 5 mls of 0.05 molar phosphate buffer solution pH 7 containing 5 mg/100 mls dithiothreitol and 2 grams/100 mls ascorbate to prevent oxidation. The flasks were wrapped with tin foil in order to shield the contents from excessive light. The 0-6 hours and 6-24 hours urine samples were collected separately as the flasks were changed at 6 hours. 0 hours denote the time of mixture of folic acid dosing while 24 hours denote the time at which the rats were killed.

Faeces were collected in empty flasks between 0-24 hours for each animal.

The livers and gut of the animals were removed immediately after killing the animals. Some of the livers were extracted for the qualitative anlysis of radioactive materials by column chromatography. The freshly removed livers were chopped into small pieces and dropped into boiling 0.05 molar phosphate buffer solution, pH 7.0, containing 5 grams/100 mls ascorbate and 5 mg/100 mls dithiothreitol. The contents were left boiling for five minutes. The solid material was discarded after centrifugation. The extract

like all other biological samples was stored in deep freeze.

Measurement of total radioactivity in the biological samples

Faeces, gut and some of the livers from each group of animals were freeze-dried and powdered. 100 mg of the powdered material, twice in each case, of faeces, gut and liver was oxidised in a Beckman biological material oxidiser and the $^{14}CO_2$ was collected in 15 mls of Fisons Absorber P. The standards, blanks and control samples were subjected to the same oxidation for comparison. The samples were counted for radioactivity in a Nuclear Enterprise liquid scintillation counter NE8310. Appropriate corrections were made for quenching and the efficiency of the machine.

The urine samples were directly counted, by putting a known fraction of the urine in a standard scintillation cocktail, repeating the same for standards and blanks.

Column chromatography of the urine samples and liver extracts

The 0-6 hours and 6-24 hours urine samples were applied separately to DEAE-cellulose columns, equilibrated with 0.05 molar phosphate buffer solution, pH 7.0, containing 5 mg/100 mls dithiothreitol and were eluted with a linear gradient of 0-1 M sodium chloride in phosphate buffer solution, pH 7.0, containing 5 mg/100 mls dithiothreitol, 5 mls fractions were collected using L.K.B.

automatic fraction collector. The radioactivity of each fraction was counted in a vial containing known quantity of the fraction in 10 mls of scintillation cocktail (Toluene 500 mls, Fisons emulsifier 250 mls and 2,5-diphenyloxazole 2.5 grams). The contents of each fraction were passed through L.K.B. Uvicord and the pressure on the column was maintained by a peristaltic pump which was connected to the column and the gradient mixer. The conductivity of the fractions was measured. The urine samples were also applied to G15 sephadex columns, equilibrated and eluted with 0.05 molar phosphate buffer, pH 7.0, containing 5 mg/100 mls dithiothreitol and 2 grams/100 mls ascorbate. The fractions were counted in the fashion of DEAE ion-exchange column fractions.

The liver extracts were also subjected to the same chromatographic analysis techniques as the urine samples.

DE52 columns were repacked after every run with a freshly equilibrated cellulose and G15 sephadex wherever necessary.

Both types of columns were calibrated with authentic materials either labelled or unlabelled, which were detected by Uvicord and a Chart Recorder. The calibrated substances include folic acid, 10-formylfolic acid, 5-formyltetrahydrofolic acid, para-aminobenzoic acid, xanthopterin, isoxanthopterin, 2-amino-4-hydroxypteridine and tetrahydrofolic acid.

The fractions under the different radioactive peaks appearing after chromatography of urine samples on DE52 ion-exchange columns, were re-chromatographed separately with the cold suspected material on both DE52 ion-exchange and G15 sephadex columns.

RESULTS

Recovery of radioactivity

Tables 1,2 and 3 give the percent of the dosed radioactivity in the urine, faeces, liver and gut after an oral dose of a mixture of 2-^{14}C folic acid and 3'-5'-9 ^{3}H folic acid to the controlled rats and the rats receiving oral doses of methotrexate 10 mg/kg and 100 mg/kg body weight respectively. Methotrexate was dosed 24 hours before the administration of folic acid.

Column chromatography of the 0-6 hours urine samples

Figures 5.1, 5.2 and 5.3 are typical DE52 ion-exchange column chromatograms of the 0-6 hours urine samples. Figure 5.1 is a chromatogram of the 0-6 hours urine sample of control rats (i.e. the rats receiving no methotrexate). Figure 5.2 is a DE52 ion-exchange chromatogram of the 0-6 hours urine sample of the rats receiving an oral dose of methotrexate 10 mg/kg body weight, while figure 5.3 is a DE52 ion-exchange chromatogram of the 0-6 hours urine sample of the rats receiving an oral dose of methotrexate 100 mg/kg body weight.

The major peak in figure 5.2 appears between 0.9 and 1.0 molar sodium chloride concentration on the gradient. This peak was identified as folic acid on the basis of co-chromatography of the radioactive fractions under this peak with authentic folic acid on DE52 ion-exchange column. This peak consists of both 2-^{14}C folic acid and 3'-5'-9' ^{3}H folic acid.

The second major peak in figure 5.2 appears at 0.4 molar sodium chloride concentration on the gradient, which is the calibrated marker position of 10-formyl-tetrahydrofolate. The radioactive fractions under this peak were re-chromatographed on a G15 sephadex column. G15 chromatogram (figure 5.4) shows that it has appeared on this column between fractions 16 to 21. 10-formyltetrahydrofolate like any folate co-enzyme, is highly susceptible to oxidation and is converted to 10-formylfolate after oxidation. This in view, the above mentioned peak fractions were freed from the antioxidants by re-chromatography on G15 sephadex column without using any antioxidants in packing or eluting the column. Oxygen was passed through the antioxidant free material of the same peak for one hour. This was then re-chromatographed on G15 sephadex column (figure 5.5). No shifts were observed in the radioactive profiles of the peak in the two columns. This suggests that the peak appearing at 0.4 molar sodium chloride concentration in figure 5.2 is not reduced folate. Hence on the basis of the evidence provided by co-chromatography and elution position, the peak appearing at 0.4 molar sodium chloride concentration on DE52 ion-exchange column chromatogram (figure 5.2) is 10-formylfolic acid. It is assumed that 10-formyltetrahydrofolate which is susceptible to oxidation, has been converted to 10-formyl-folate in the course of subsequent chromatography.

Figure 5.2 shows that the peaks appearing between 0.9 and 1.0 molar and at 0.4 molar sodium chloride

are associated with both ^{14}C and ^{3}H, while the peak appearing between 0.3 and 0.4 molar sodium chloride concentration is only ^{3}H labelled. The peak between 0.3 and 0.4 molar sodium chloride concentration appears just before 10-formyltetrahydrofolate and is at the marker position of para-aminobenzoyl-L-glutamate. The contents of the fractions under this peak were re-chromatographed on G15 sephadex column. Figure 5.9 shows that it has appeared between fractions 17 and 18, which again is the calibrated site of para-aminobenzoyl-L-glutamate on G15 sephadex column. Further analysis of this isolated tritium peak was carried out in the subsequent course of the present experiments by applying isotope dilution test.

In contrast to the above mentioned results of finding an isolated peak of tritium in figure 5.2, no prominent sign of tritium labelled peak was found in figure 5.1. Figure 5.1 is a DE52 ion-exchange column chromatogram of 0-6 hours urine of rats which did not receive methotrexate, but received an oral dose of a mixture of ^{14}C and ^{3}H labelled folic acid.

Figure 5.3 shows a DE52 ion-exchange column chromatogram of 0-6 hours urine sample of the rats receiving an oral dose of 100 mg/kg body weight methotrexate 24 hours before an oral dose of a mixture of ^{14}C and ^{3}H labelled folic acid. The labelled metabolites appearing were identified to be the same after a similar course of analysis as in 0-6 hours urine of the rats receiving an oral dose of 10 mg/kg body weight methotrexate (figure 5.2).

The only difference on detectable scale was the higher amounts of the labelled folic acid in the chromatogram (figure 5.3). Table 4 gives the distribution of the radio-activity amongst the metabolites in 0-6 hours urine samples as resolved by DE52 ion-exchange columns. The amounts are expressed as a percentage of the total radio-activity subjected to chromatography on each column.

Figures 5.11 and 5.12 are G15 sephadex column chromatograms of the 0-6 hours urine samples of control rats and the rats receiving 10 mg/kg body weight methotrexate respectively.

Isotope dilution test of the isolated tritium peak

In addition to the re-chromatography of the isolated peak with authentic materials on DE52 ion-exchange and G15 sephadex columns, isotope dilution analysis was carried out for the identification of the above mentioned peak.

0-6 hours urine from the rats dosed with 100mg/kg methotrexate was applied to DE52 ion-exchange columns. The fractions were counted each time and the isolated ^{3}H peaks, all appearing just before 0.4 molar concentration of sodium chloride on the gradient, were mixed together. To the resulting solution, authentic unlabelled para-amino-benzoyl-L-glutamate was added to saturation at 70^{o}C. The solution was left overnight for crystallization in the fridge, the crystals so formed were isolated by decantation and filtration and washed with cold distilled water before final filtration. The crystals were dried under vacum

and two 5 mg portions were weighed accurately and put in two counting vials and dissolved in 1 ml of distilled water, 10 mls of Fisons Dioxan D was added to each vial and counted for radioactivity in the counter. The remaining crystals were re-dissolved in minimum amount of distilled water at 70°C, left overnight in the fridge and 5 mg of crystals counted after similar treatment as the first batch of crystals. The treatment was repeated five times. The result is shown in figure 5.10. Almost constant specific activity was obtained in all the batches of the re-crystallization.

Column chromatography of 6-24 hours urine samples

Figures 5.6, 5.7 and 5.8 are typical DE52 ion-exchange column chromatograms of the three groups of rats. Figure 5.6 is that of controls, figure 5.7 is of the rats receiving 10 mg/kg methotrexate and figure 5.8 is of the rats receiving 100 mg/kg body weight methotrexate. Table 4 gives the distribution of the radioactivity involved with each metabolite of the three columns, expressed as a percentage of the total radioactivity applied to the specific column.

The radioactive fractions of the peak appearing between 0.9 and 1.0 molar sodium chloride concentration in figures 5.6, 5.7 and 5.8 were separately pooled together and re-chromatographed on three freshly packed DE52 ion-exchange columns with authentic unlabelled folic acid. The radioactivity and UV trace of authentic folic acid did

not separate in all three above mentioned cases. This suggests that the peak eluting between 0.9 and 1.0 molar sodium chloride concentration of the gradient in the three chromatograms (figures 5.6, 5.7 and 5.8) is folic acid.

Figure 5.6, a DE52 ion-exchange column chromatogram of 6-24 hours urine of control rats, shows a prominent peak at about 0.6 to 0.7 molar sodium chloride concentration of the gradient. This peak was identified as 5-methyltetrahydrofolate on the basis of elution position and co-chromatography with authentic 5-methyltetrahydrofolate on DE52 ion-exchange column.

The 6-24 hours urine ion-exchange column chromatograms of rats receiving 10 mg/kg and 100 mg/kg methotrexate 24 hours before folic acid administration are shown in figures 5.7 and 5.8 respectively. These chromatograms show that the peak eluting at the calibrated site of 5-methyltetrahydrofolate has diminished to a considerable extent. This peak, however, is more prominent in figure 5.8 as compared to figure 5.7.

The ^{14}C and ^{3}H labelled peak appearing approximately between 0.4 and 0.45 molar sodium chloride concentration on DE52 ion-exchange column (figure 5.6) is at the site where 10-formyltetrahydrofolate appears on this type of column. A tritium labelled peak, which is not quite clear of 10-formyltetrahydrofolate peak, is perhaps para-aminobenzoyl glutamate as it appeared at the calibrated site of the same compound (figure 5.6). No isotope dilution test

could be applied for its identification as it was difficult to isolate due to partial overlapping of the adjacent peaks. The peak appearing around 0.25 molar sodium chloride concentration (figure 5.6) has been identified as 4a-hydroxy-5-methyltetrahydrofolate. It has been claimed that this compound is a long term metabolite of folic acid in the urine of rats dosed with folic acid (145).

Figures 5.7 and 5.8 show that apart from folic acid which is the major peak in these chromatograms, there are only two other prominent peaks. The peak which contains both ^{14}C and ^{3}H appears at about 0.4 molar sodium chloride concentration and has been identified as 10-formyltetrahydrofolate on the basis of its elution position. The other peak, which is only tritium labelled, has already been identified as para-aminobenzoyl-L-glutamate in the corresponding 0-6 hours urine sample chromatogram (figures 5.2 and 5.3).

The amounts of radioactivity in the different metabolites, expressed as a percentage of chromatographed radioactivity, present in figures 5.6, 5.7 and 5.8 is given in table 4.

Column chromatography of liver extracts

Low amounts of radioactivity present in the liver and hence in their extracts, of the rats treated with methotrexate, offered difficulties in the column chromatographic techniques of identification. Moreover the unavailability of the desired authentic materials,

i.e. specific polyglutamates, for the calibration of the columns, in the course of the experiments, made the task even more difficult.

Figures 5.13 and 5.14 are the DE52 ion-exchange column chromatograms of control and methotrexate treated (10 mg/kg body weight) animal liver extracts respectively.

Figures 5.15 and 5.16 are G15 sephadex chromatograms of the same liver extracts, which are similar in the outlook of appearance as far as the elution positions are concerned. The polyglutamates appear at the void volume of sephadex column. On the other hand, polyglutamates on ion-exchange columns employed in the present experiments appear at higher concentrations of sodium chloride concentrations of sodium chloride on the gradient.

In the light of above mentioned characteristics of the polyglutamates, it may be difficult to particularise the specific polyglutamate but can safely be assumed that some or other polyglutamates are present in the liver extracts of both control and methotrexate treated animals, which may or may not have any relation with actual form of their existence in the intact liver before extraction.

Rat No	Urine				Gut		Liver		Faeces		Total	
	0-6 hrs		6-24 hrs									
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1	12.0	14.3	8.3	15.2	7.2	3.3	11.2	12.8	34.0	29.6	72.7	75.2
2	6.1	6.2	10.9	7.4	6.7	2.5	9.4	15.2	38.8	51.4	71.9	82.7
3	4.9	6.0	12.1	13.3	3.4	0.9	13.2	13.7	32.5	49.0	66.1	82.9
4	8.5	7.3	18.3	20.9	7.2	5.5	10.5	14.1	30.1	25.4	74.6	73.2
Average	7.9	8.4	12.4	14.2	6.1	3.0	11.0	13.9	33.8	38.8	71.3	78.5

Table 1 Distribution of radioactivity in urine, gut, liver and faeces (expressed as a percentage of a mixture of 2-^{14} folic acid and 3'-5'-9 ^{3}H folic acid dosed) of control animals killed 24 hours after dual labelled folic acid administration.

Rat No	Urine				Gut		Liver		Faeces		Total	
	0-6 hrs		6-24 hrs									
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1	11.2	14.1	8.6	13.1	3.2	2.9	3.3	7.5	40.3	55.0	66.6	92.6
2	18.1	21.9	9.4	10.9	2.2	3.9	2.9	8.8	-	-	-	-
3	20.3	30.4	15.8	15.2	1.5	3.1	4.1	4.2	32.7	30.5	74.4	83.4
4	20.2	16.5	10.3	18.7	2.5	1.0	2.6	5.3	41.8	46.0	77.4	87.5
Average	17.4	20.7	11.0	14.4	2.3	2.7	3.2	6.4	38.3	43.8	72.2*	88.0*

Table 2 Distribution of radioactivity in urine, gut, liver and faeces (expressed as percentage of a mixture of 2-^{14}C folic acid and 3'-5'-9 ^{3}H folic acid dosed) of methotrexate treated animals (10 mg/kg body weight) killed 24 hours after dual labelled folic acid administration.

* The figures denote sum of the individual averages.

Rat No	Urine				Gut		Liver		Faeces		Total	
	0-6 hrs		6-24 hrs									
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1	28.2	31.9	20.1	27.0	8.1	11.5	4.7	8.3	-	-	-	-
2	19.7	21.0	14.5	18.4	11.7	10.3	3.0	6.2	-	-	-	-
3	30.6	22.5	5.8	7.7	7.6	9.0	2.2	2.9	-	-	-	-
4	20.2	21.4	8.6	2.1	4.9	2.0	1.9	3.3	46.1	51.0	81.7	79.8
5	15.2	16.0	13.5	14.0	2.1	1.8	1.6	2.8	53.9	55.5	86.3	90.1
6	13.1	18.3	8.1	10.2	5.3	6.6	3.1	3.0	45.2	49.2	74.8	87.3
Average	21.2	21.8	11.7	13.2	6.6	6.8	2.7	4.4	48.4	51.9	90.7*	98.4*

Table 3 Distribution of radioactivity in urine, gut, liver and faeces (expressed as a percentage of a mixture of 2-^{14}C folic acid and 3'-5'-9 ^{3}H folic acid dosed) of methotrexate treated animals (100 mg/kg body weight) killed 24 hours after dual labelled folic acid administration.

* The figures denote sum of the individual averages.

Chromatogram	1		2		3		4		5	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Figure 5.1	71.1 (60-78)	51.7	16.9 (42-52)	12.3	7.7 (35-42)	13.0	4.9 (28-35)	10.6	-	-
Figure 5.2	83.6 (73-82)	64.3	2.5 (50-60)	3.2	15.3 (42-49)	13.8	-	-	-	9.6 (36-42)
Figure 5.3	90.1 (64-75)	77.4	-	-	5.3 (36-42)	6.9	-	-	-	11.1 (32-36)
Figure 5.6	45.6 (70-81)	30.5	19.2 (49-56)	16.9	13.2 (38-49)	13.2	18.0 (27-38)	11.0	-	10.0 (35-38)
Figure 5.7	45.7 (70-80)	30.0	3.0 (50-60)	3.0	18.2 (41-47)	15.3	-	-	-	27.1 (35-41)
Figure 5.8	84.2 (65-75)	58.5	5.0 (48-56)	6.1	6.5 (36-42)	7.5	-	-	-	15.0 (30-37)

Table 4 Distribution of radioactivity in various urinary metabolites as resolved by DE52 ion-exchange columns, expressed as a percentage of total radioactivity chromatographed on each column.

Metabolites 1 to 5 appear at the calibrated sites of folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate, 4a-hydroxy-5-methyltetrahydrofolate and p-aminobenzoyl-L-glutamate respectively on DE52 ion-exchange column.

Figures in parentheses denote sample numbers at which the respective peaks appear.

	0-6 hours Urine		6-24 hours Urine	
	^{14}C	^{3}H	^{14}C	^{3}H
Control animals	0.6	1.0	1.6	1.8
Methotrexate treated animals (10 mg/kg)	2.7	2.8	2.0	2.2
Methotrexate treated animals (100 mg/kg)	1.1	1.5	0.8	1.0

Table 5 Amounts of radioactivity present in the peak, appearing at the calibrated site of 10-formyl-tetrahydrofolate on DE52 column, expressed as a percentage of the dosed folic acid radioactivity.

Note: Amounts given in the above table have been calculated from the peak appearing at about 0.4 molar sodium chloride concentration on DE52 ion-exchange chromatograms; Figures 5.1 and 5.6 for control animals; Figures 5.2 and 5.7 for methotrexate (10 mg/kg) treated animals; Figures 5.3 and 5.8 for methotrexate (100 mg/kg) treated animals.

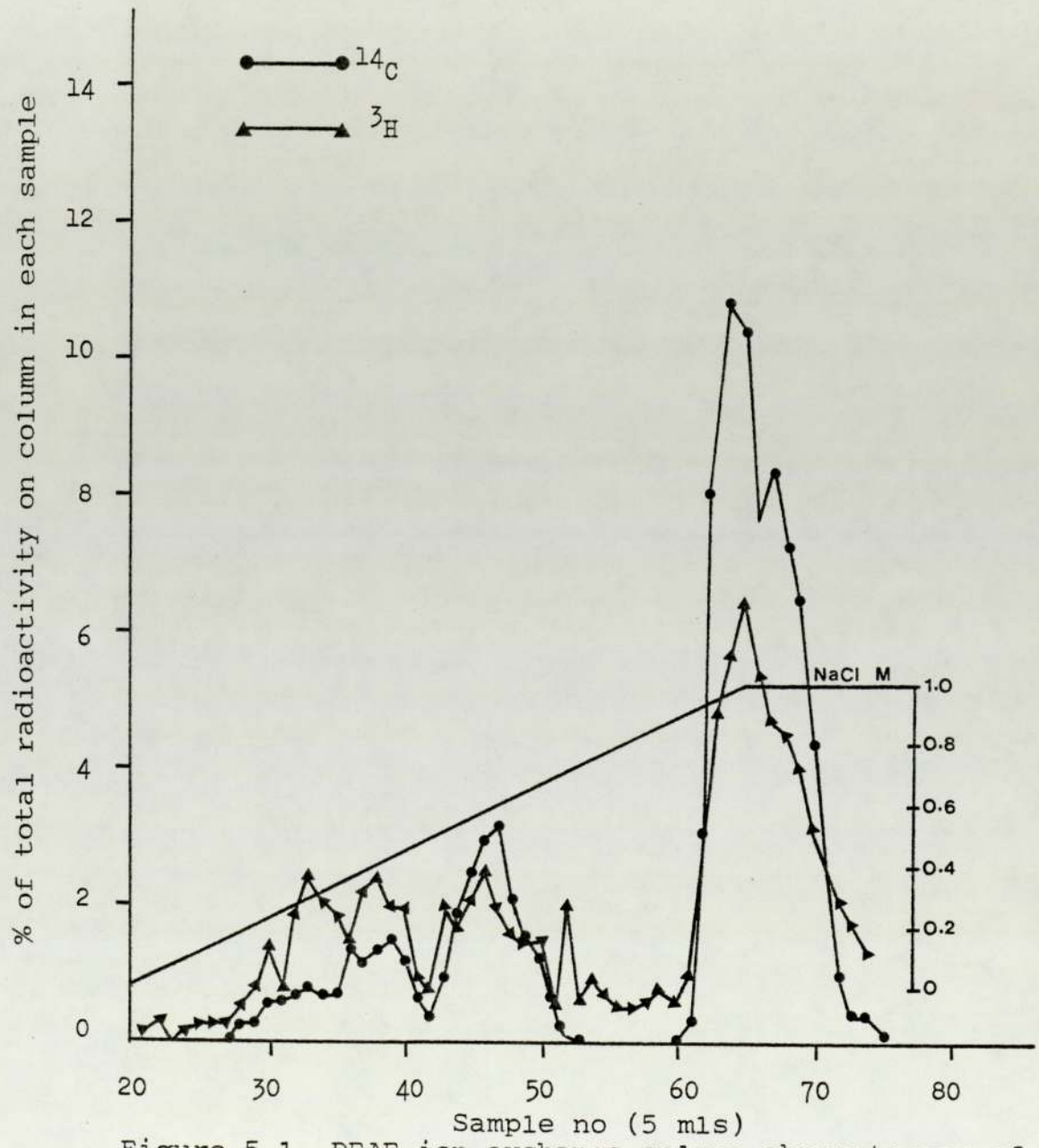


Figure 5.1 DEAE ion-exchange column chromatogram of 0-6 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid.

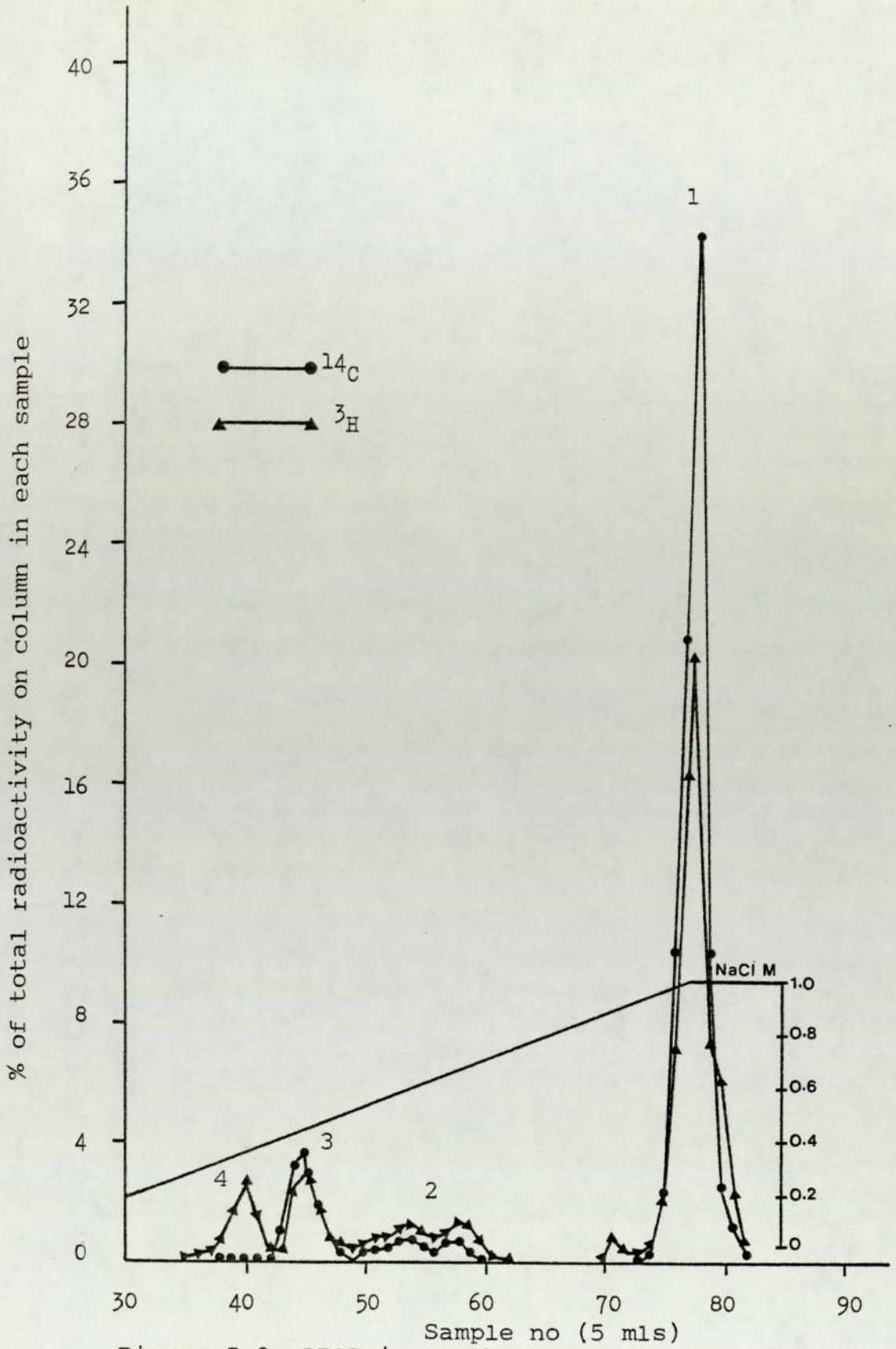


Figure 5.2 DEAE ion-exchange column chromatogram of 0-6 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate (10mg/kg) administration.

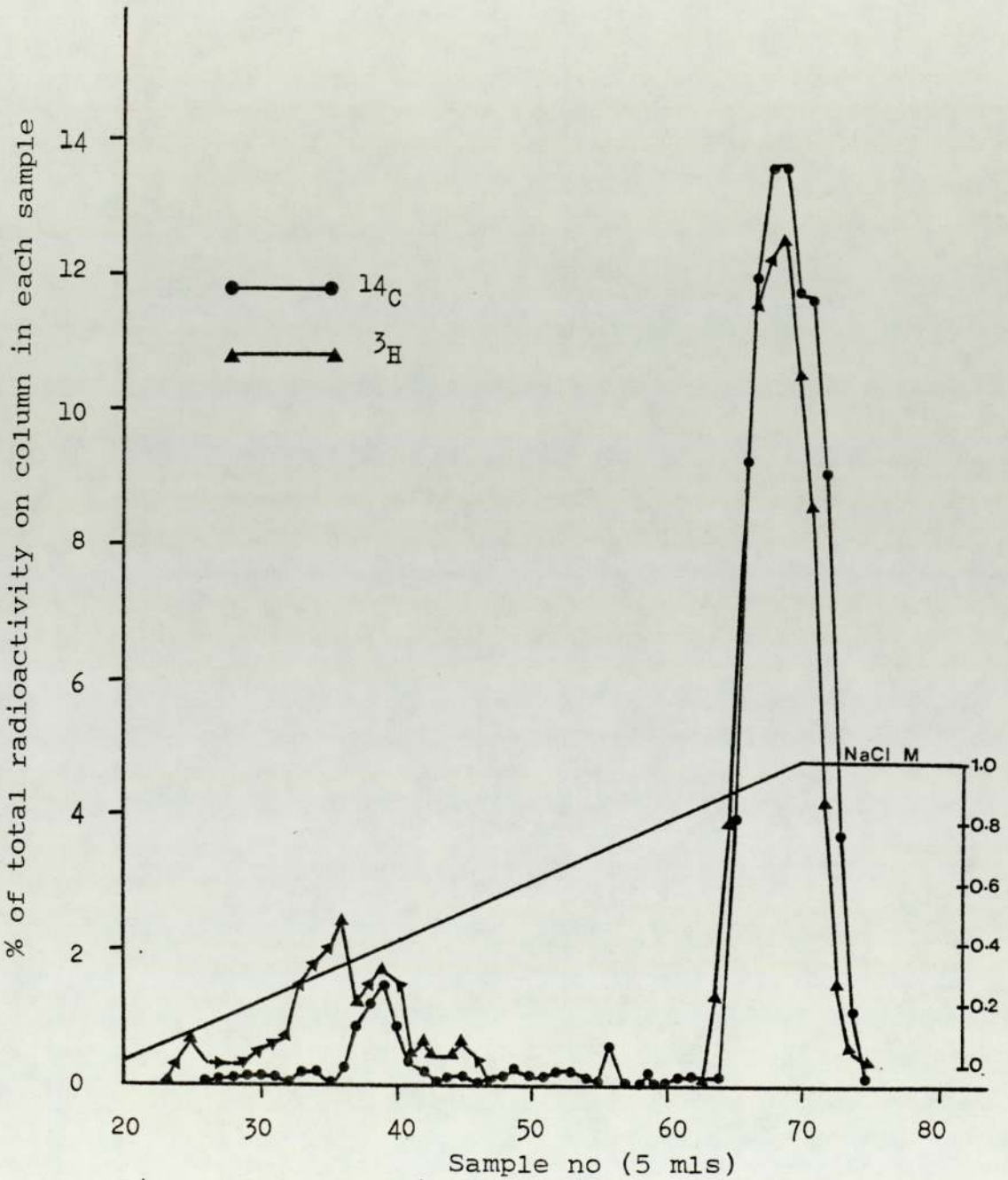


Figure 5.3 DEAE ion-exchange column chromatogram of 0-6 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate (100mg/kg) administration.

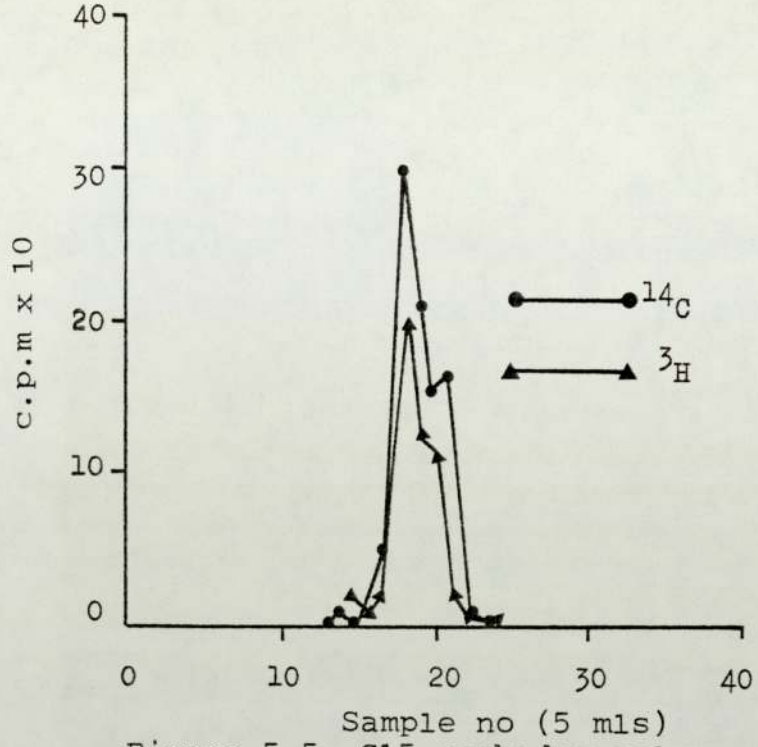


Figure 5.5 G15 sephadex column chromatogram re-chromatography of peak 3 on DEAE ion-exchange column chromatogram (fig 5.2) after oxidation.

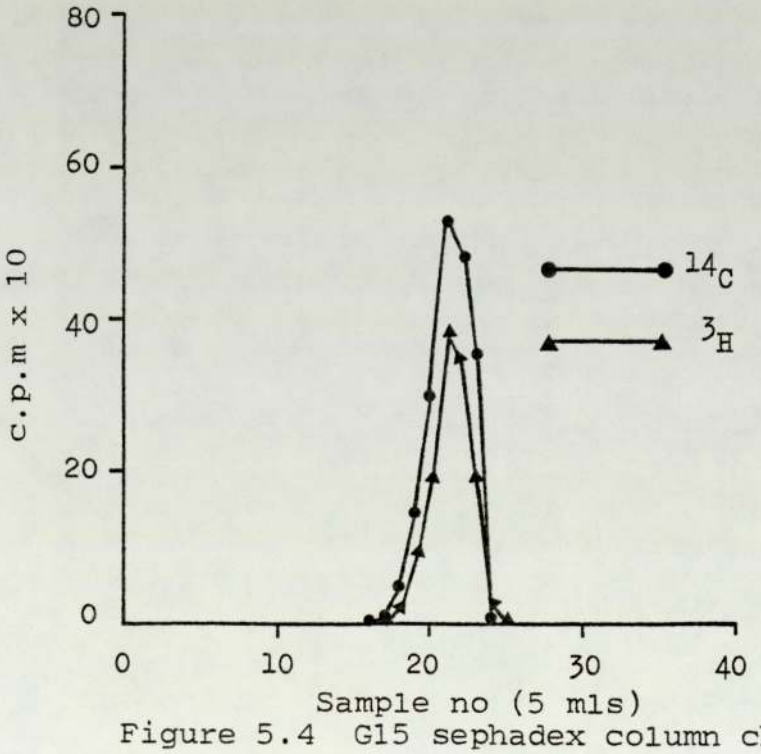


Figure 5.4 G15 sephadex column chromatogram re-chromatography of peak 3 on DEAE ion-exchange column chromatogram (fig 5.2).

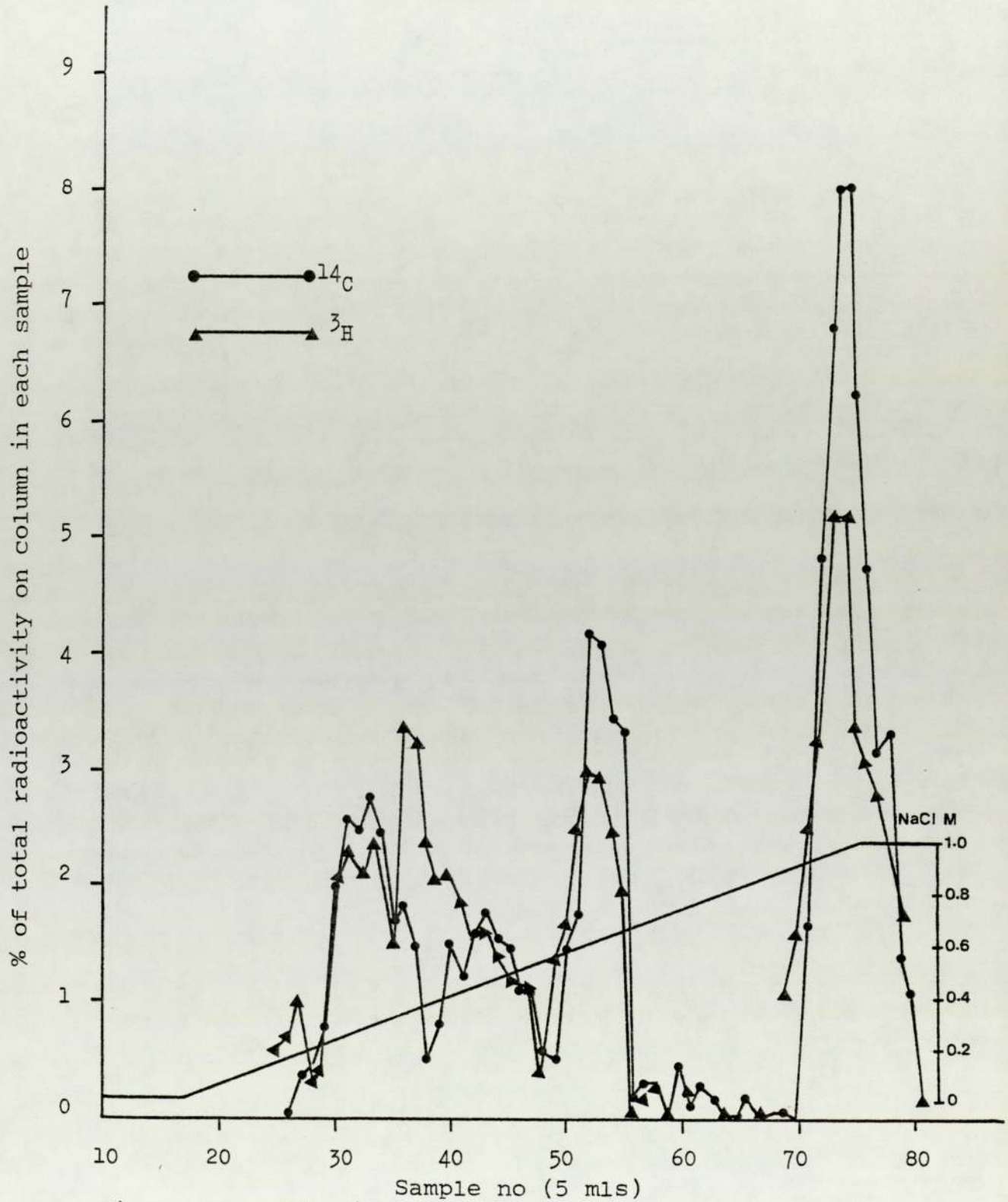


Figure 5.6 DEAE ion-exchange column chromatogram of 6-24 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid.

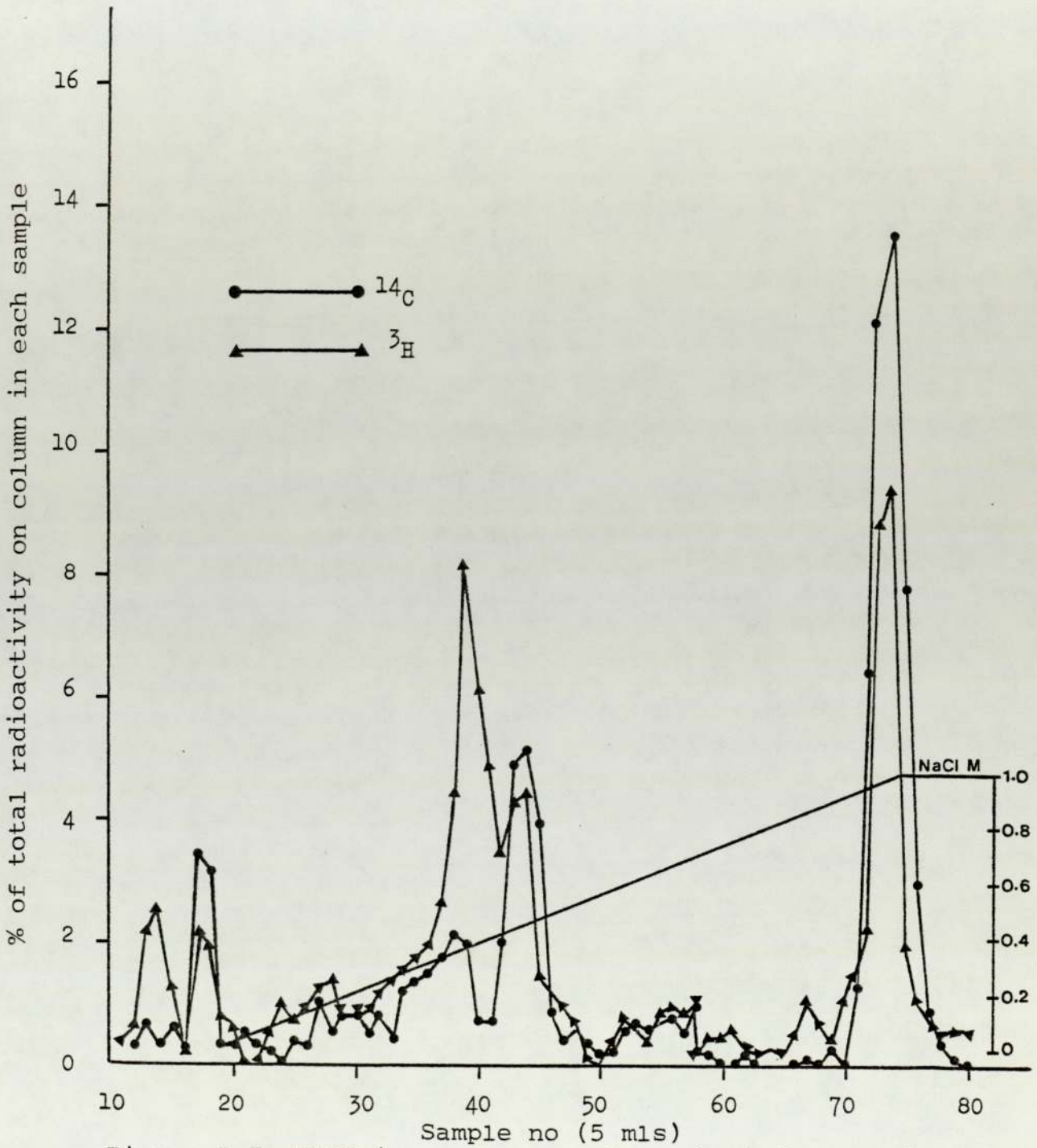


Figure 5.7 DEAE ion-exchange column chromatogram of 6-24 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate (10mg/kg) administration.

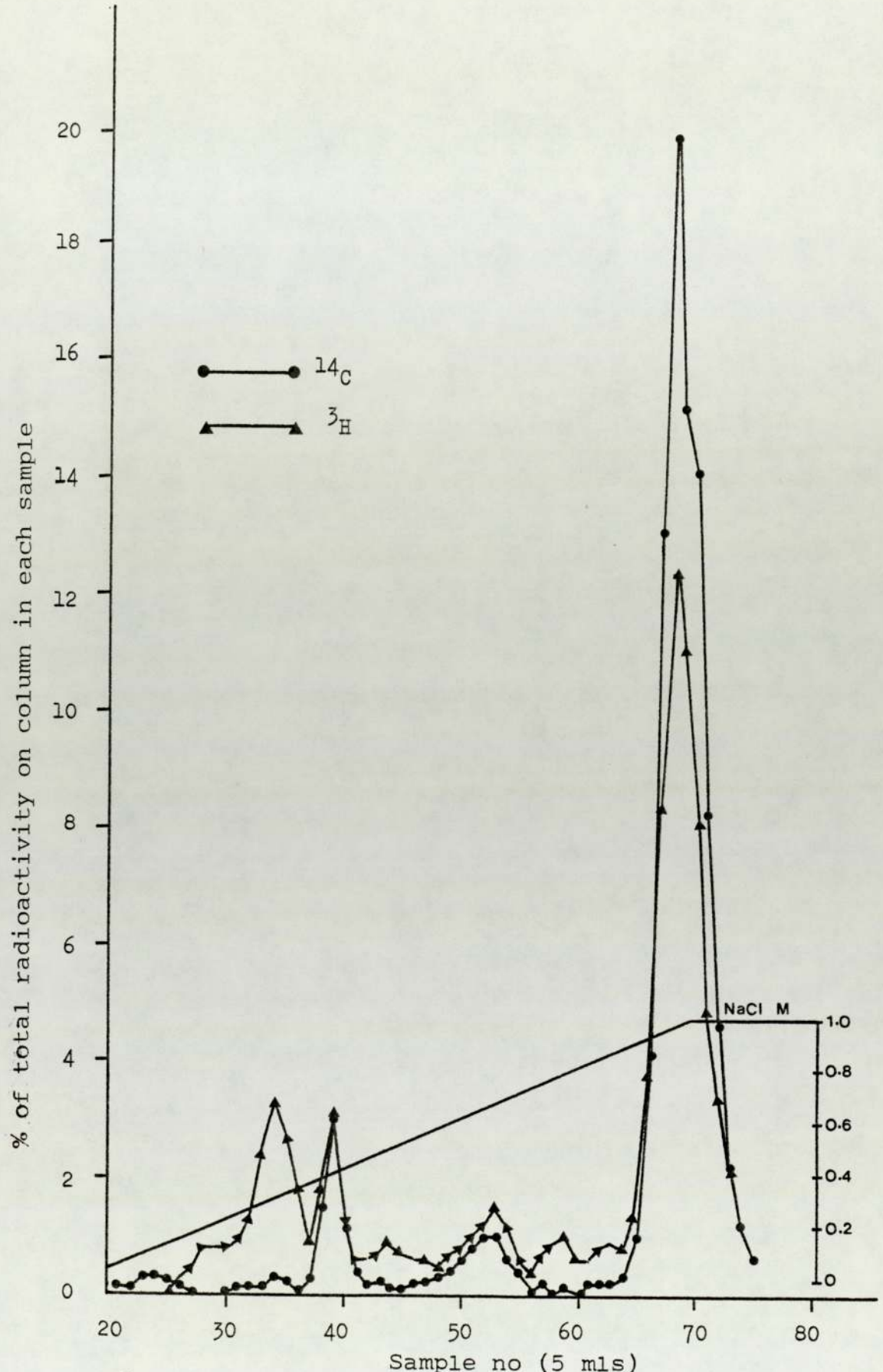


Figure 5.8 DEAE ion-exchange column chromatogram of 6-24 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate (100mg/kg) administration.

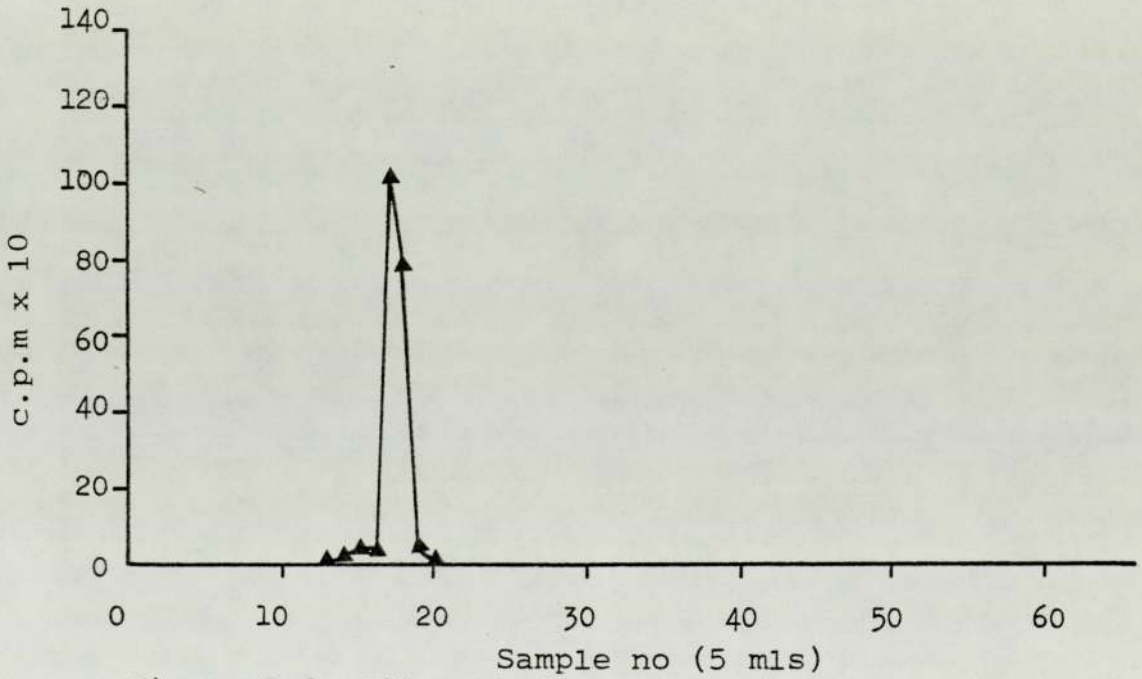


Figure 5.9 G15 sephadex column chromatogram re-chromatography of peak 4 on DEAE ion-exchange column chromatogram (fig 5.2).

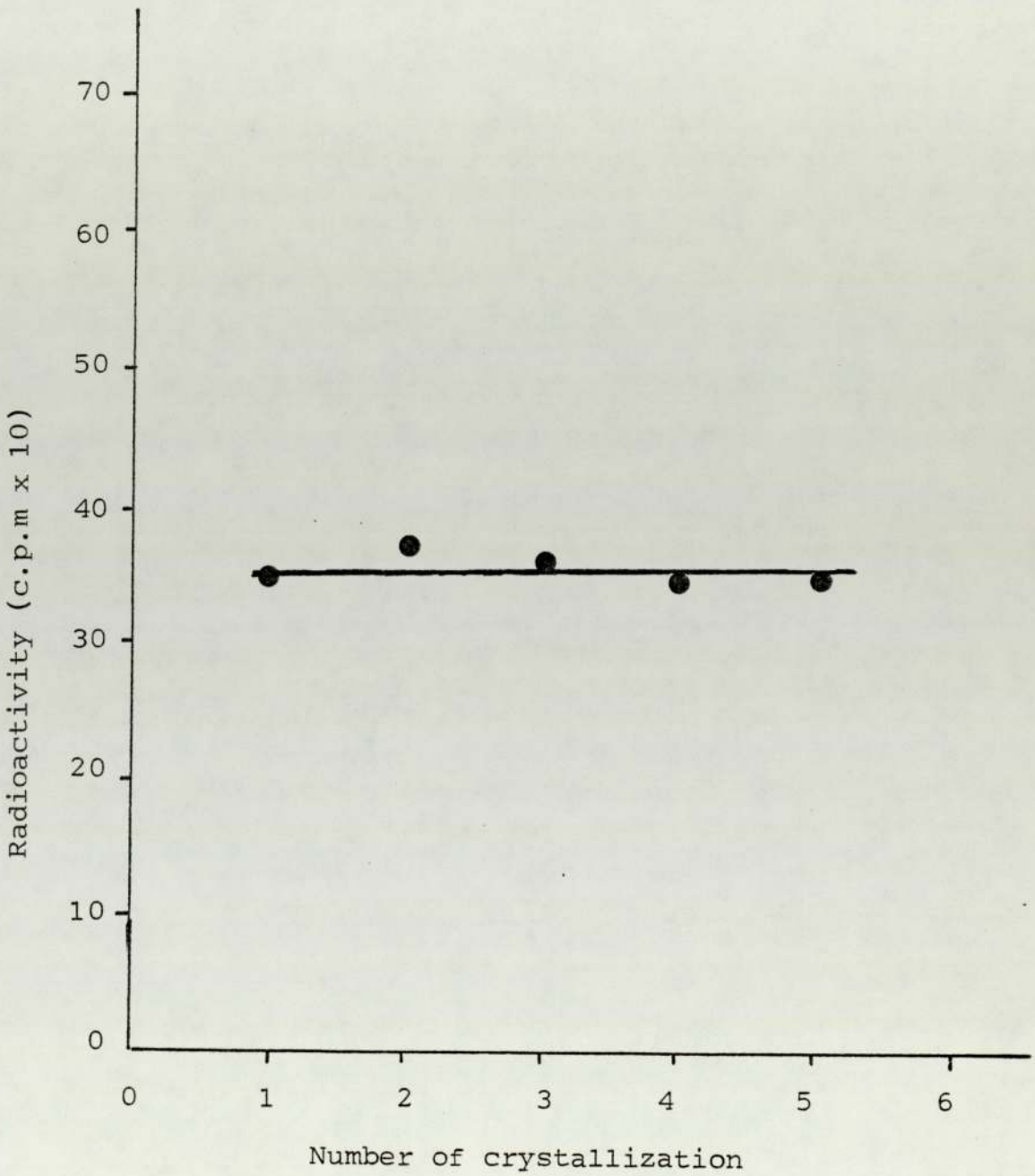


Figure 5.10 Isotope dilution test applied to isolated tritium peak with p-amino-benzoyl-L-glutamate.

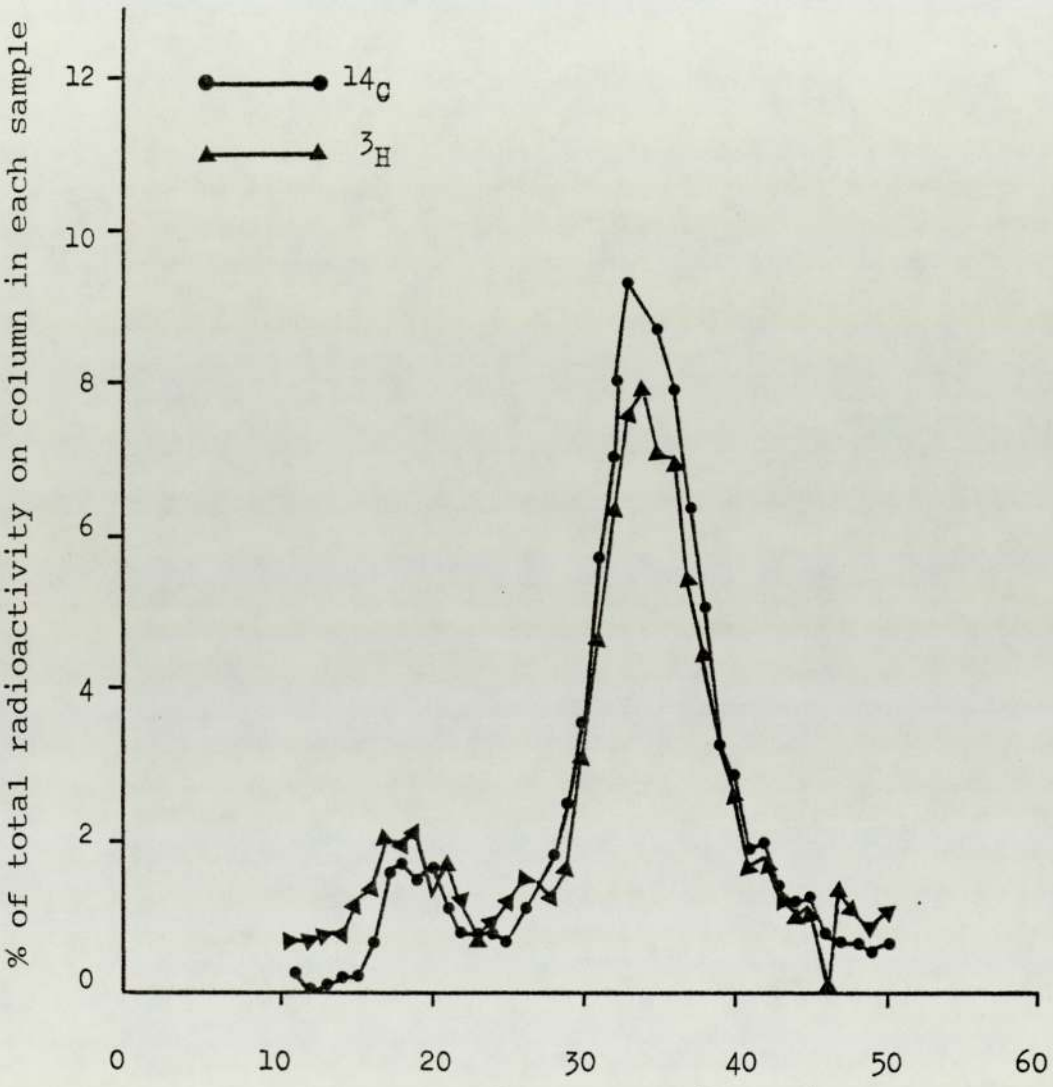


Figure 5.11 G15 sephadex column chromatogram of 0-6 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid.

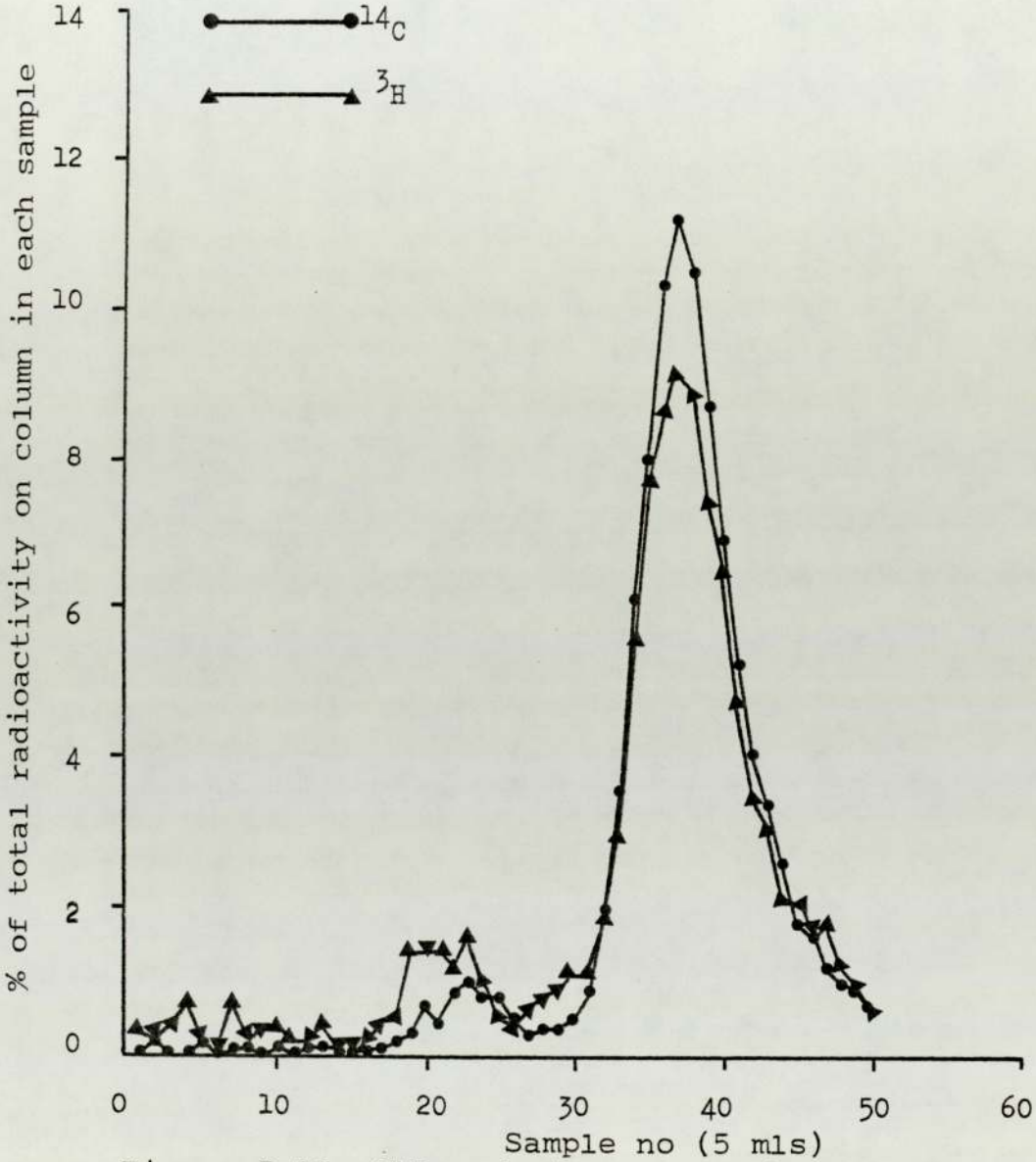


Figure 5.12 G15 sephadex column chromatogram of 0-6 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate (10mg/kg) administration.

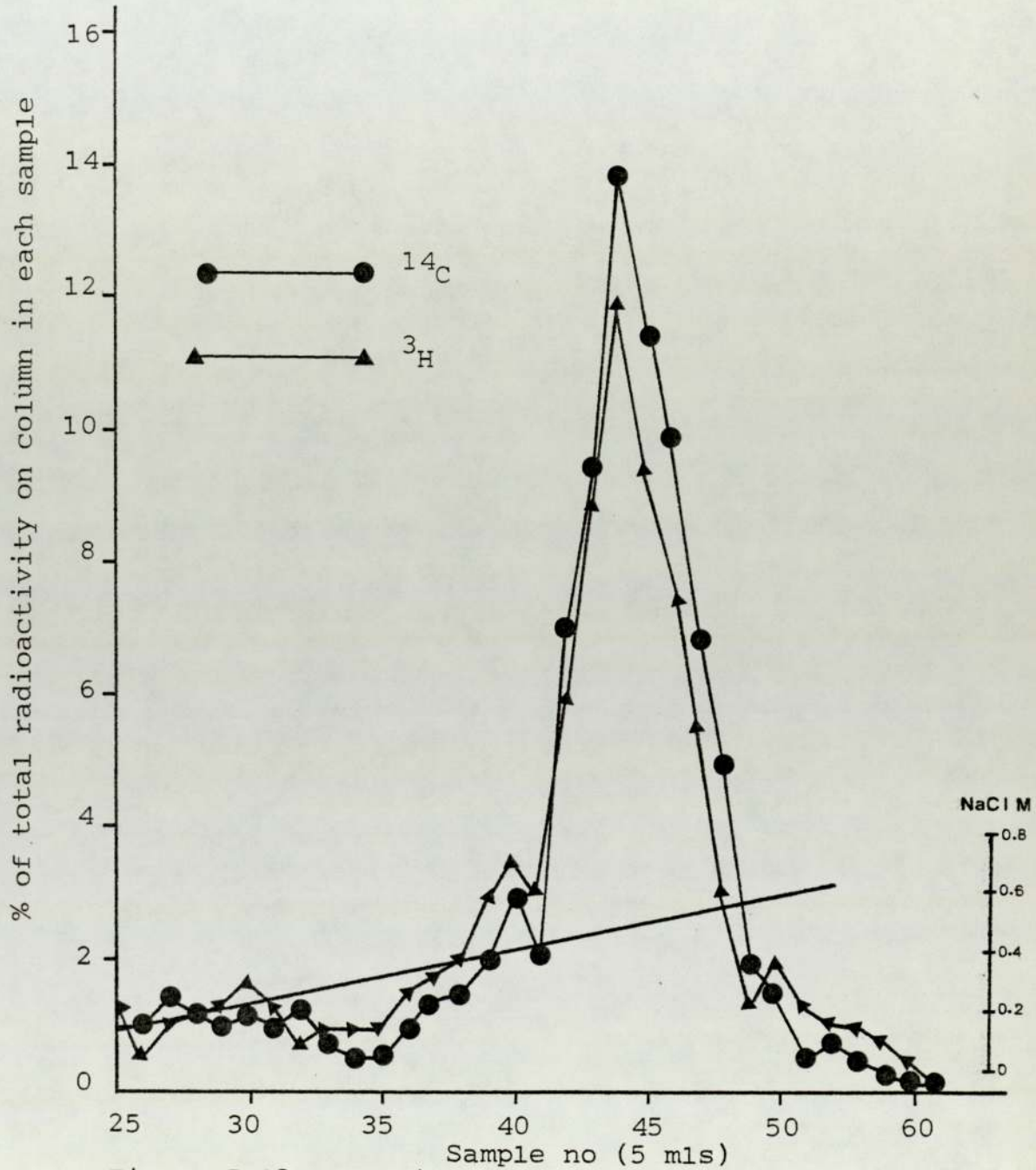


Figure 5.13 DEAE ion-exchange column chromatogram of 'boiled' liver extract of rats receiving a mixture of tritium and carbon-14 labelled folic acid.

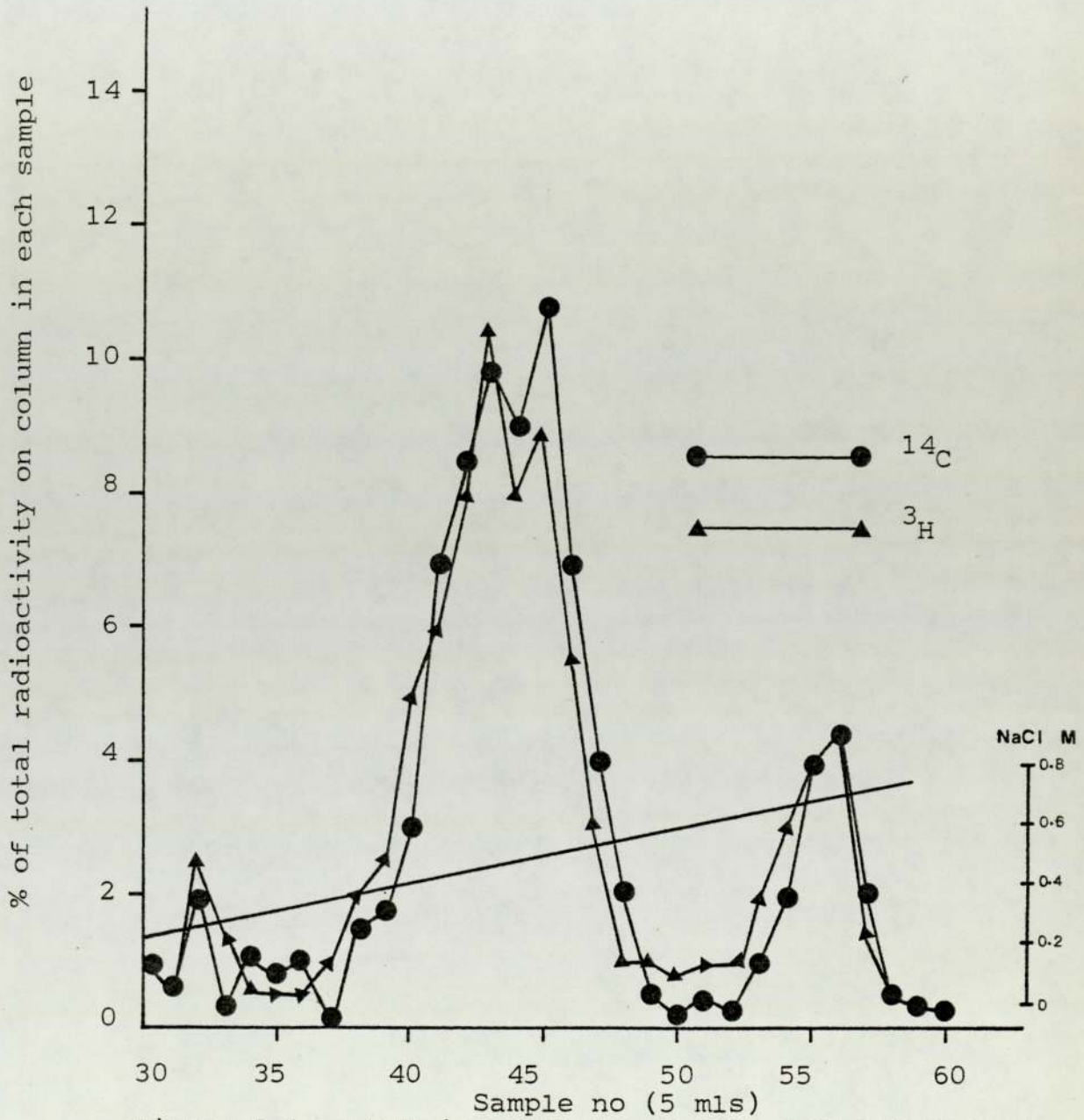


Figure 5.14 DEAE ion-exchange column chromatogram of 'boiled' liver extract of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate (10 mg/kg) administration.

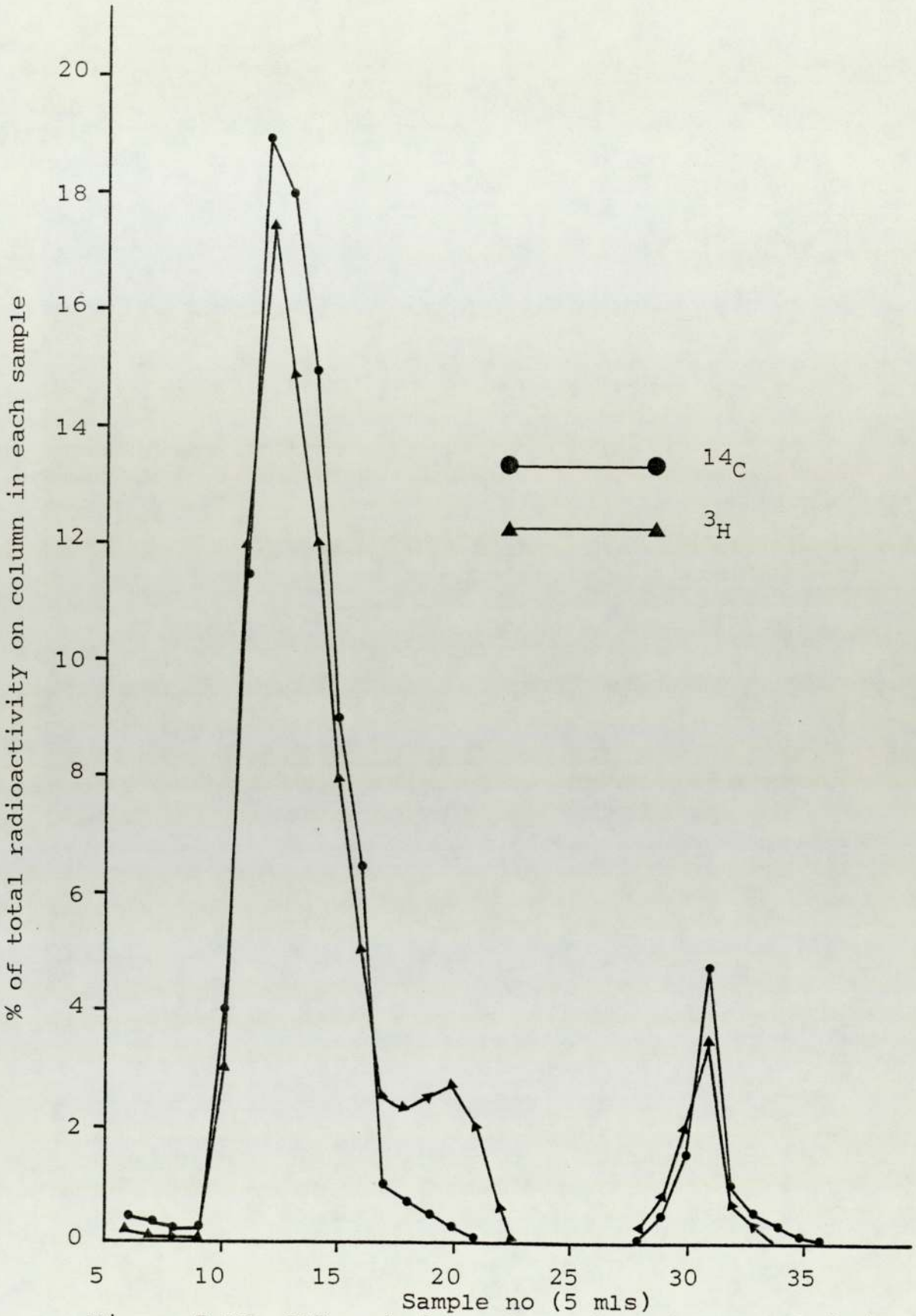


Figure 5.15 G15 sephadex column chromatogram of 'boiled' liver extract of rats receiving a mixture of tritium and carbon-14 labelled folic acid.

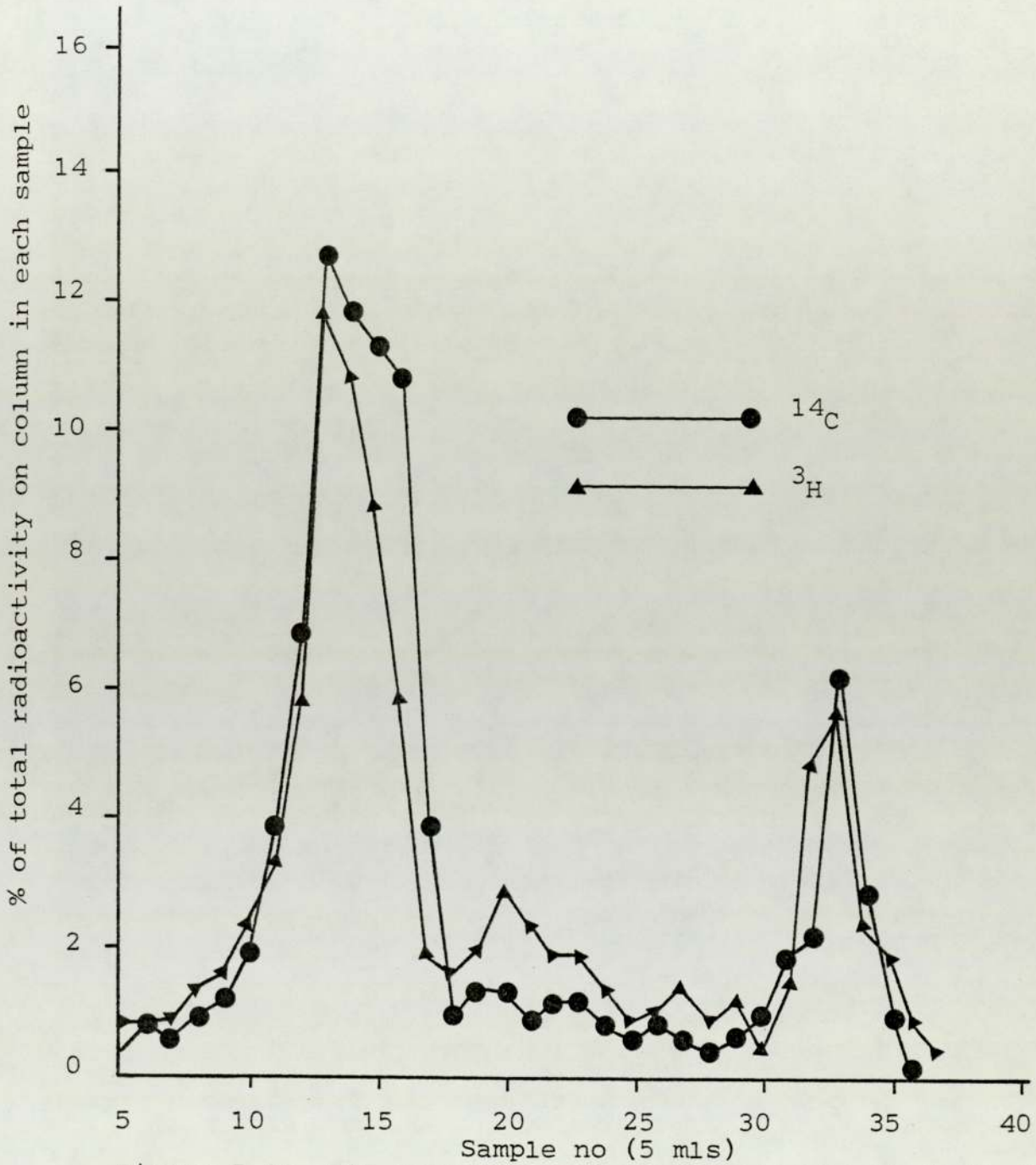


Figure 5.16 G15 sephadex column chromatogram of 'boiled' liver extract of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate (10 mg/kg) administration.

Discussion

Table 1 gives the distribution of radioactivity contained in an oral dose of 3'-5'-9' ^{3}H folic acid and 2-^{14}C folic acid in urine, gut, liver and faeces of control animals, expressed as a percentage of the dose given. It shows that in 0-6 hours urine, on average, 7.9% and 8.4% of the dosed radioactivity was excreted in the form of ^{14}C and ^{3}H labelled materials respectively. When the same urine was chromatographed on a DE52 ion-exchange column (figure 5.1), four radioactive peaks were observed. Table 4 gives the distribution of chromatographed radioactivity in the peaks. The major peak which appears after 0.9 molar sodium chloride concentration on the gradient was identified as folic acid. Table 4 shows that it contains 71.1% of the chromatographed radioactivity in the form of 2-^{14}C folic acid. This means that 5.6% of the dosed 2-^{14}C folic acid has been excreted as such in the 0-6 hours urine. Similarly it appears that 4.3% of the dosed 3'-5'-9' ^{3}H folic acid has been excreted in the same urine.

Table 2 shows that the rats receiving 10 mg/kg body weight methotrexate, 24 hours before receiving same oral dose of 3'-5'-9 ^{3}H folic acid and 2-^{14}C folic acid, excreted 17.4% and 20.7% of the dosed radioactivity in the form of ^{14}C and ^{3}H labelled materials in 0-6 hours urine. The DE52 ion-exchange chromatogram of the same urine (figure 5.2) shows that 83.6% of the chromatographed ^{14}C radioactivity is due to unmetabolised 2-^{14}C folic acid dosed, and 64.3% of the chromatographed ^{3}H radioactivity

is due to unmetabolised 3'-5'-9 ^{3}H folic acid dosed. This means that about 14.5% of the dosed 2-^{14}C folic acid and 13.3% of the dosed 3'-5'-9 ^{3}H folic acid have been excreted unchanged in 0-6 hours urine. These recoveries are much higher than the corresponding recoveries of unchanged dosed folic acid in the urine of control animals collected during the same period. Hence these experiments suggest that administration of methotrexate increases the excretion of unmetabolised folic acid in the urine in first six hours.

The 0-6 hours urine of rats receiving 100 mg/kg body weight methotrexate contained 21.2% radioactivity due to 2-^{14}C folic acid dosed, and 22% radioactivity due to 3'-5'-9 ^{3}H folic acid dosed (table 3). DE52 ion-exchange chromatography of the same urine (figure 5.3) shows that the unmetabolised folic acid peak contains 90.1% of the chromatographed ^{14}C radioactivity and 77.4% of the chromatographed ^{3}H radioactivity. In other words, 19.1% of the dosed 2-^{14}C folic acid and 17% of the dosed 3'-5'-9 ^{3}H-folic acid have appeared unchanged in the 0-6 hours urine. The unmetabolised folic acid excretion level in this case, which is higher than the excretion level of unmetabolised folic acid in rats receiving 10 mg/kg body weight methotrexate, suggests that higher dose of methotrexate increases the excretion level of unmetabolised folic acid in urine in first six hours.

Similar analysis of the 6-24 hours urine samples show that control animals excreted 5.6% of the dosed

2-^{14}C folic acid and 4.3% of the dosed 3'-5'-9' ^{3}H folic acid as unmetabolised folic acid. Comparing this with the urine of rats receiving 10 mg/kg body weight methotrexate, the latter excreted about 5% of the dosed 2-^{14}C folic acid and 4.3% of the dosed 3'-5'-9 ^{3}H folic acid as unmetabolised folic acid in 6-24 hours urine. This indicates that there is no significant difference in the excretion levels of unmetabolised folic acid in 6-24 hours urine of controlled rats and rats receiving 10 mg/kg body weight methotrexate. However, the 6-24 hours urine of rats receiving 100 mg/kg body weight methotrexate contained 9,9% of the dosed 2-^{14}C folic acid and 7.7% of the dosed 3'-5'-9 ^{3}H folic acid as unmetabolised folic acid, which is higher than the corresponding amounts of unmetabolised folic acid present in 6-24 hours urine of rats receiving 10 mg/kg body weight methotrexate and controlled rats.

Figure 5.1 shows that 5-methyltetrahydrofolate is the second major peak, which is both ^{14}C and ^{3}H labelled. ^{14}C radioactivity in this peak is 16.9% of the chromatographed radioactivity, while ^{3}H radioactivity in the same peak is 12.3%. Table 1 shows that on average 7.9% and 8.4% of the dosed ^{14}C and ^{3}H radioactivity respectively is excreted in 0-6 hours urine of controlled rats. Hence ^{14}C labelled 5-methyltetrahydrofolate in the above mentioned peak is about 1.3% of the dosed 2-^{14}C folic acid, while ^{3}H labelled 5-methyltetrahydrofolate is about 1% of the dosed 3'-5'-9 ^{3}H folic acid. The corresponding DE52 ion-exchange chromatograms of rats receiving 10 mg/kg and

100 mg/kg body weight methotrexate (figures 5.2 and 5.3 respectively) show that 5-methyltetrahydrofolate is present in very minute quantity.

The 6-24 hours control urine, however, contains a large peak due to 5-methyltetrahydrofolate (figure 5.6). Table 4 shows that in this peak 19.2% of the chromatographed radioactivity is due to ^{14}C and 16.9% due to ^{3}H. Hence ^{14}C labelled 5-methyltetrahydrofolate is about 2.4% of the dosed radioactivity. The tritium peak also contains about 2.4% of the dosed tritium radioactivity. The corresponding DE52 ion-exchange column chromatograms of 6-24 hours urine of rats receiving 10 mg/kg methotrexate (figure 5.7) and 100 mg/kg methotrexate (figure 5.8) show that 5-methyl-tetrahydrofolate peak in each acse is less than 1% of the dosed radioactivity for both ^{14}C and ^{3}H labelled compounds.

The second major peak, containing both ^{3}H and ^{14}C, in DE52 ion-exchange column chromatograms of 0-6 hours and 6-24 hours urines of rats receiving either 10 mg/kg or 100 mg/kg body weight methotrexate (figures 5.2,5.7,5.3,5.8) was identified as 10-formyltetrahydrofolate by virtue of its elution position on DE52 column, though it might have been oxidised to 10-formylfolate during collection and re-chromatography of urine samples.

It is evident from table 5, which gives the amounts of 10-formyltetrahydrofolate present in the urine of control and methotrexate treated rats,that the excretion level of 10-formyltetrahydrofolate is significantly higher in urine of methotrexate treated animals than in control.

The next identified compound in the urine of methotrexate treated animalswas para-aminobenzoyl glutamate, which shows that the dosed folic acid molecule has been split. Whether this happens in the course of urine collection and re-chromatography or occurs in the animal system is not clear.

Table 1 shows that the gut of control animals retained, on average, 6.1% of the dosed 2-^{14}C folic acid radioactivity and 3% of the dosed 3'-5'-9 ^{3}H folic acid radioactivity. Table 2 shows the corresponding retention of radioactivity (2.3% and 2.7% of the dosed 2-^{14}C folic acid and 3'-5'-9 ^{3}H folic acid respectively) in the gut of animals receiving 10 mg/kg methotrexate. The lower amount of radioactivity retained in the gut of methotrexate treated animals indicates that the presence of methotrexate has reduced the uptake and retention of folic acid or its metabolites. Table 3 shows, however, that the rats receiving 100 mg/kg methotrexate have retained higher amounts of the dosed folic acid or its metabolites in the gut, which contradicts the above statement.

The retained radioactivity in the liver of control animals is much higher than the animals receiving 10 mg/kg or 100 mg/kg methotrexate (tables 1, 2, and 3). This is consistent with the previous results documented in chapter 4. Thus methotrexate decreases the uptake of folates equally after low or high doses. Methotrexate has also effected the uptake of folic acid in the gut which is evident from the higher percentage of radioactivity recovered in the faeces.

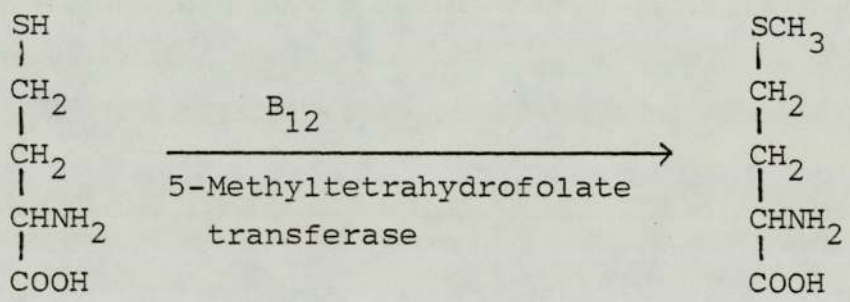
CHAPTER 6

METABOLISM OF 5-^{14}C METHYLTETRAHYDROFOLATE IN RAT IN PRESENCE AND ABSENCE OF METHOTREXATE

Introduction

Figure 1 shows the formation of 5-methyltetrahydrofolate, which is ultimately formed from tetrahydrofolate and serine as a one carbon atom donor, the tetrahydrofolate on the other hand is formed by the enzymic reduction of dihydrofolate and folic acid using the enzyme dihydrofolate reductase by the steps shown in figure 1.

5-Methyltetrahydrofolate transfers the N5 methyl group to homocysteine forming methionine in a reaction catalysed by the B_{12} containing enzyme homocysteine 5-methyltetrahydrofolate transferase (38,173,174,175).



Homocysteine Methionine

This enzymic reaction uses monoglutamate form only, on the other hand, in bacteria a non B_{12} requiring pathway for the methylation of homocysteine to form methionine has been demonstrated to have an absolute requirement for a polyglutamate and is inhibited by the monoglutamates (176,177). However, no evidence for this system has been recorded for animals.

Methionine is then activated by ATP to S-adenosylmethionine in presence of methionine adenosyltransferase.

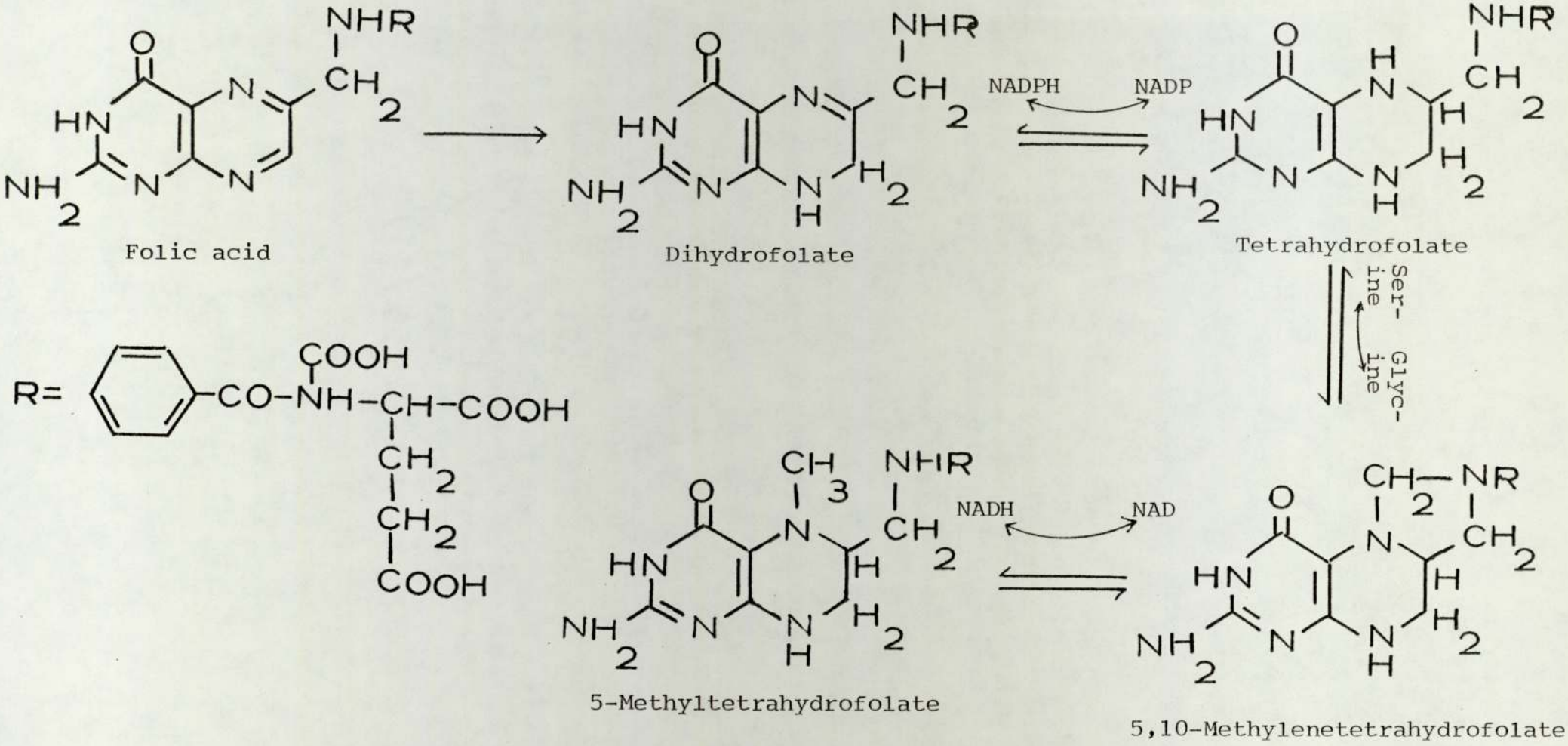
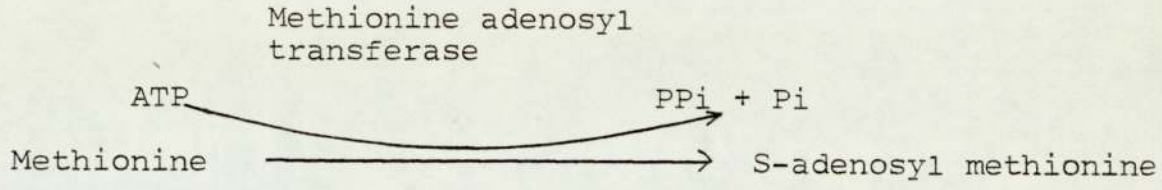
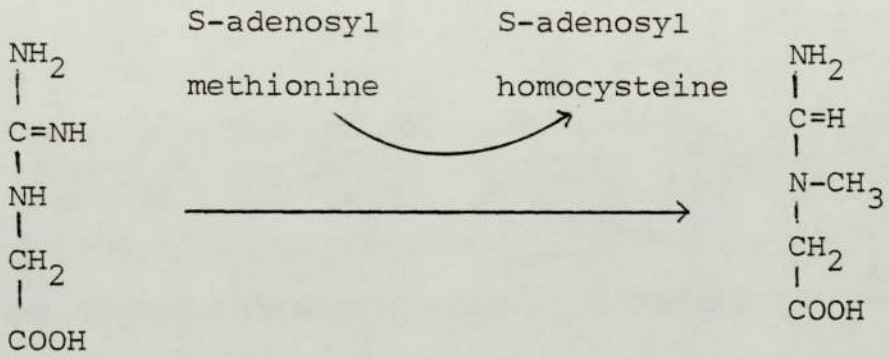


Figure 1 Formation of 5-methyltetrahydrofolate.



The methyl group of S-adenosyl methionine is then transfered to various purine bases present in messenger and transfer ribonucleic acids and to creatine.



Guanidinoacetic acid

Creatine

Thus the methylation of tetrahydrofolate and the demethylation of 5-methyltetrahydrofolate provides a route for the de novo synthesis of methyl groups from serine and ultimately glucose required for the biosynthesis of S-adenosyl methionine, creatine and methylated RNAs.

However, it is well established that a rat requires an exogenous supply of methionine for the maintenance of normal health. Therefore, the exogenous supply of methyl groups synthesized by de novo route is not adequate.

It has been shown that in normal adult man on an adequate diet, the proportion of methyl groups used in biosynthesis reactions obtained from diet is about 74% and that

obtained by de novo pathway is about 26% (178).

The transfer of the N5 methyl group from 5-methyltetrahydrofolate releases tetrahydrofolate which then participates in a series of one carbon transfer reactions forming the co-enzymes 10-formyltetrahydrofolate, 5,10-methenyltetrahydrofolate and 5,10-methylenetetrahydrofolate. These co-enzymes then supply the one carbon atom fragments required for the biosynthesis of purine and thymidine (see chapter 1).

Any interference with supply and metabolism of 5-methyltetrahydrofolate would therefore have marked effects on the metabolism of the mammal. The most extensively studied example of this to date is in human B_{12} deficiency arising from pernicious anaemia or dietary deficiency (179-183). Thus lack of vitamin B_{12} reduces the rate of 5-methyltetrahydrofolate ('methyl trap hypothesis') demethylation and slows down the formation of purines, thymidine, RNA and DNA. Reduction in this biosynthetic processes by this method results in megaloblastic anaemias readily reversible by B_{12} administration.

It has been suggested that the loss of the methyl group from 5-methyltetrahydrofolate can occur by an alternative process, the reversal of its formation from tetrahydrofolate and serine (184). Other authors have however rejected this and that (a) it is thermodynamically unlikely and (b) argued that where this process has been observed in tissue extracts it is an analytical artifact caused by the incorporation of strong electron acceptors (185).

In view of the ease by which megaloblastic anaemias can be induced by B_{12} deficiency, it seems unlikely that if this alternative pathway operates it makes a major contribution to the release of tetrahydrofolate from 5-methyltetrahydrofolate.

Although methotrexate has been widely used in the treatment of cancer and psoriasis and its biochemical effects on folate metabolism extensively studied in isolated biological systems (186,187,196-199), little work has been described on its action on folate metabolism in the intact animal. This chapter describes some experiments directed towards this end. 5-Methyltetrahydrofolate labelled with ^{14}C in the methyl group makes it possible to follow the metabolic fate of this group and to investigate if methotrexate has any effect on the de novo synthesis of methyl groups and metabolic sequences dependent on this. It was hoped that use of this labelled compound would provide some information on the suggested reversal of the 5-methyltetrahydrofolate formation pathway by studying the possible appearance of ^{14}C labelled 10-formylfolate in the urine. It has been proposed that 4a-hydroxy-5-methyltetrahydrofolate a known oxidation product of 5-methyltetrahydrofolate is a long term metabolite of folates (145).

Further information on this possibility could be obtained from an analysis of the products found in the urine after 5-^{14}C methyltetrahydrofolate administration. An earlier claim that 5-^{14}C methyltetrahydrofolate forms liver polyglutamate is reinvestigated. A previous statement that the

two diastereoisomers of 5-methyltetrahydrofolate are absorbed through the intestine at different rates is reexamined.

Materials and Methods

5-^{14}C methyltetrahydrofolate was purchased from the Radio-chemical Centre, Amersham. It was a 50-50 mixture of the two diasterioisomers epimeric at the C-6 position. Methotrexate was obtained from the Sigma Chemical Company Limited.

Four male adult Wistar rats weighing 230-250 grams received methotrexate 100 mg/kg body weight in water suspension after mild ether anaesthesia.

Four Wistar rats of the same weight, age and sex were given same quantity of water without methotrexate under mild anaesthesia.

Twenty four hours later both groups of rats received 5-^{14}C methyltetrahydrofolate 70 microgram/kg body weight in water orally. Animals were then immediately housed in metabolic cages, Jencons Metabowls, specially designed to collect urine and faeces separately.

Collection of biological samples

All urines were collected in flasks containing 5 mls of 0.05 molar phosphate buffer solution, pH 7, containing 2% ascorbate and 5 mg/100 mls of dithiothreitol. The flasks were changed after the first six hours and at twenty four hours. Faeces were collected for 0-24 hours in empty flasks.

The animals were killed after twenty four hours and the livers were taken out immediately. Two livers from each group of rats were chopped, separately put in 20 mls

of boiling phosphate buffer 0.05 molar, pH 7, with 2% ascorbate and left boiling for fifteen minutes to inactivate the conjugases. Solid material was discarded after centrifugation and the extract stored in deep freeze for chromatographic analysis. Another two livers from each group were freeze dried and powdered for oxidation in Beckman biological material oxidiser for the determination of the total radioactivity in each rat liver. Faeces was also freeze dried and subjected to the same means of the determination of radioactivity as livers. All the samples were counted in a liquid Scintillation Counter type NE 8310.

Chromatography of biological samples and extracts

All the urines and liver extracts were chromatographed on DE52 ion-exchange columns and G15 sephadex columns.

DE52 ion-exchange columns were equilibrated with 0.05 molar phosphate buffer, pH 7, containing 5 mg of dithiothreitol /100 mls as antioxidant and were eluted with a linear gradient of 0-1 molar sodium chloride in 0.05 molar phosphate buffer, pH 7, containing 5 mg/100 mls dithiothreitol. 5 mls fractions were collected by automatic LKB fraction collector. Each fraction was counted separately for total radioactivity, in scintillation cocktail. 100% radioactivity was recovered from most of the columns. Sephadex G15 columns were equilibrated with 0.05 molar phosphate buffer, pH 7, containing 5 mg of dithiothreitol per 100 mls and were eluted with the same buffer solution.

RESULTS

Recovery of radioactivity

The recovered radioactivity as a percentage of the administered dose of 5-^{14}C methyltetrahydrofolate (70 microgram/kg body weight) in the urine, faeces and liver of control and methotrexate treated animals is given in tables 1 and 2 respectively.

Table 1 shows that 31.1% of the total dose of 5-^{14}C methyltetrahydrofolate (70 microgram/kg) radioactivity is excreted in the urine in 24 hours, 23.9% of the dosed radioactivity is excreted in faeces, while 9.7% of the dosed radioactivity is excreted in liver. The total radioactivity excreted through these biological products, on average, is 64.7% of the dose.

In methotrexate treated animals, however, it appears that less radioactivity has been excreted in the urine and more in the faeces than control animals (table 2). The amount of radioactivity recovered in the urine and faeces of methotrexate treated animals, on average, is 21.4% and 33.0% respectively (table 2).

Comparison of tables 1 and 2 also shows that retention of radioactivity in the liver of methotrexate treated animals is much less than in control animals. The percentage recovery of radioactivity from the liver of methotrexate treated animals was only 4.3% (on average) as compared to 9.7% from the liver of control animals.

Column chromatography of 0-6 hours urine of control rats

A typical DE52 column chromatogram of 0-6 hours urine is shown in figure 6.1. The major peak 1 is eluted between 0.6 and o.7 molar sodium chloride concentration and is about 84.5% of the total radioactivity in the urine put on the column. Peak 2 appearing at 0.4 to 0.5 molar sodium chloride concentration constitutes 5.5% of the total radioactivity put on the column. Peak 3 is between 0.25 and 0.35 molar sodium chloride concentration and represents 1.7% of the total radioactivity. Peak 4, though not very much separated from peak 5 yet seems to be a different metabolite from compound of peak 5, represents less than 1% of radioactivity on the column. Peak 5 appears at the start of gradient and is about 4% of the total radioactivity on the column.

Table 3 gives the distribution of radioactivity as a percentage of the total radioactivity put on the column.

Sephadex G15 chromatography of the 0-6 hours urine sample is shown in figure 6.2. Sephadex G15 resolves the urine in two peaks, the major one extending from tube fraction number 20 to 40 and other very small peak appearing between tube fractions 13 and 18. This chromatogram produces less resolution of the metabolites found in 0-6 hours urine than the DE52 column.

Identification of peaks appearing in DE52 column chromatogram of 0-6 hours urine (figure 6.1)

Peak 1

The major peak in figure 6.1 appears at a similar position to authentic 5-methyltetrahydrofolate on the same column. When the fractions composing this peak were pooled and re-chromatographed with authentic 5-methyltetrahydrofolate on DE52 ion-exchange column, a single radioactive peak appeared at the same concentration of sodium chloride gradient, which was not separable from the UV peak representing the authentic 5-methyltetrahydrofolate. Figure 6.3 shows the result of re-chromatography.

Other evidence of this peak being 5-methyltetrahydrofolate was obtained when the radioactive contents of the peak were mixed with authentic cold 5-methyltetrahydrofolate and the resulting solution re-chromatographed on DE52 ion-exchange column after oxidation with hydrogen peroxide. The radioactivity and UV absorption was equally resolved into two peaks. The major peak appearing at 0.3 molar sodium chloride concentration where peak 3 of the first urine chromatogram (figure 6.1) appeared. Second minor peak appeared at the same position as peak 1 in figure 6.1. This indicates that peak 3 in figure 6.1 might be the oxidation product of peak 1 in figure 6.1.

Peak 2

The pooled fractions under this peak were re-chromatographed with authentic 10-formylfolate on a DE52 ion-exchange column. The UV peak of 10-formylfolate

separated from the radioactivity peak (figure 6.4).

Similarly the re-chromatography of the contents of this peak with authentic 5-formyltetrahydrofolate separated the two compounds. Hence peak 2 in figure 6.1 is not 10-formylfolate or 5-formyltetrahydrofolate.

The possibility of this peak being 10-formyl-tetrahydrofolate was eliminated when the contents of the peak were oxidised with air and re-chromatographed with authentic 10-formylfolate on DE52 ion-exchange column; the UV and radioactivity peaks separated.

Peak 3

Peak 3 in figure 6.1 appears at the marker site of 4a-hydroxy-5-methyltetrahydrofolate on DE52 ion-exchange column. As mentioned before, when 5-methyltetrahydrofolate was oxidised with hydrogen peroxide as described by Gapski et.al (190) and re-chromatographed on DE52 ion-exchange column, two radioactive peaks appeared. One at the original site of 5-methyltetrahydrofolate and the second at the same position as peak 3 in figure 6.1. The description of the oxidation product given by Gapski et.al (190) are those of 4a-hydroxy-5-methyltetrahydrofolate. Therefore, peak 3 is most probably 4a-hydroxy-5-methyl-tetrahydrofolate.

Peak 4

No attempt was made to identify this peak.

Peak 5

The contents of the fractions appearing under this peak were mixed with authentic creatine and

re-chromatographed on DE52 ion-exchange column. Creatine was identified by Jaffe's reaction after converting it to creatinine. The radioactivity was found only in the fractions showing positive Jaffe's reaction. The UV absorption after the development of pinkish colour, of each fraction, matched the radioactive peak 5 (figure 6.5). The re-chromatography of peak 5 on G15 sephadex column with creatine also gave similar result.

The methyl group of creatine and creatinine comes from the "active methionine" which inturn gets its methyl group from 5-methyltetrahydrofolate, its immediate precursor, therefore creatine in the urine of rats dosed with 5-methyltetrahydrofolate is not unexpected.

Chromatography of 6-24 hours urine of control animals

The chromatography of 6-24 hours urine also resulted in similar results as 0-6 hours urine.

Figure 6.6 is a DE52 ion-exchange chromatogram of 6-24 hours urine of control rats. All metabolites found in 0-6 hours urine (figure 6.1) are also present in 6-24 hours urine.

Figure 6.7 is a G15 sephadex column chromatogram of the same urine.

Chromatography of 0-6 hours and 6-24 hours urine samples of methotrexate treated animals

Figure 6.8 shows a DE52 ion-exchange column chromatogram of 0-6 hours urine of methotrexate treated animals. It shows the same number of peaks as in the

corresponding control animals urine chromatogram (figure 6.1). Figure 6.9 is a G15 sephadex column chromatogram of 0-6 hours urine of rats dosed with methotrexate. It also shows almost same two peaks found in the corresponding control animals chromatogram (figure 6.2).

The 6-24 hours urine samples of methotrexate treated animals also gave similar pattern of various metabolites as in the corresponding urine of control animals. Figures 6.10 and 6.11 show DE52 ion-exchange and G15 sephadex column chromatograms of 6-24 hours urine of methotrexate treated animals respectively.

The qualitative anlysis carried out for the identification of metabolites appearing in DE52 ion-exchange column chromatograms (figures 6.8 and 6.10) consisted of the same procedure of chromatography and re-chromatography of pooled fractions under the peaks concerned with authentic materials. The identified peaks were exactly the same found in control animals urine.

Chromatography of liver extracts

A DE52 ion-exchange column chromatogram of control liver extract is shown in figure 6.12. Figure 6.13 shows the G15 sephadex column chromatogram of the same extract.

Peak 1 in DE52 chromatogram of liver extract of control animals (figure 6.12) appears at the same position as peak 5 in figure 6.1. The re-chromatography of this peak with authentic creatine showed that Jaffe's reaction colour was developed by the fractions containing radio-active material. The UV absorption of the contents did not help owing to the coloured material already present in the extract. However, in the above mentioned peak creatine is probably present.

Rat No	Urine		Faeces	Liver	Total
	0-6 hrs	6-24 hrs			
1	16.6	14.8	20.2	-	-
2	25.8	19.8	23.8	7.9	77.3
3	5.4	18.5	29.1	11.5	64.5
4	7.1	16.5	22.8	-	-
Average	13.7	17.4	23.9 ± 3.75	9.7	64.7*

Table 1 Distribution of radioactivity in urine, faeces and liver (expressed as a percentage of 5-^{14}C methyltetrahydrofolate dosed) of control animals killed 24 hours after 5-^{14}C methyltetrahydrofolate administration.

* The figures denote sum of the individual averages.

Rat No	Urine		Faeces	Liver	Total
	0-6 hrs	6-24 hrs			
1	19.4	6.4	33.3	-	-
2	11.2	14.8	31.2	4.7	61.9
3	8.6	17.1	34.7	3.8	64.2
4	3.0	5.3	-	-	-
Average	10.5	10.9	33.0	4.2	58.7*

Table 2 Distribution of radioactivity in urine, faeces and liver (expressed as a percentage of 5-^{14}C methyltetrahydrofolate dosed) of methotrexate treated animals killed 24 hours after 5-^{14}C methyltetrahydrofolate administration.

* The figures denote sum of the individual averages.

Chromatogram	Metabolites				
	1	2	3	4	5
Fig 6.1	84.5	5.5	1.7	0.7	4.2
Fig 6.6	69.8	6.9	5.2	1.1	11.0
Fig 6.8	90.0	2.6	1.4	1.4	2.0
Fig 6.10	68.3	20.0	1.8	1.4	6.3

Table 3 Distribution of radioactivity in metabolites expressed as a percentage of the total radioactivity chromatographed.

The metabolites numbered as 1, 3 and 5 in the above table appear at authentic marker sites of 5-methyltetrahydrofolate, 4a-hydroxy-5-methyltetrahydrofolate and creatine respectively. The metabolites numbered as 2 and 4 have not been identified.

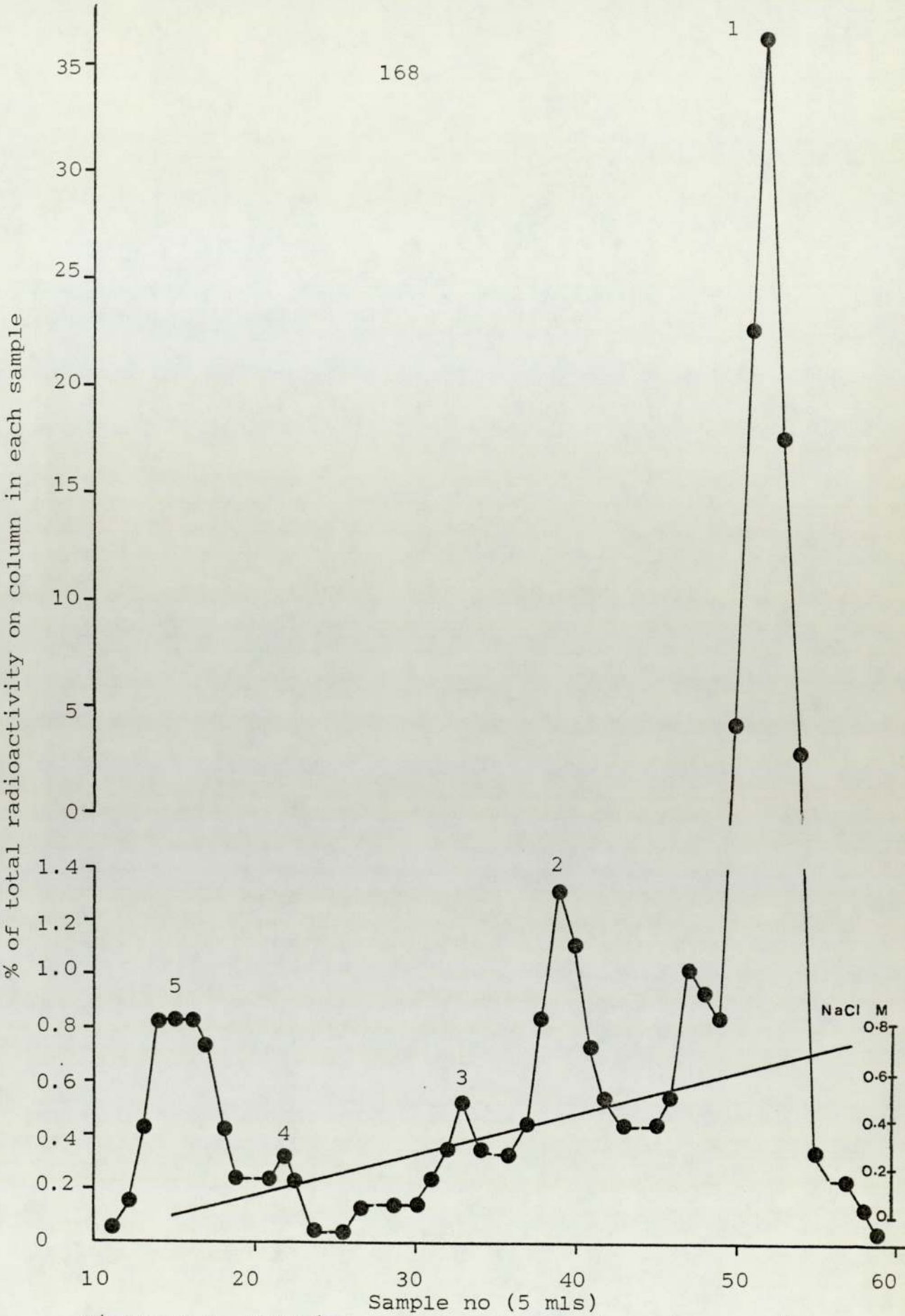


Figure 6.1 DEAE ion-exchange column chromatogram of 0-6 hr urine of rats receiving 5-methyl-tetrahydrofolate, labelled with carbon-14 at methyl group.

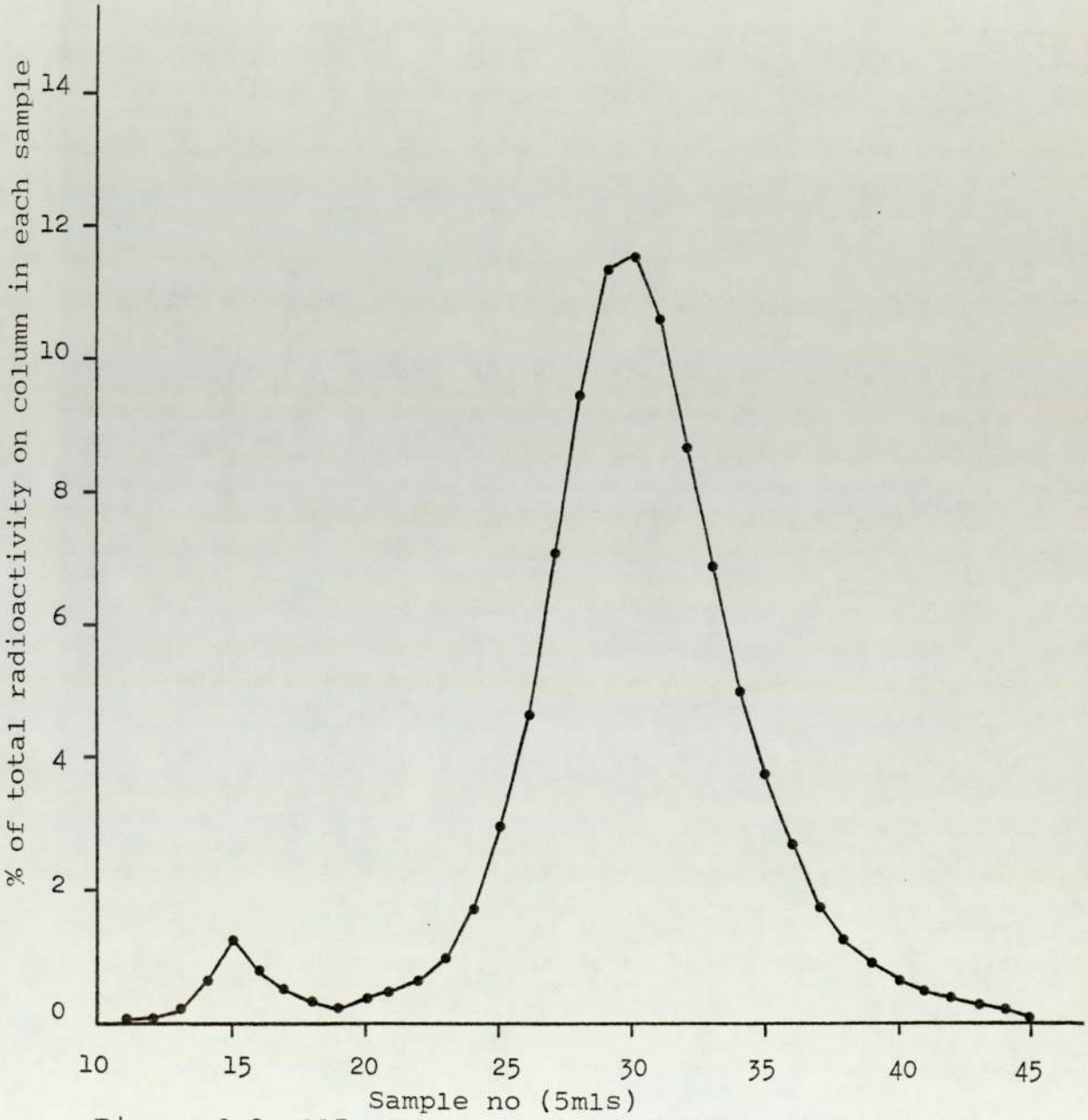


Figure 6.2 G15 sephadex column chromatogram of 0-6 hr urine of rats receiving 5-methyltetrahydrofolate, labelled with carbon-14 at methyl group.

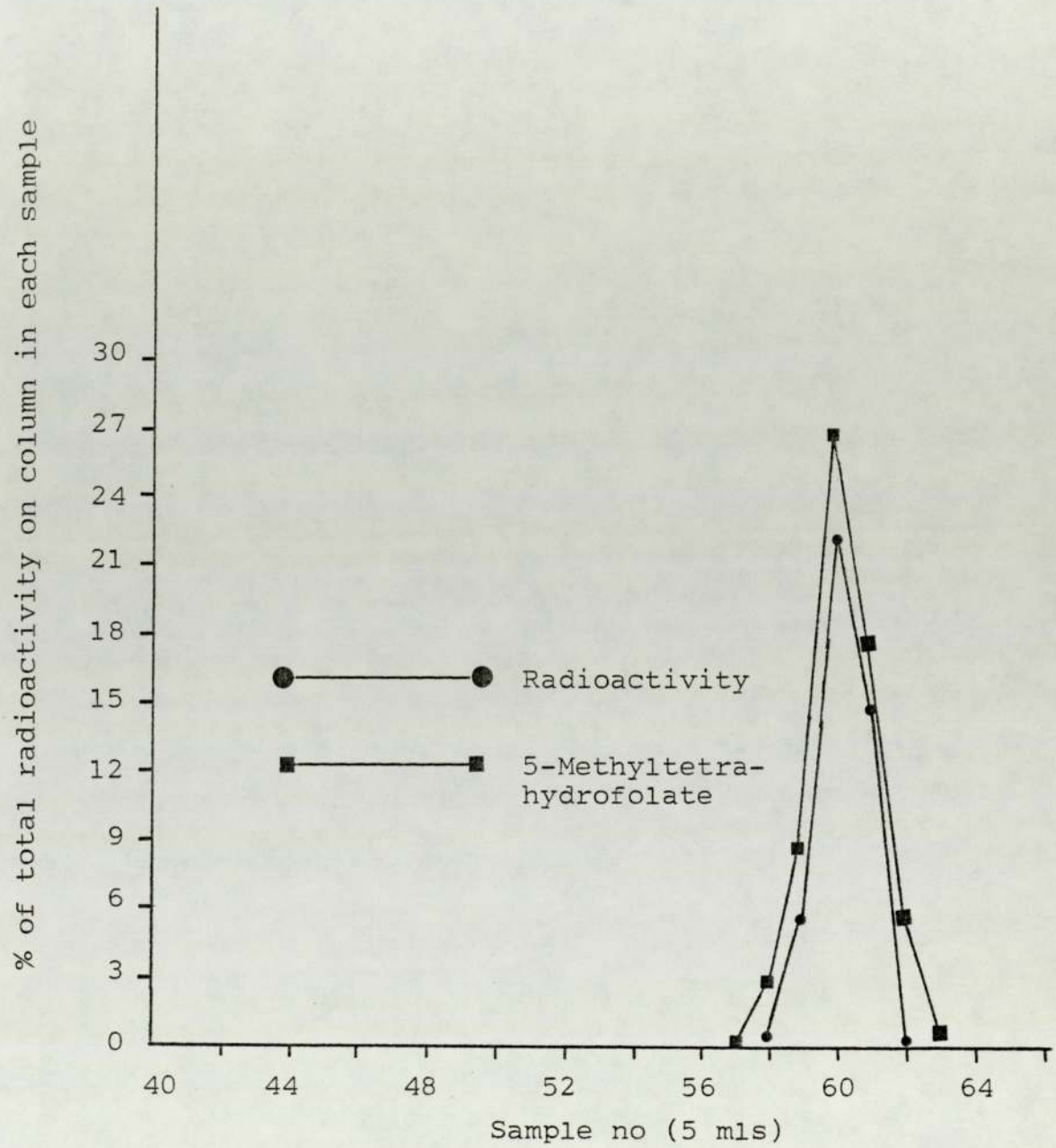


Figure 6.3 DEAE ion-exchange column chromatogram of peak number 2 in figure 6.1 mixed with unlabelled 5-methyltetrahydrofolate.

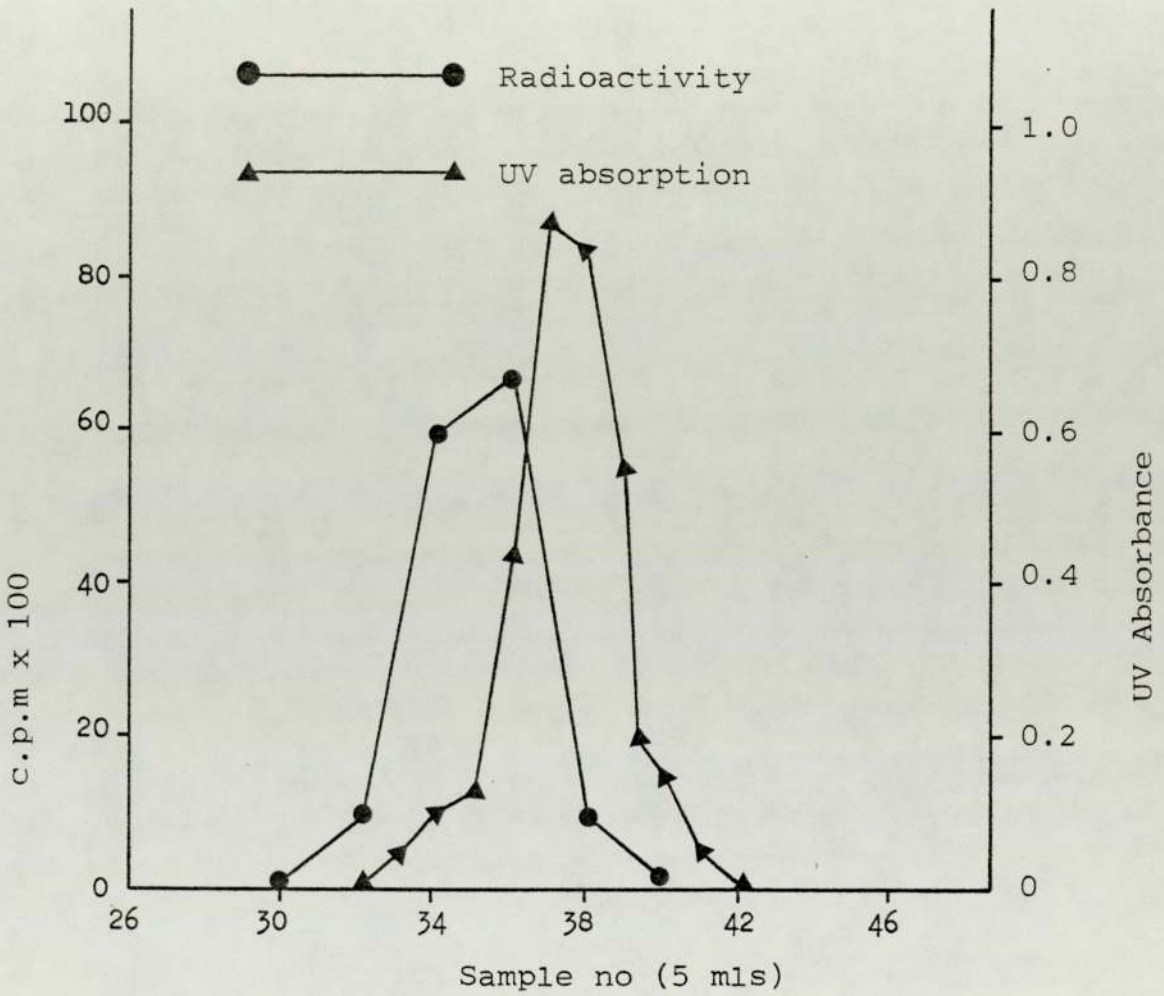


Figure 6.4 DEAE ion-exchange column chromatogram of of peak number 2 in figure 6.1 mixed with unlabelled 10-formyltetrahydrofolate.

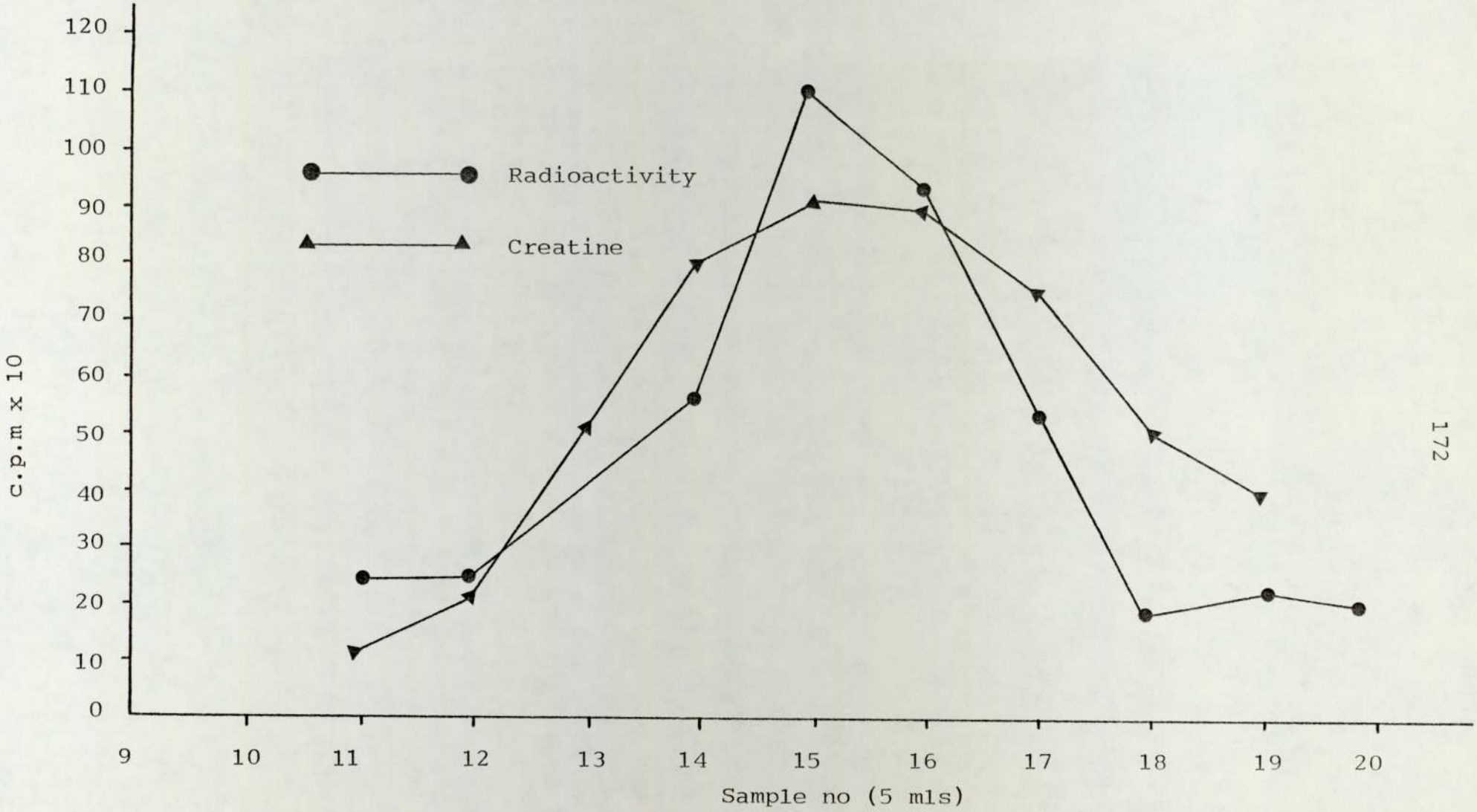


Figure 6.5 DEAE ion-exchange column chromatogram of peak number 5 in figure 6.1 mixed creatine.

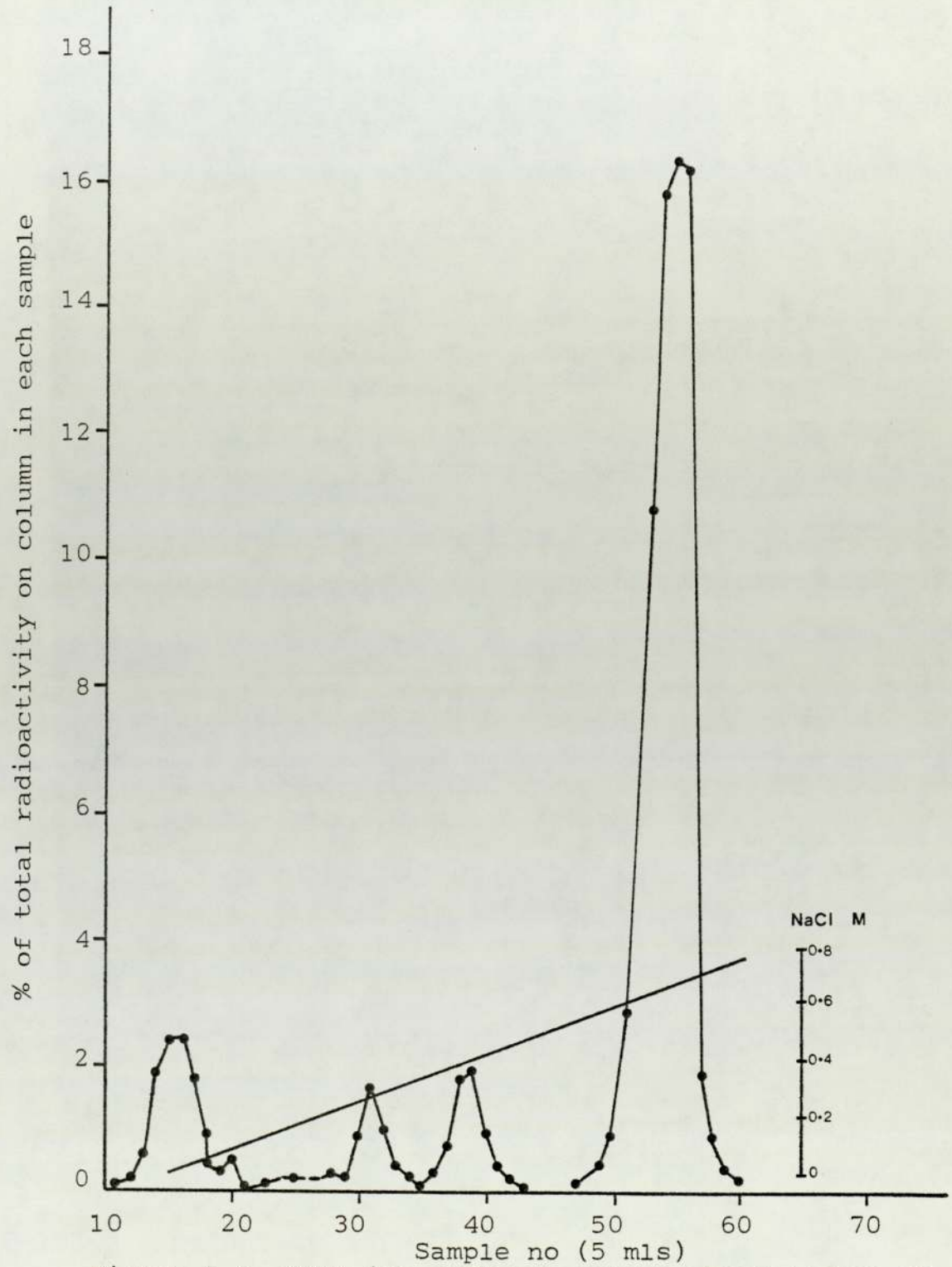


Figure 6.6 DEAE ion-exchange column chromatogram of 6-24 hr urine of rats receiving 5-methyl-tetrahydrofolate, labelled with carbon-14 at methyl group.

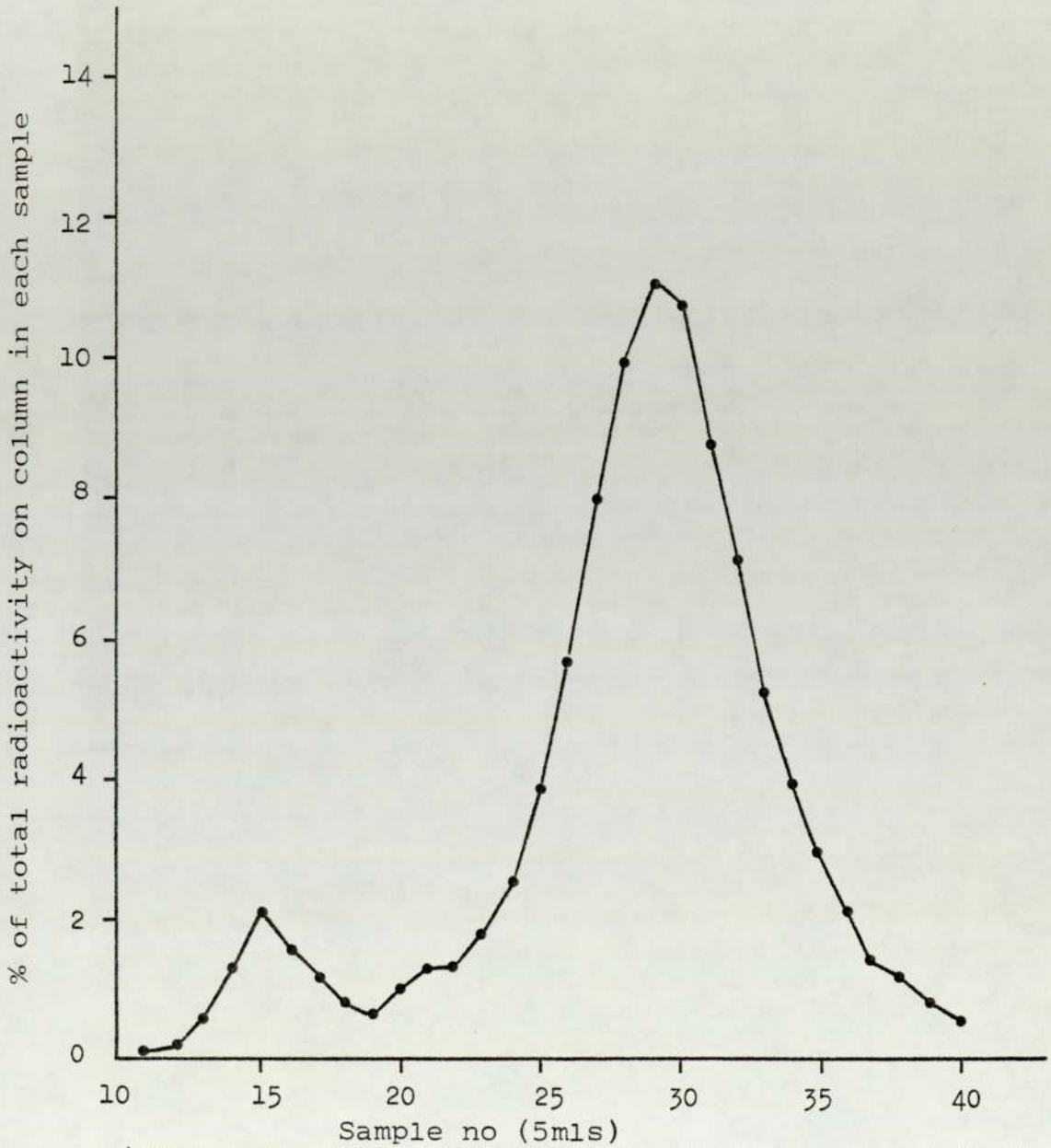


Figure 6.7 G15 sephadex column chromatogram of 6-24 hr urine of rats receiving 5-methyltetrahydrofolate, labelled with carbon-14 at methyl group.

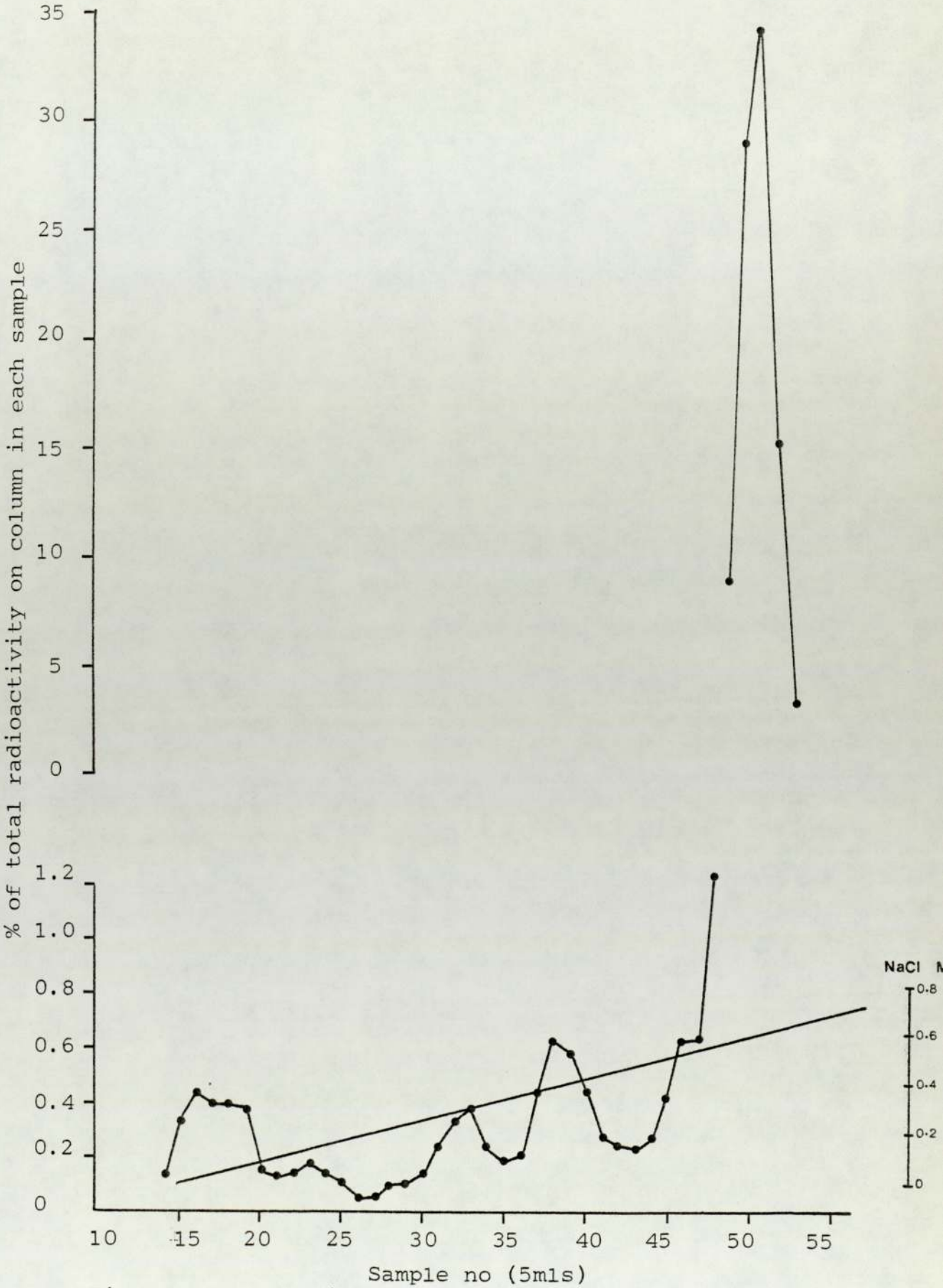


Figure 6.8 DEAE ion-exchange column chromatogram of 0-6 hr urine of rats receiving 5-methyltetrahydrofolate, labelled with carbon-14 at methyl group, 24 hours after methotrexate administration.

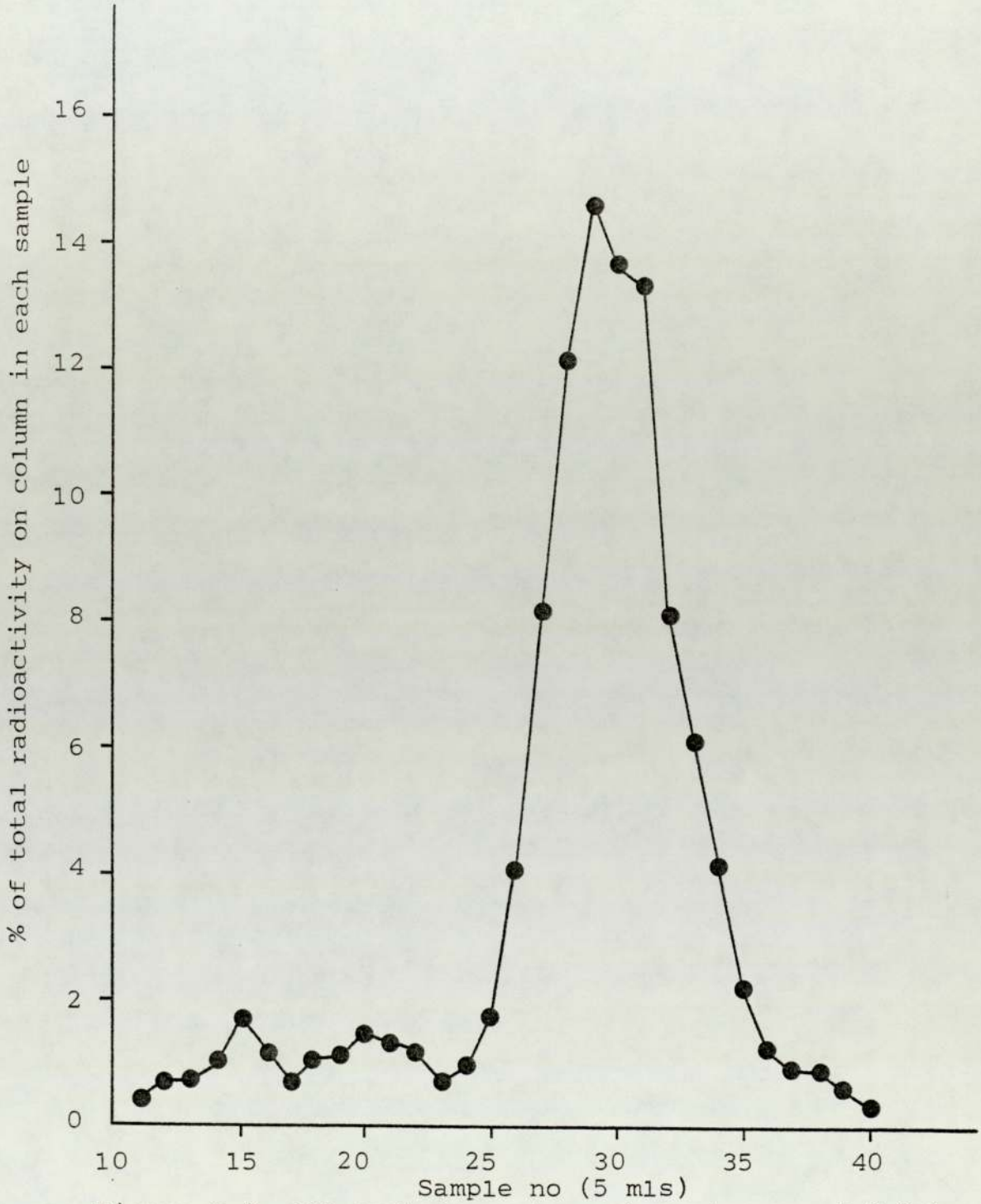


Figure 6.9 G15 sephadex column chromatogram of 0-6 hr urine of rats receiving 5-methyltetrahydrofolate, labelled with carbon-14 at methyl group, 24 hours after methotrexate administration.

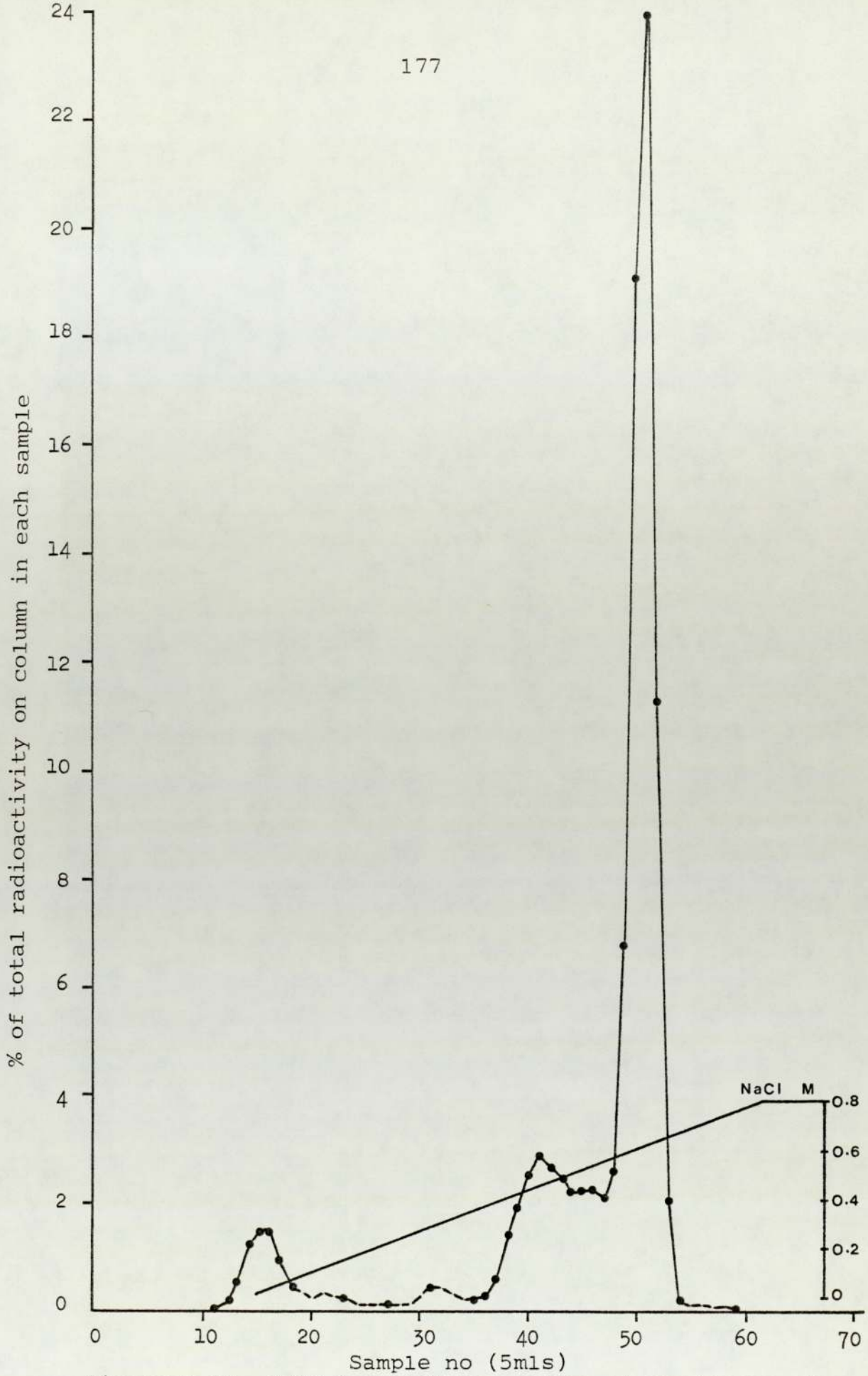


Figure 6.10 DEAE ion-exchange column chromatogram of 6-24 hr urine of rats receiving 5-methyl-tetrahydrofolate, labelled with carbon-14 at methyl group, 24 hours after methotrexate administration.

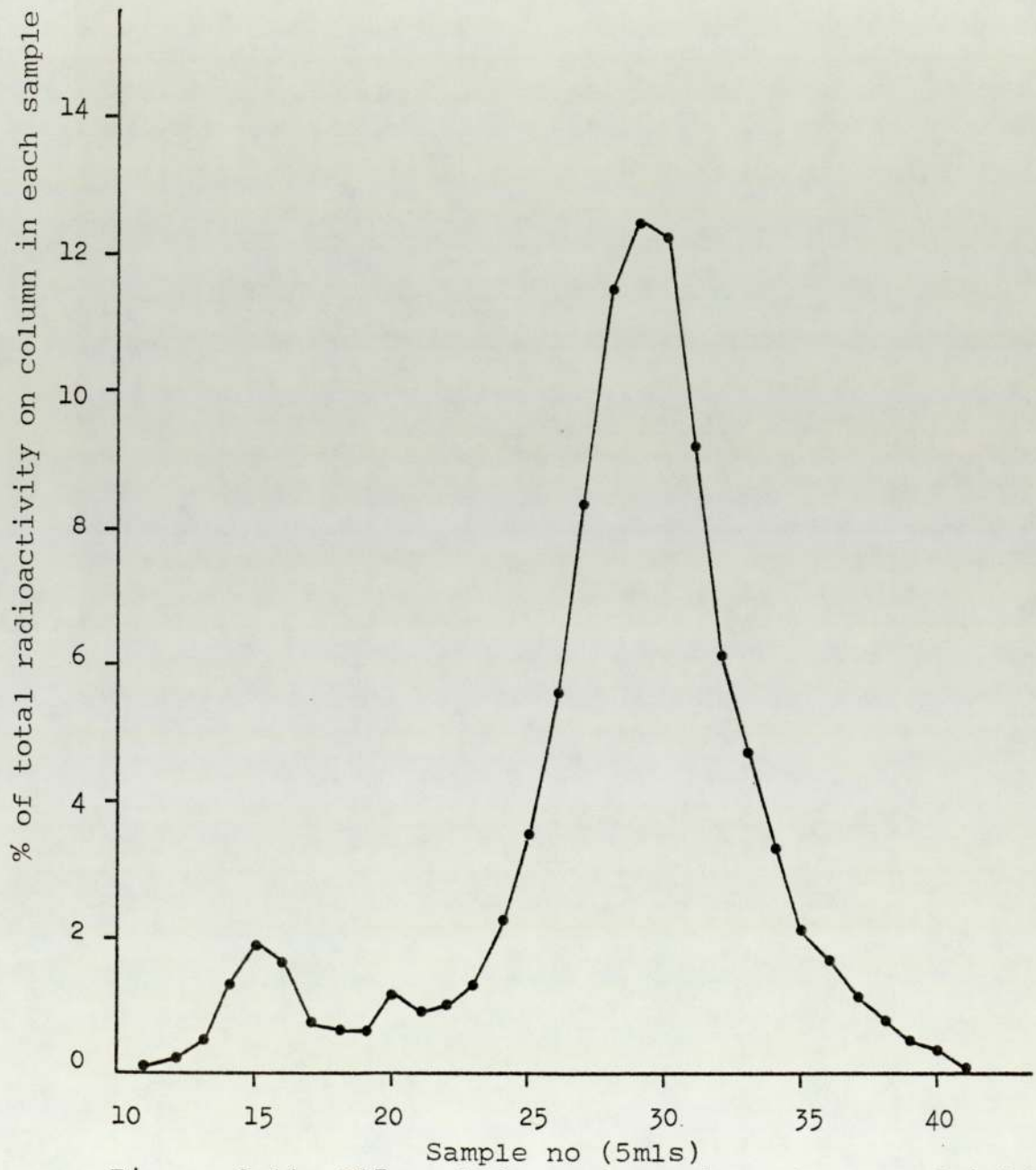


Figure 6.11 G15 sephadex column chromatogram of 6-24 hr urine of rats receiving 5-methyltetrahydrofolate, labelled with carbon-14 at methyl group, 24 hours after methotrexate administration.

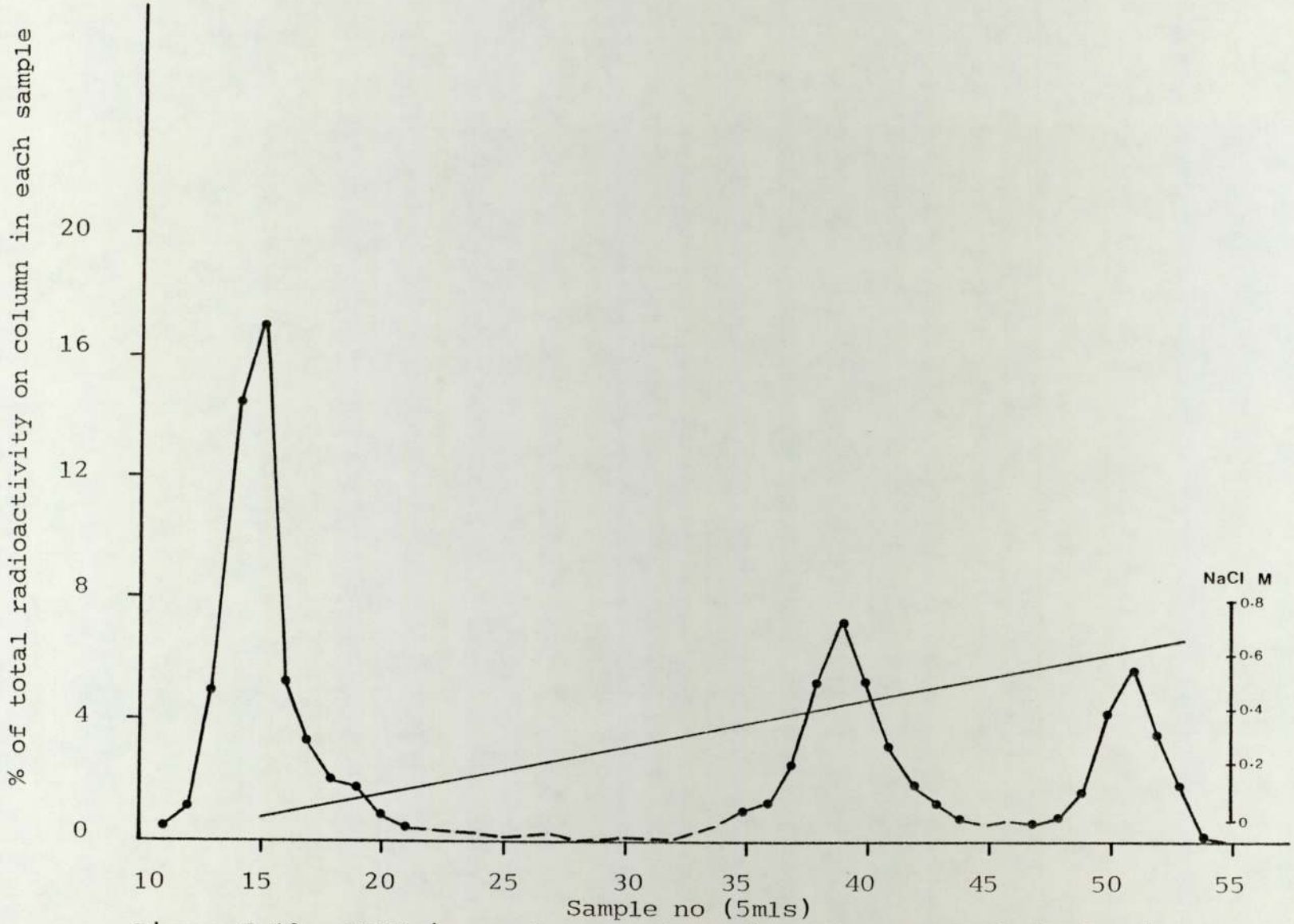


Figure 6.12 DEAE ion-exchange column chromatogram of 'boiled' liver extract of rats receiving 5-methyltetrahydrofolate, labelled with carbon-14 at methyl group.

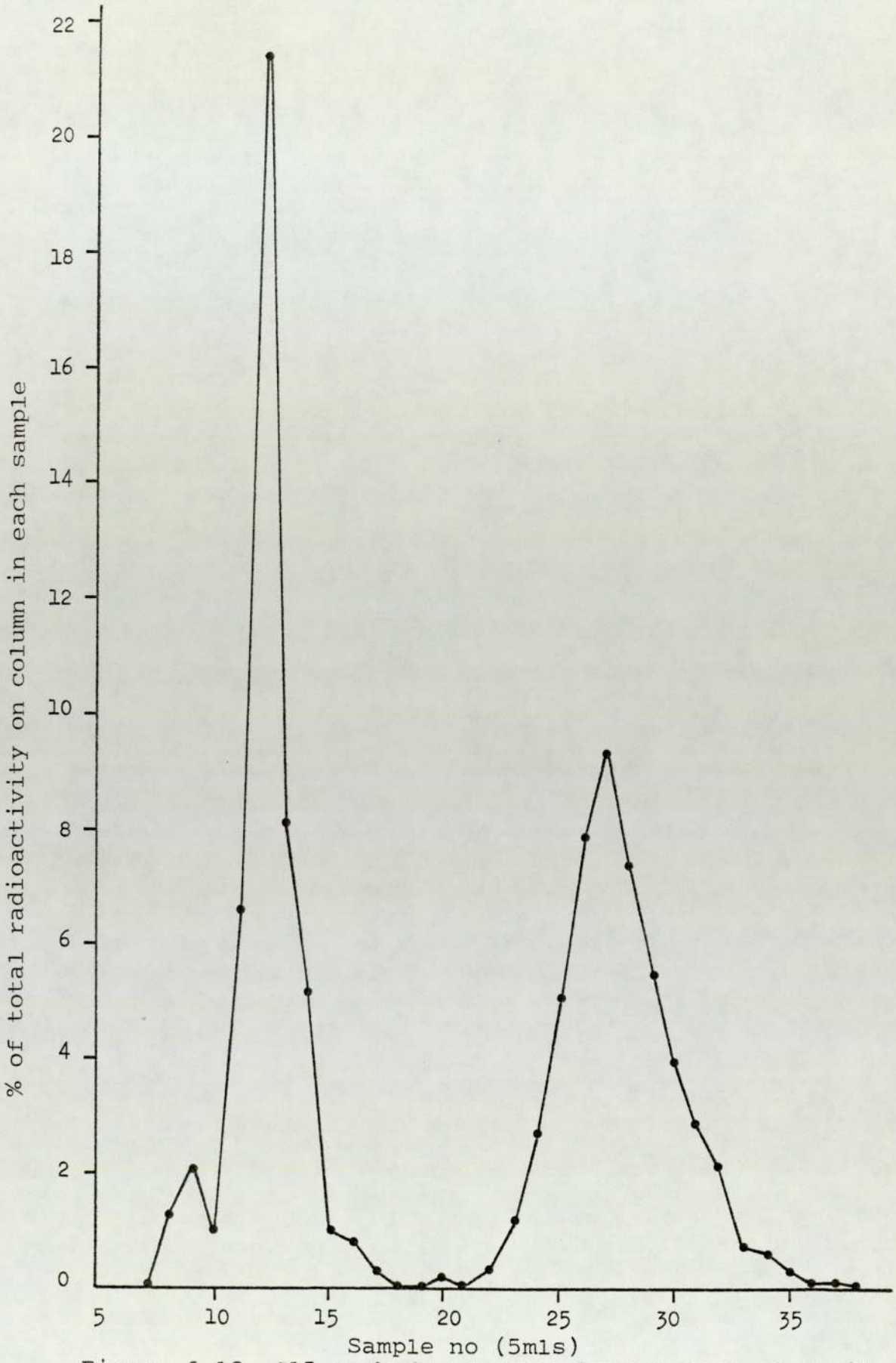


Figure 6.13 G15 sephadex column chromatogram of 'boiled' liver extract of rats receiving 5-methyl-tetrahydrofolate, labelled with carbon-14 at methyl group.

Discussion

When 5-^{14}C methyltetrahydrofolate is orally administered (70 microgram/kg body weight) to adult male Wistar rats, radioactivity during the next 24 hours in urine, faeces and liver was 31%, 24% and 9.7% respectively (table 1) accounting for 64.7% of the material given. At least 76% of the methyltetrahydrofolate is absorbed from the intestine. This is comparable to the radioactivity distribution and amount absorbed when 2-^{14}C folic acid is given orally (144).

Wistar rats given a similar dose of 5-^{14}C methyl-tetrahydrofolate 24 hours after an oral dose of methotrexate (100 mg/kg) had a slightly lower recovery of radioactive material from the faeces, urine and liver after 24 hours of dose (58.7% ; table 2). However much less was found in the urine (21.5%) and liver (4.3%) and much more in the faeces (33.0%). The change in distribution pattern between urine and faeces, especially the significant increase in faeces suggest that after a large oral dose of methotrexate the absorption of 5-methyltetrahydrofolate (at least 67%) from the intestine is reduced. This is supported by previous work (188) which showed that 24 or 48 hours after oral dose of methotrexate the surface acidity of the jejunum was more alkaline than normal and that this was paralleled by the reduction in intestinal transport of 2-^{14}C folic acid (71).

The smaller amount appearing in the liver after

methotrexate dosing may be due to the reduced intestinal absorption and/or interference with the uptake and retention processes in the liver. That methotrexate competes with folates for transport into isolated mammalian and bacterial cells has been claimed previously (68).

Ion-exchange chromatography of the radioactive urine samples after an oral dose of 5-^{14}C methyltetrahydrofolate showed presence of five compounds. The major peak, about 84% of the radioactivity in the 0-6 hours urine and about 70% in the 6-24 hours urine, was identified as 5-methyltetrahydrofolate by (a) its elution profile and co-chromatography with an authentic standard on ion-exchange column and (b) oxidation with hydrogen peroxide to 4a-hydroxy-5-methyltetrahydrofolate and co-chromatography on ion-exchange columns with an authentic standard. Since the biologically active diastereoisomer of 5-methyltetrahydrofolate rapidly loses the methyl group by exchange (189), the 5-methyltetrahydrofolate appearing in the urine must be the inactive diastereoisomer. As this is about 23% of the dose given, 46% of the dose of inactive diasterioisomer appears in the urine. The appearance of large amounts of 5-methyltetrahydrofolate in urine disagrees with the earlier work of Beavon and Blair (191) who failed to find this compund. This may have been caused by the ready oxidation of 5-methyltetrahydrofolate to 4a-hydroxy-5-methyltetrahydrofolate (190,194).

It also refutes the proposal that the biologically

inactive diastereoisomer may be converted to the biologically active diastereoisomer by sequential procees of oxidation, keto-enol tautomerism and reduction (192).

Of an intraperitoneal dose of 5-methyltetrahydrofolate (60 microgram/kg), 33% appeared in the first day urine as the inactive diastereoisomer of 5-methyltetrahydrofolate. Only trace amounts were found in liver and faeces (193). Thus 67% of a dose of inactive 5-methyltetrahydrofolate was retained within the body. Applying these figures to the oral dose of 5-methyltetrahydrofolate shows that at least 70% of the inactive diastereoisomer was absorbed through the intestine. To this must be added a further 4% to make the total absorption of the inactive diastereoisomer to 74%. As 76% of the oral dose was absorbed the rates of intestinal transport of the biologically active and inactive diastereoisomers at these levels must be the same. This disagrees with an earlier work (189), which claimed that the two diastereoisomers of 5-methyltetrahydrofolate were absorbed at different rates in man. However, this work (189) was carried out using tritium labelled compound and recent work (144) has shown this to be an unreliable indicator of folate absorption as the tritium labelled species appearing in the urine are not necessarily folates.

A minor peak about 1.7% of 0-6 hours urine radioactivity and about 5% of 6-24 hours urine radioactivity was identified as 4a-hydroxy-5-methyltetrahydrofolate by

its elution position and co-chromatography with an authentic standard. As this compound is readily formed from 5-methyltetrahydrofolate by oxidation, it could have been formed as analytical artifact during collection. However, since all collection and storage was done in ascorbate this is perhaps unlikely, and most probably this compound is formed by oxidation of the biologically inactive diastereoisomer within the body.

A peak about 5.5% of the urinary radioactivity in 0-6 hours urine sample and 7% of the urinary radioactivity in 6-24 hours urine sample chromatographed in a similar position to formylfolates. Co-chromatography of this material with authentic 10-formylfolate and 5-formyltetrahydrofolate showed a clear separation. Hence this radioactive peak was not 10-formylfolate, 10-formyltetrahydrofolate or 5-formyltetrahydrofolate. In a study of the metabolism of 5-^{14}C methyltetrahydrofolate in three patients with kidney deficiency, a similar compound in urine which was identified as formylfolate on the basis of elution position only (195). Since no radioactive formylfolate was found, this experiment provides no evidence for the reversal of 5-methyltetrahydrofolate with the direct formation of 10-formyltetrahydrofolate from the former compound.

A fourth material accounting for 4% in 0-6 hours urine and 11% in 6-24 hours urine was identified as creatine by its elution position and co-chromatography with

authentic sample on DE52 ion-exchange column and G15 sephadex gel permeation column. It represented about 2% of the total dose given and 4% of the active diastereoisomer. It was presumably derived from 5-^{14}C methyltetrahydrofolate by transmethylation to form S-adenosylmethionine which transfers its methyl group to creatine.

A fifth metabolite was not charecterized but it accounted for only about 1% of the urinary radioactivity.

The 0-6 hours and 6-24 hours urines, obtained from adult male Wistar rats fed 5-^{14}C methyltetrahydrofolate 24 hours after methotrexate administration, on DE52 ion-exchange column gave five radioactive compounds identical with those of untreated animals. However, in 6-24 hours urine there were quantitative differences. 68% of the urinary radioactivity was 5-methyltetrahydrofolate and 6% was creatine as compared to 70% and 11% in control urines. 20% of the urinary radioactivity instead of 7% now appeared as the unknown compound chromatographing in a similar fashion to formylfolates. Thus prior administration of methotrexate increased the urinary excretion of this compound and decreased that of 5-methyltetrahydrofolate and creatine.

Less radioactivity from the administered dose was found in the liver of animals pretreated with methotrexate (4%) than in control animals (9.7%). This may have been due to the reduced intestinal absorption or a combination of that and reduced liver uptake. Chromatographic

analysis of the livers of the control animals extracted by hot extraction procedures on DE52 ion-exchange columns gave three radioactive peaks. The first peak accounting for 48% of the liver radioactivity was identified as creatine by its elution position and co-chromatography with an authentic standard on ion-exchange column. The second material eluting at a similar position to one of the unidentified urine metabolites accounted for about 31% of the radioactivity. A third peak accounting for about 17% of the liver radioactivity was identified as 5-methyl-tetrahydrofolate by its elution position. From the reason advanced before it was the inactive diasterioisomer.

Gel permeation chromatography of the same extracts on G15 gave a large peak of radioactivity (44.5%) chromatographing in the void volume in a position similar to polyglutamates. As this is the elution position of creatine on these columns, this compound is most probably creatine. The remainder of the radioactivity chromatographed in the same position as 5-methyltetrahydrofolate and corresponds to the two smaller peaks in figure 6.12. This shows that the unidentified metabolite and 5-methyltetrahydrofolate in figure 6.12 have not separated on G15 sephadex column.

Chromatography on DE52 ion-exchange columns of hot liver extracts obtained by hot extraction from rats pretreated with methotrexate gave a large peak corresponding to creatine (60-70%), and lesser corresponding to

5-methyltetrahydrofolate (20%) and an unidentified material.

From the experiments reported here methotrexate has little effect on the transfer, by several steps, of the methyl group from 5-methyltetrahydrofolate to creatine. However, methotrexate appears to reduce the intestinal absorption and liver uptake of 5-methyltetrahydrofolate and this is reflected in the lesser amount of creatine appearing in the liver and urine of the treated animals. Thus because of the reduced folate levels the de novo synthesis of methyl groups may be reduced in methotrexate treated animals.

CHAPTER 7

METABOLISM OF 2-^{14}C 5-METHYLTETRAHYDROFOLATE IN RAT IN PRESENCE AND ABSENCE OF METHOTREXATE

Introduction

The study of metabolism of folates in mammal using reduced folates is direct approach towards this end, as folic acid itself as such does not exist in natural foods. On the other hand use of reduced folates suffers substantially on account of their susceptibility towards oxidation, as slightest mistake in handling might render whole conclusions irrelevant.

The only ^{14}C labelled 5-methyltetrahydrofolate available (Radio-chemical Centre, Amersham) is one with ^{14}C in the methyl group of the molecule and is 50-50 mixture of two diastereoisomers, one of which is biologically inactive. There is no practicable means of separating the two isomers chemically before use; as biologically active substance is the desired folate for experiments such as documented in this chapter. Moreover, demethylation (transfer) of 5-^{14}C-methyltetrahydrofolate in the biological system would inenvitably leave us with a folate or its derivative undetectable if excreted as such. A method was therefore designed to yeild a biologically active 5-methyltetrahydrofolate labelled with ^{14}C at 2-position of the pteridine ring of the molecule, to ensure the possible presence of metabolites containing the more stable ring structure, or any derivative of the whole folate in the urine of rats fed with the above mentioned 2-^{14}C 5-methyltetrahydrofolate. Furthermore, the comparison of the metabolism of 5- ^{14}C-methyltetrahydrofolate and 2- ^{14}C 5-methyltetrahydrofolate in rat would provide an opportunity to look into the problem

from different angles and validity of results.

Adequate details of 5-methyltetrahydrofolate and its functions are described in the introductory pages of chapter 6 in this thesis. Although experiments in the present chapter were carried out long before those in chapter 6, but have been documented later in this chapter.

The 2-^{14}C 5-methyltetrahydrofolate was isolated from the urine of the rats dosed with 2-^{14}C folic acid as described below. Further details are given in the materials and methods section of this chapter.

If Wistar rats are dosed orally with 2-^{14}C folic acid, the labelled substances appear in the urine. Among these materials unmetabolised 2-^{14}C folic acid and 2-^{14}C-5-methyltetrahydrofolate are the major components of the total radioactivity in the urine collected within 24 hours of the dose. The radioactively labelled substances are resolved if the urine is applied to DE52 ion-exchange column, separating 2-^{14}C folic acid and 2-^{14}C 5-methyltetrahydrofolate quite efficiently, though some mixing takes place in the middle fractions of formylfolate and 5-methyltetrahydrofolate peaks, which appear closer on the column. But such fractions can be rejected and the fractions containing 5-methyltetrahydrofolate can be further purified by repeated chromatography on G15 sephadex columns. Though such a method of isolating the required substance is lengthy and difficult, yet is practicable. The most difficult part of the experiment is to get substantial amount of the labelled folate in the minimum volume of the solvent and to

ensure the purity before use. It is also important to use anti-oxidants throughout the isolation procedure, excessive amounts of anti-oxidants can be removed by chromatography before use, as the total amount of anti-oxidants would not guarantee the purity of the reduced folate.

A single oral dose of methotrexate 100 mg/kg body weight was administered 24 hours before an oral dose of 2-^{14}C 5-methyltetrahydrofolate isolated as described above to some rats and some rats received only 2-^{14}C 5-methyltetrahydrofolate. The animals were killed 24 hours after folate dosing. Urine and faeces were collected separately. The livers were taken out immediately and extracted. The details of the experiment are described in the following pages.

Materials and Methods

2-^{14}C folic acid was purchased from the Radiochemical Centre, Amersham. Unlabelled 5-methyltetrahydrofolate was obtained from E. Prova, Switzerland. Adult male Wistar rats weighing 200-250 grams were obtained from Messrs Bantaw and King. The chemicals used as anti-oxidants and for making buffer solutions were of purest grade. Methotrexate was gift from Lederle Laboratories.

Isolation of 2-^{14}C 5-methyltetrahydrofolate

A Dosing and collection of urine samples

Six male adult Wistar rats weighing 200-250 grams were orally dosed with 76 microgram/kg body weight 2-^{14} folic acid in saline water. The animals were then caged separately in metabolic cages. The urine and faeces were collected separately. The urine was collected in flasks, each flask containimg 5 mls of 0.05 molar phosphate buffer, pH 7. The solution contained 5 gms/100 mls ascorbate and 5 mg/100 mls dithiothreitol. The animals were fed and watered ad libitum throughout the course of their caging.

The urine samples were collected between 0-24 hours, where 0 hour is the time of folic acid dosing. A fraction of each urine sample from every individual animal was counted for the estimation of radioactivity content. All the samples were then pooled together and stored in deep freeze.

The same rats were dosed second time orally with 2-^{14}C folic acid receiving same amounts as the first dosing.

The urine samples were collected and the time of collection was again 0-24 hours, where 0 hour is the time of second folic acid dosing. The animals were killed 24 hours after second dosing. Both batches of urine samples collected after first and second dosing of 2-^{14}C folic acid were pooled together. The radioactivity recovered from the urine of individual rat in the two batches of urine expressed as a percentage of the radioactivity dosed is given in table 1.

B Column chromatography of urine samples and recovery of 2-^{14}C 5-methyltetrahydrofolate

DE52 ion-exchange cellulose was equilibrated with 0.05 molar sodium phosphate buffer, pH 7, containing 5 mg/100 mls dithiothreitol, degased and packed in columns (2 sq.cm x 50 cm). The columns were eluted with linear gradient of 0-1 molar sodium chloride in 0.05 molar phospahte buffer, pH 7, containing 5 mg/100 mls dithiothreitol.

The pooled urine sample (15 mls) from the above mentioned batches was applied each time to freshly packed DE52 ion-exchange column and 10 such columns were run. 5 mls fractions were collected by LKB automatic fraction collector. The recovery of radioactivity was complete in most of the cases. Each fraction was counted. Figure 7.1 is a typical chromatogram. The peaks are numbered as 1, 2, 3, 4 and 5 on the chromatogram. Peak 1 was identified as 2-^{14}C folic acid and peak 2 as 2-^{14}C 5-methyltetrahydrofolate on the basis of co-chromatography of the fractions under the peaks with unlabelled authentic materials on G15 sephadex and

DE52 ion-exchange columns.

The fractions under peak number 2, which were well clear of peak number 3, were pooled together. The fractions were collected from 10 such above mentioned columns, 15 mls of urine sample applied each time. Ascorbic acid was added each time to the pooled fractions of the peak.

The pooled fractions of peak number 2 from all the above mentioned ion-exchange columns were lyophilized in presence of anti-oxidants to 20 mls of solution, which again was re-chromatographed on DE52 ion-exchange column for further purification. The radioactively labelled material appearing at the authentic site of 5-methyltetrahydrofolate on the gradient was preserved and the rest of the fractions rejected.

The final purification was achieved by applying the lyophilized material on G15 sephadex column, as the excessive amounts of anti-oxidants were removed by this column.

Dosing of methotrexate and isolated 2-^{14}C 5-methyltetrahydrofolate to the animals of the experiment

Ten male adult Wistar rats weighing 200-250 grams wwre obtained for the actual experiments. Six of the rats were orally dosed with 100 mg/kg body weight methotrexate each in water suspension. Rest of the four were orally dosed with the same amount of water only. Twenty four hours later all ten rats were dosed with 1.5 mls fraction of isolated 2-^{14}C 5-methyltetrahydrofolate solution per rat. The amount

of dose was estimated to be approximately 7 microgram/kg body weight by comparing the recovered activity present in the isolated material to the dosed 2-^{14}C folic acid to all previous rats.

Two rats were caged in one metabowl, so that each pair belonged to either methotrexate treated animals or controlled ones. The animals were fed and watered ad libitum and were killed after 24 hours 2-^{14}C 5-methyltetrahydrofolate administration.

Collection of biological samples

(1) Urine samples

The urine samples for each pair of rats were collected in flasks between o-24 hours, where 0 hour is the time of 2-^{14}C 5-methyltetrahydrofolate dosing. The flasks contained 0.05 molar phosphate buffer, pH 7, containing 5 mg/100 mls dithiothreitol and 2 gm/100 mls ascorbate. A fraction of urine collected in each flask was counted in the counter for the total recovery of radioactivity. The samples were chromatographed on DE52 ion-exchange and G15 sephadex columns.

(2) Faeces

The faeces was collected in empty flasks. It was freeze dried and powdered and 0.1 gram sample from each flask was oxidised in biological material oxidiser. $^{14}CO_2$ was absorbed in 15 mls of Fisons absorber. The same was done with the"blanks" and standards. The samples were counted for radioactivity in liquid scintillation counter.

(3) Gut and liver

Some of the gut and liver of the animals were freeze dried and subjected to similar oxidation and counting, as faeces, for the determination of radioactivity retained by the above mentioned organs.

Preparation of liver extract

Some of the livers, from both methotrexate treated and control animals were extracted separately. The freshly taken out livers were chopped in small peices and dropped in boiling sodium phospahte buffer solution, pH 7, containing 5 mg/100 mls dithiothreitol and 2 gm/100 mls ascorbate. Boiled for 5 minutes, centrifuged after cooling to room temperature. The extracts were stored in deep freeze for column chromatography.

RESULTS

Recovery of radioactivity

Table 2 gives the amounts of radioactivity recovered in urine, faeces, gut and liver of control and methotrexate treated animals, expressed as a percentage of total radioactivity of 2-^{14}C 5-methyltetrahydrofolate dosed. Since two rats were housed in one metabowl, the figures in table 2 stand for two rats.

Analysis of urine and liver extract by column chromatography

The urine samples from the controlled and methotrexate treated animals were chromatographed separately on DE52 ion-exchange and G15 sephadex columns. Figure 7.2 is a DE52 ion-exchange column chromatogram of rats dosed with 2-^{14}C 5-methyltetrahydrofolate only isolated from the urine of rats dosed with 2-^{14}C folic acid. The figure shows three radioactive peaks marked as A, B and C in the chromatogram. The major peak A appears around 0.5 molar sodium chloride concentration on the gradient. Number of attempts were made to identify this peak. Many DE52 ion-exchange columns were run to isolate the above mentioned peak and the fractions under the peak were pooled together and partially lyophilized fractions were re-chromatographed with authentic 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 10-formyltetrahydrofolate and xanthopterin. The radioactive peak in all cases separated from the UV absorption peaks indicating that the compound represented by peak A could not be identified with any of the compounds it was re-chromatographed with.

The second peak B on chromatogram (figure 7.2) appears at the calibrated site of 4a-hydroxy-5-methyltetrahydrofolate. Peak C contained very small amount of radioactivity in its fractions and therefore could not be isolated in appropriate amounts to be re-chromatographed with unlabelled materials for identification.

Figure 7.3 shows a G15 sephadex column chromatogram of the same urine of controlled animals.

Figure 7.4 is a DE52 ion-exchange column chromatogram of the urine of rats receiving 100 mg/kg body weight methotrexate orally, 24 hours before 2-^{14}C 5-methyltetrahydrofolate administration. The picture of radioactive peaks essentially remains the same as in the corresponding chromatogram of controlled animals urine. Again the major peak in figure 7.4 did not co-chromatograph with authentic 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 10-formyltetrahydrofolate and 10-formylfolate on DE52 ion-exchange column.

Figure 7.5 is a G15 sephadex column chromatogram of the urine of methotrexate treated animals.

Column chromatography of liver extracts

The liver extracts of methotrexate treated animals failed to show any prominent peaks on gel permeation or ion-exchange columns due to very small amounts of radioactivity associated with the liver. However, liver extract of the controlled rats show three prominent peaks on DE52 ion-exchange column chromatogram (figure 7.6). The same extract on G15 sephadex column showed two peaks (figure 7.7).

	Urines collected after first dose of 2-^{14}C folic acid		Urines collected after second dose of 2-^{14}C folic acid	
Rat No	Volume (mls)	% radio-activity	Volume (mls)	% radio-activity
1	19.3	8.2	11.6	11.0
2	39.1	10.5	20.4	10.2
3	13.7	11.5	7.6	8.8
4	11.1	16.9	12.4	24.5
5	15.4	8.2	10.0	11.1
6	39.0	13.0	19.0	21.1
Average	22.9	11.4	13.5	14.5

Table 1 Recovary of radioactivity in urine of rats receiving 2-^{14}C folic acid, expressed as a percentage of total 2-^{14}C folic acid radioactivity dosed.

	Rat No	Urine 0-24 hrs	Liver	Gut	Faeces	Total
Control rats	1 + 2	16.1	13.5	6.0	31.3	66.9
	3 + 4	18.6	-	5.3	37.5	61.4
	Average	17.4	13.5	5.6	34.4	64.2
*MTX treated rats	1 + 2	20.6	6.5	4.4	40.4	71.9
	3 + 4	19.6	4.4	3.5	36.0	63.5
	5 + 6	19.1	-	2.7	38.8	60.0
	Average	19.8	5.5	3.5	38.4	65.3

Table 2 Distribution of radioactivity in urine, liver, faeces and gut of control and methotrexate treated rats. Control rats received 2-^{14}C-5-methyltetrahydrofolate only. The distribution has been expressed as a percentage of 2-^{14}C-5-methyltetrahydrofolate dosed.

*MTX denotes Methotrexate.

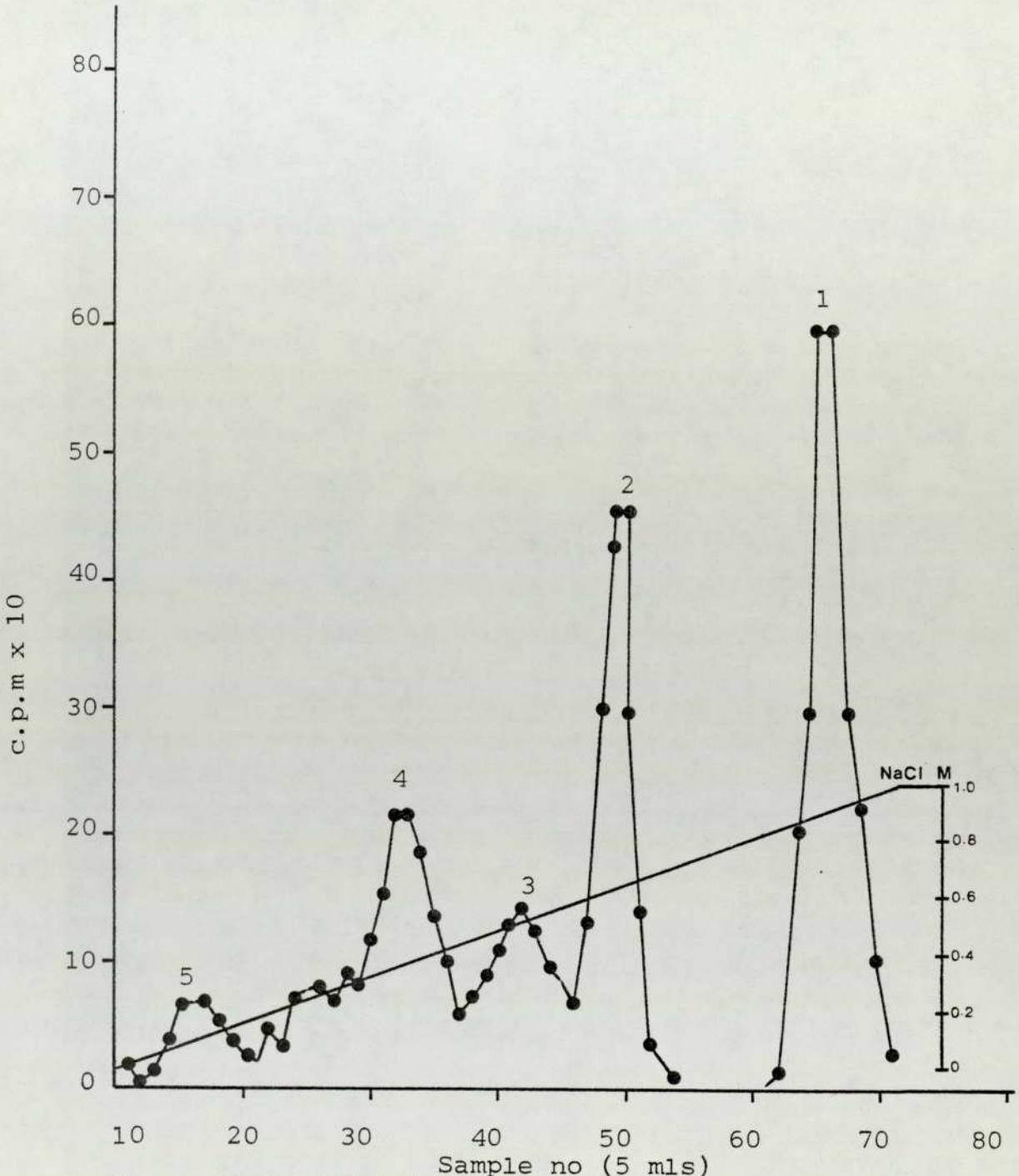


Figure 7.1 DEAE ion-exchange column chromatogram of 0-24 hr urine of rats receiving folic acid labelled with carbon-14 at 2-position.

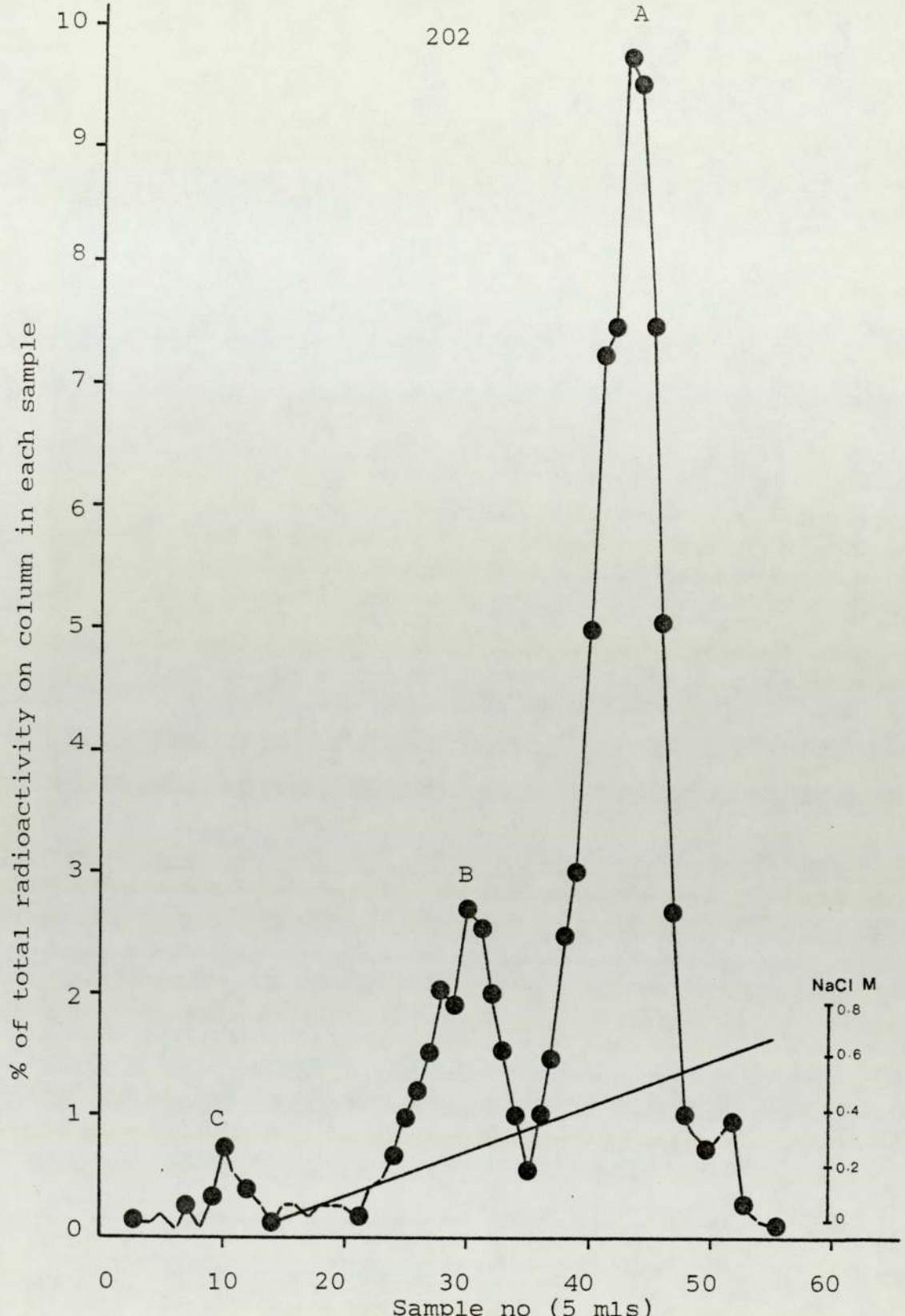


Figure 7.2 DEAE ion-exchange column chromatogram of 0-24 hr urine of rats receiving 5-methyltetrahydrofolate labelled with carbon-14 at 2-position.

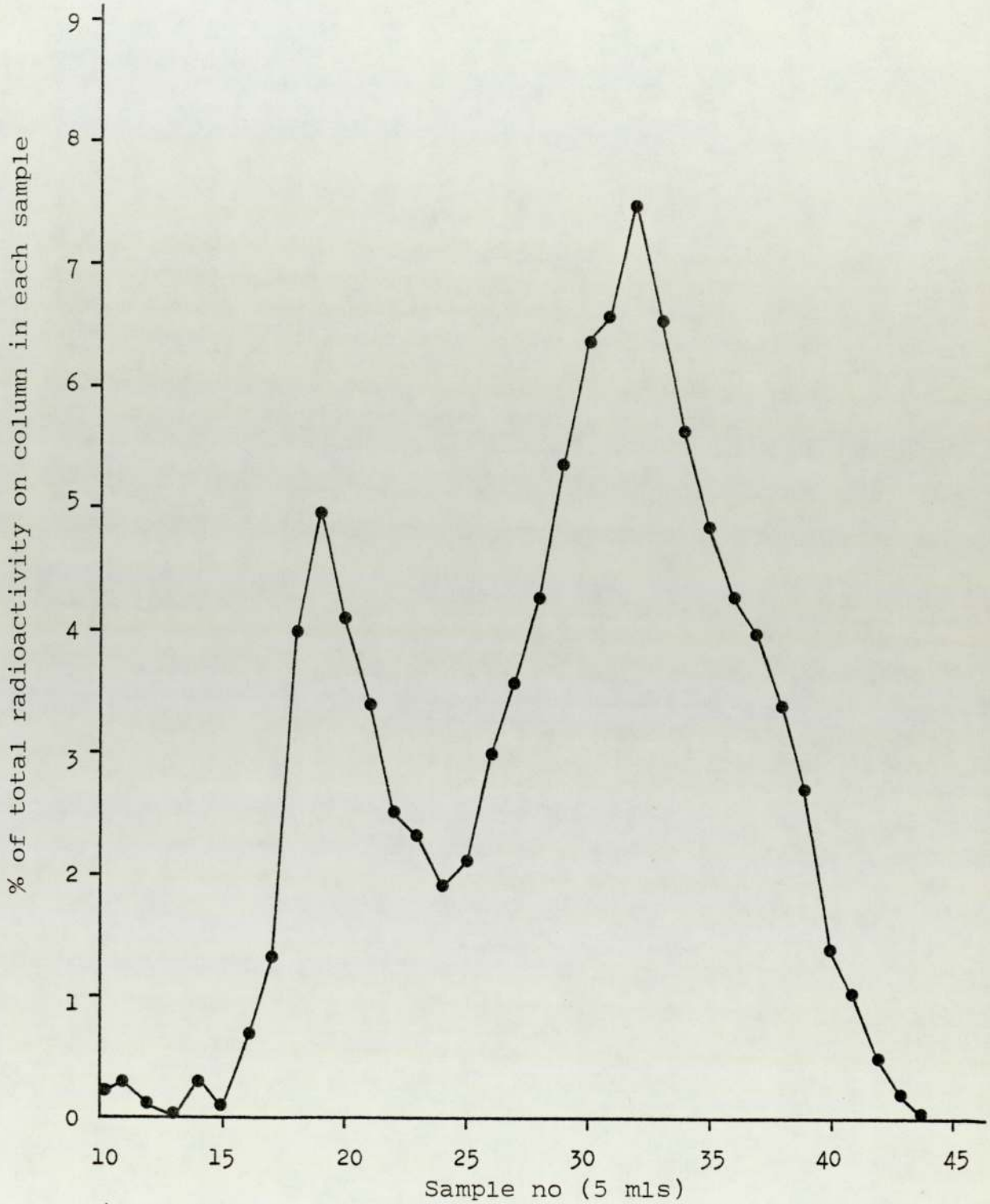


Figure 7.3 G15 sephadex column chromatogram of 0-24 hr urine of rats receiving 5-methyltetrahydrofolate labelled with carbon-14 at 2-position.

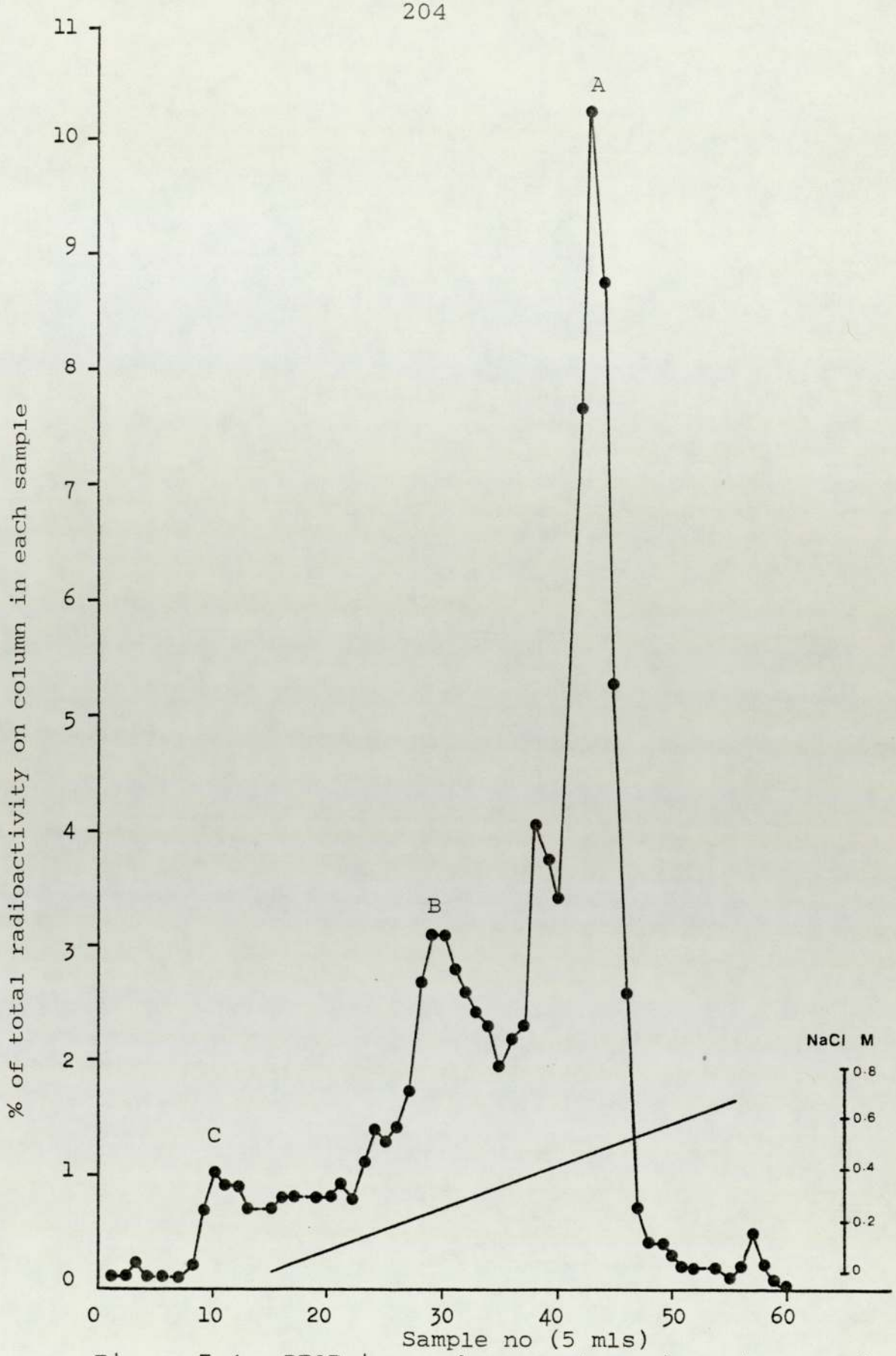


Figure 7.4 DEAE ion-exchange column chromatogram of 0-24 hr urine of rats receiving 5-methyl-tetrahydrofolate labelled with carbon-14 at 2-position. The rats were dosed with methotrexate 24 hours before 5-methyl-tetrahydrofolate administration.

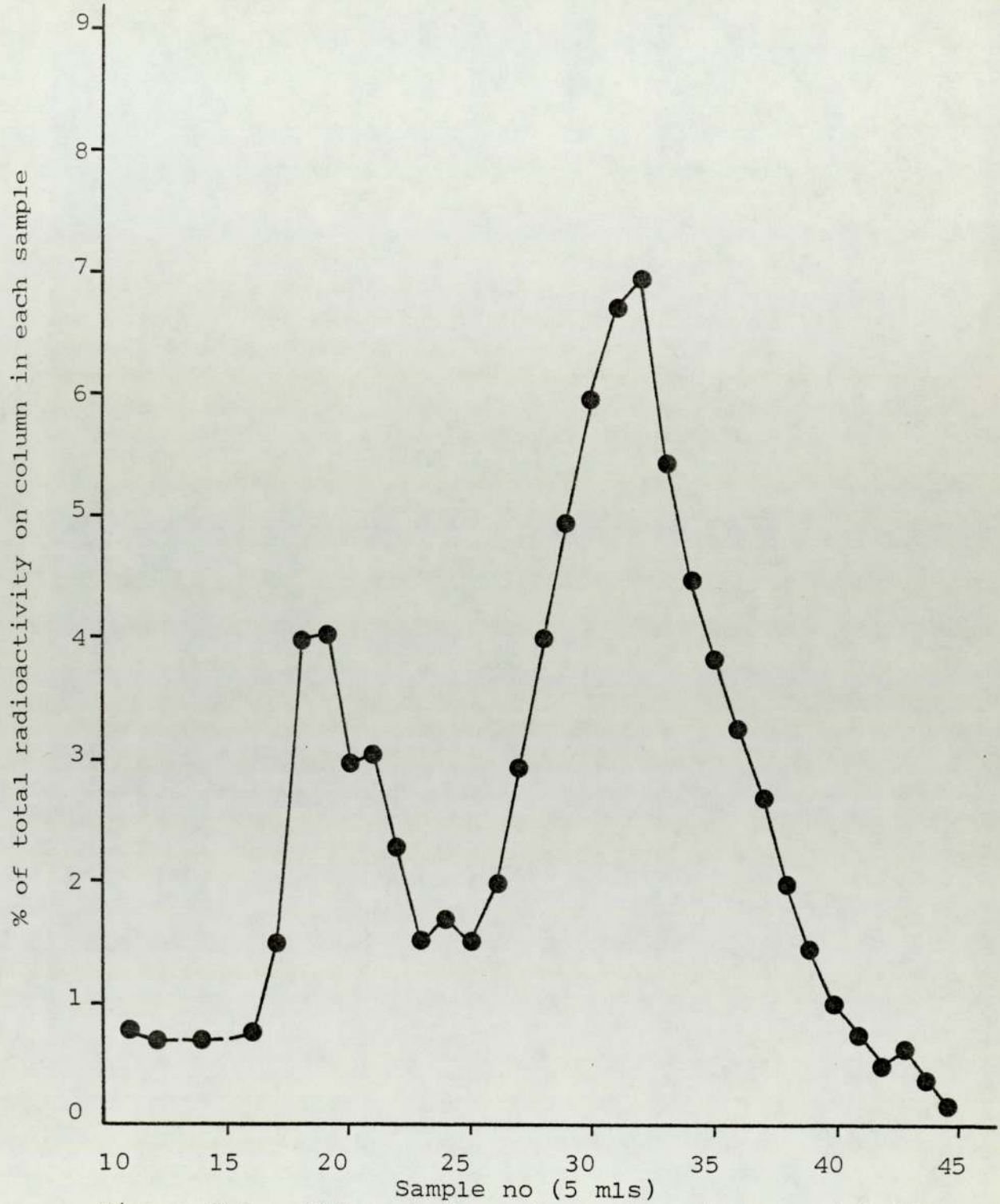


Figure 7.5 G15 sephadex column chromatogram of 0-24 hr urine of rats receiving 5-methyltetrahydrofolate labelled with carbon-14 at 2-position. The rats were dosed with methotrexate 24 hours before 5-methyltetrahydrofolate administration.

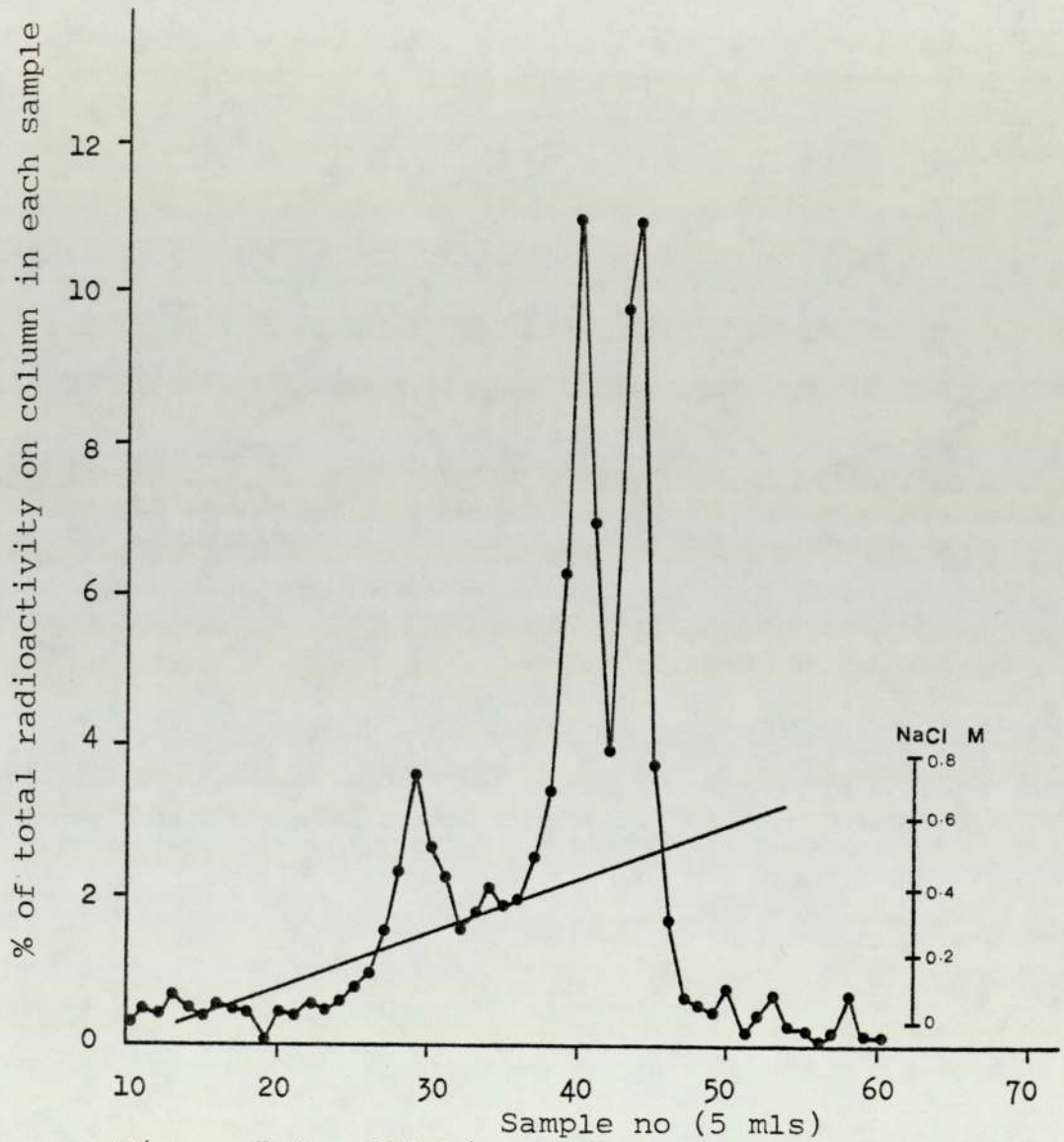


Figure 7.6 DEAE ion-exchange column chromatogram of 'boiled' liver extract of rats receiving 5-methyltetrahydrofolate labelled with carbon-14 at 2-position.

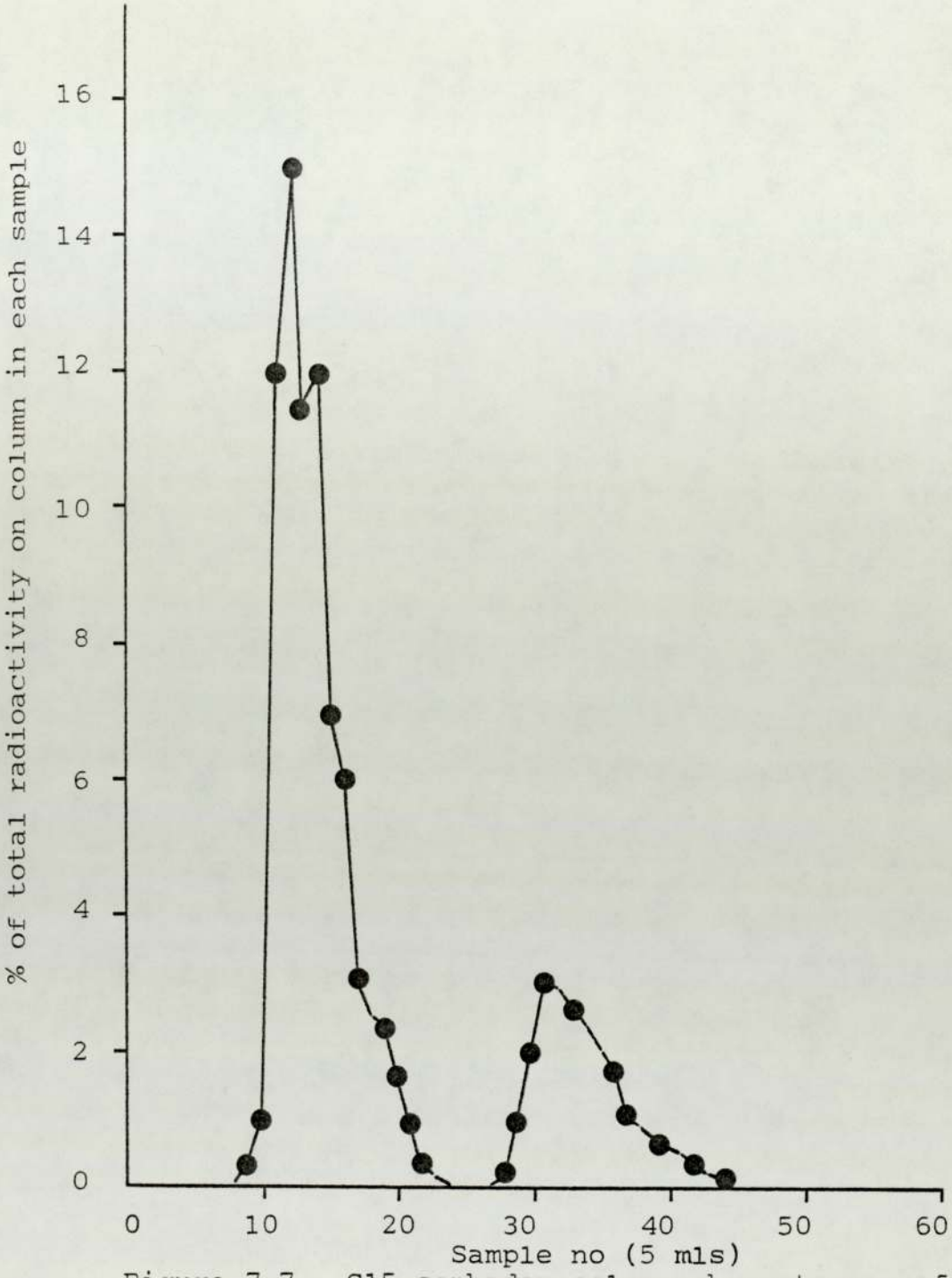


Figure 7.7 G15 sephadex column chromatogram of 'boiled' liver extract of rats receiving 5-methyltetrahydrofolate labelled with carbon-14 at 2-position.

Discussion

A Distribution of radioactivity

Distribution of radioactivity in urine, liver, gut and faeces of controlled and methotrexate treated rats is given in table 2. Since two rats were dosed in one metabowl, the distribution shown is average of two rats. Table 2 shows that methotrexate (100 mg/kg) treated rats have excreted 19.8% of the dosed 2-^{14}C-5-methyltetrahydrofolate radioactivity in 0-24 hours urine, while controlled animals excreted 17.4% in the same time period. This observation is not in agreement with previous results which indicated that methotrexate treated animals excreted lesser amount of radioactivity in urine when dosed with 5-^{14}C methyltetrahydrofolate, 24 hours after methotrexate (100 mg/kg) administration, as compared to rats receiving 5-^{14}C methyltetrahydrofolate only. However, previous experiments were carried out using an oral dose of 70 microgram/kg 5-^{14}C methyltetrahydrofolate (a 50-50 mixture of two diasterioisomers epimeric at C-6 position), while in present experiments animals received an oral dose of about 7 microgram/kg 5-methyltetrahydrofolate labelled at C-2 position in the pteridine ring. Moreover, this was urinary metabolite isolated after 2-^{14}C folic acid administration.

The total radioactivity recovered in controlled and methotrexate treated animals is comparable (64.2% in controlled animals and 65.4% in methotrexate treated animals).

The radioactivity recovered from the liver of the two groups of rats, however, differs substantially.

Table 2 shows that liver of methotrexate treated animals contained 5.4% of dosed radioactivity, while liver of controlled animals contained 13.5% of dosed radioactivity. Thus methotrexate reduced the uptake of 2-^{14}C-5-methyltetrahydrofolate in the liver considerably.

B Urinary metabolites

The DE52 ion-exchange column chromatograms of 0-24 hours urine samples of methotrexate treated and controlled rats (figures 7.2 and 7.4) show no remarkable difference. Both groups of rats did not excrete unchanged 5-methyltetrahydrofolate in the urine. Majority of the urinary radioactivity was associated with peak A, which could not be identified, but it was not 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and 5-formyltetrahydrofolate.

The absence of 5-methyltetrahydrofolate in the urine may be due to the small amount of 2-^{14}C-5-methyltetrahydrofolate (7 microgram/kg) dosed, or the high biological activity of 2-^{14}C-5-methyltetrahydrofolate, which was isolated from the urine of rats dosed with 2-^{14}C folic acid.

The reason for not finding 10-formyltetrahydrofolate in the urine could be that 2-^{14}C-5-methyltetrahydrofolate is not converted to 5-10-methylenetetrahydrofolate and thus to 10-formyltetrahydrofolate. This suggests that the formation of 5-methyltetrahydrofolate is irreversible in the biological system.

These experiments also suggest that methotrexate has no remarkable effect on the urinary metabolites. This can be explained by considering the property of methotrexate as a

inhibitor of dihydrofolate reductase, the enzyme responsible for converting dihydrofolate and folate to tetrahydrofolate. Thus it is suggested that 5-methyltetrahydrofolate bypasses the inhibitory action of methotrexate and is metabolised.

The effect of methotrexate on the metabolites of 5-methyltetrahydrofolate in the liver, however, could not be established in this experiment as the chromatography of liver extracts of methotrexate treated animals failed to give any prominent radioactive peaks.

CHAPTER 8

METABOLISM OF 3'-5'-9 ^{3}H-2-^{14}C FOLIC ACID IN RAT AFTER ORAL 6-MERCAPTOPURINE

Introduction

It has been claimed that methotrexate blocks the conversion of folate to tetrahydrofolate by binding almost irreversibly to the enzyme dihydrofolate reductase (83) and that the binding of methotrexate to the active centres of enzyme is so tight that a single dose of the drug remains in the tissues for many weeks after administration (161). These observations suggest that perhaps folic acid will not be converted to folate co-enzymes in the presence of methotrexate in the animal system.

It was noticed in previous chapters that oral methotrexate given to the rat prior to folic acid administration increased the excretion of unmetabolised folic acid in the urine. This is explainable if we consider the activity of the drug as an inhibitor of dihydrofolate reductase, the enzyme responsible for the synthesis of reduced folates in the mammal. At the same time it was also noticed that despite the high dose of methotrexate,24 hours before folic acid administration,to Wistar rats the urine contained all reduced folates identified in the urine of rats dosed with folic acid only. The only difference was that the amounts of reduced folates was very small in the urine of methotrexate treated animals, which suggests that a single dose of methotrexate (10 mg/kg or 100 mg/kg) has not completely blocked the formation of these folate co-factors.

Moreover, increased levels of 10-formylfolate in the urine of methotrexate treated animals indicated some

additional aspect of the drug apart from the dihydrofolate reductase inhibition. Since folate co-enzyme dependent reactions in the synthesis of purines are essential in the biosynthesis of nucleic acids in the cell, the increased formylfolate in the urine indicates that methotrexate may have blocked the de novo biosynthesis of purine in an as yet unknown inhibitory fashion. To explore and confirm this secondary site of methotrexate action, it is p 105 necessary to study folate metabolism in the rat in presence of another purine biosynthesis inhibitor. Unfortunately it is difficult to find such a drug with specific action at one site inhibition of purine biosynthesis, required for such a study, as most of the drugs have more than one loci of action.

This chapter describes some aspects of folate metabolism in normal adult male Wistar rats in the presence of 6-mercaptopurine, a classical anti-metabolite of purines. Some aspects of metabolism and sites of action of 6-mercaptopurine have already been described (chapter 1).

6-Mercaptopurine shows considerable selectivity among neoplastic diseases and is useful chiefly in the treatment of acute leukaemia in childhood and chronic myelocytic leukaemia (207). It may give complete control of the disease for sometime causing no toxicity to the host (207).

6-Mercaptopurine is metabolised in vivo to 6-mercaptopurine ribotide, 6-methylmercaptopurine ribotide and the oxidation product 6-thiouric acid (200,201,202).

Earlier studies claimed that 6-mercaptopurine inhibited the purine biosynthesis by inhibiting formate incorporation (127,128,129,130,131). It was reported that inhibition caused by 6-mercaptopurine is reversed by 4-amino-5-imidazole carboxamide or hypoxanthine in cultured cells (138,139). It was stated that 6-mercaptopurine inhibits the de novo synthesis of purine in DNA and RNA equally (129). It has also been presumed that ^{35}S-6-mercaptopurine incorporated into the RNA and DNA of tumors (208), though the radioactivity found in the RNA fraction was equivalent to only one unit in 8000 of the purine units and the DNA contained slightly higher amounts.

It has been observed that 6-mercaptopurine delays G1 and S phases of cell division and probably kills G1 cells (203). Methotrexate on the other hand, delays S and G2 phases of cell division (204). The S phase denotes the period of cell division when cells are actively undergoing DNA synthesis, while G1 is presynthetic period. It is generally assumed that end of G1 phase contains preparations for DNA synthesis (205). Thus it is clear that 6-mercaptopurine interferes with DNA synthesis. However, no definite proof of its site of action has been reported in the literature.

Materials and Methods

6-mercaptopurine was purchased from the Sigma Chemicals. 2-^{14}C folic acid and 3'-5'-9' ^{3}H folic acid was obtained from the Radio-chemical Centre, Amersham. Wistar rats were purchased from Messrs Bantan and King.

5 Male adult normal Wistar rats weighing 200-250 grams were dosed orally with 6-mercaptopurine in water, each rat received 25 mg/kg body weight of 6-mercaptopurine in 5 mls of water after mild ether anaesthesia. 24 hours later rats were orally dosed with a mixture of potassium salts of 2-^{14}C folic acid and 3'-5'-9 ^{3}H folic acid such that each rat was administered with 8.8 microgram/kg body weight of 3'-5'-9 ^{3}H folic acid and 76 microgram/kg body weight of 2-^{14}C folic acid in 0.4 mls of water.

Animals were housed separately in Jencons metabowls, fed and watered ad libitum.

Collection of biological samples

The urine samples were collected between 0-6 hours and 6-24 hours in flasks containing 5 mls of 0.05 molar phosphate buffer solution, pH 7, containing 5 mg/100 mls dithiothreitol and 2 grams/100 mls ascorbate. The urine samples were collected in subdued light by wrapping the flasks with tin foil.

The faeces were collected in empty flasks between time of folic acid dosing (0 hours) and 24 hours when the rats were killed.

The liver and gut of the rats were taken out

immediately after killing. Three livers were extracted (hot extraction- see chapter 2) for the chromatographic analysis and the remaining two were freeze dried for the measurement of radioactivity.

Measurement of total radioactivity in the biological samples

The urine samples were directly counted with a known amount of urine sample in vials containing 10 mls of scintillation cocktail.

The faeces, liver and gut were freeze dried, weighed and powdered. A known amount of each sample (0.1 gram) was oxidised in Beckman biological material oxidiser. The oxidation process was repeated twice. $^{3}H_2O$ and $^{14}CO_2$ were collected separately (see chapter 2). $^{14}CO_2$ was absorbed in 15 mls of Fisons Absorber P in the 'trap' and tritium was condensed as $^{3}H_2O$ in empty trap immersed in cooling mixture of methanol and dry ice.

Column chromatography of urine samples and liver extracts

The pooled urine samples of 0-6 hours and 6-24 hours were chromatographed separately on DE52 ion-exchange and G15 sephadex columns. The liver extracts were also chromatographed on the two types of columns mentioned above.

RESULTS

Table 1 gives the distribution of radioactivity in urine, liver and gut of animals receiving an oral dose of 25 mg/kg 6-mercaptopurine 24 hours before 2-^{14}C folic acid (76 microgram/kg) and 3'-5'-9 ^{3}H folic acid (8.8 microgram/kg) administration. The distribution is expressed as a percentage of total folic acid dosed. The animals were killed 24 hours after folic acid administration and the gut and liver were taken out immediately after killing.

Column chromatography of urine samples

Figure 8.1 shows a DE52 ion-exchange column chromatogram of 0-6 hours urine sample. It shows five prominent peaks. A peak which appears at about 0.9 molar sodium chloride concentration on the gradient was identified as folic acid by its elution position and co-chromatography with authentic folic acid. The peak is composed of both ^{14}C and ^{3}H radioactivity, hence of both kinds of labelled folic acid dosed.

The second peak appearing around 0.6 molar sodium chloride concentration on the gradient was similarly identified as 5-methyltetrahydrofolate.

Third peak which appears between 0.3 and 0.5 molar sodium chloride concentration on the gradient perhaps shows presence of more than one metabolite. Most probably it contains 4a-hydroxy-5-methyltetrahydrofolate, p-aminobenzoyl glutamate and 10-formyltetrahydrofolate which have not been resolved properly.

The fourth peak between 0.1 and 0.2 molar sodium chloride concentration on the gradient consists mainly of tritium radioactivity and has not been identified, but is not p-aminobenzoyl glutamate. Table 2 gives the distribution of radioactivity in the metabolites as resolved by DE52 ion-exchange column, expressed as a percentage of the total chromatographed radioactivity.

Figure 8.2 is DE52 ion-exchange column chromatogram of 6-24 hours urine. Essentially it is similar to figure 8.1, except the tritium labelled metabolite appearing between 0.1 and 0.2 molar sodium chloride concentration on the gradient in figure 8.1 is absent. Furthermore the chromatogram (figure 8.2) shows better resolution of the metabolites. The distribution of radioactivity in the metabolites is given in table 2.

Figures 8.3 and 8.4 are the G15 sephadex column chromatograms of 0-6 hours and 6-24 hours urine samples respectively.

Figure 8.5 is DE52 ion-exchange column chromatogram of liver extract (extracted by "boiling" procedure-see chapter 2) and figure 8.6 is G15 sephadex column chromatogram of the same extract.

Rat No	Urine				Liver		Gut		Total	
	0-6 hrs		6-24 hrs							
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1	10.9	18.9	9.4	11.4	8.3	11.6	4.9	4.9	33.5	46.8
2	14.2	15.4	13.2	14.2	7.9	7.3	8.4	7.3	43.7	44.2
3	16.0	16.1	12.7	18.7	15.1	18.5	5.6	5.3	49.4	58.6
4	12.0	14.5	23.0	30.9	13.2	16.0	9.9	8.7	58.1	70.1
5	30.1	36.0	19.2	23.5	3.0	5.5	2.1	3.2	54.4	68.2
Average	16.6	20.2	15.5	19.7	9.5	11.8	6.2	5.9	47.8	57.6

Table 1 Distribution of radioactivity in urine, liver and gut (expressed as a percentage of a mixture of 2-^{14}C folic acid and 3'-5'-9 ^{3}H folic acid dosed) of rats receiving an oral dose of 6-mercaptopurine (25 mg/kg) 24 hours before folic acid administration.

Chromatogram	Metabolites									
	A		B		C		D		E	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Figure 8.1	62.3	49.1	12.3	16.0	16.6*	19.0*	-	-	-	-
Figure 8.2	38.9	32.3	24.2	21.8	18.7	15.3	6.9	5.8	-	20.1

Table 2 Distribution of radioactivity in various urinary metabolites as resolved by DE52 ion-exchange column, expressed as a percentage of total radioactivity chromatographed on each column.

Metabolites A to E appear at the calibrated sites of folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate, 4a-hydroxy-5-methyltetrahydrofolate and p-aminobenzoyl-L-glutamate on DE52 ion-exchange column respectively.

* These figures may not be due to 10-formyltetrahydrofolate only, since other metabolites 4a-hydroxy-5-methyltetrahydrofolate and p-aminobenzoyl-L-glutamate do not appear separately in DE52 ion-exchange column chromatogram of 0-6 hours urine sample (figure 8.1).

Rat group	Urine 0-6 hrs		Urine 6-24 hrs		Liver		Faeces		Gut		Total	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1 (4)	7.9	8.4	12.4	14.2	11.0	13.9	33.8	38.8	6.1	3.0	71.2	78.3
2 (6)	21.2	21.8	11.7	13.2	2.7	4.4	48.4	51.9	6.6	6.8	90.7	98.4
3 (5)	16.6	20.2	15.5	19.7	9.5	11.8	-	-	6.2	5.9	47.8	57.6

Table 3 Average distribution of radioactivity in biological samples of various groups of rats following an oral dose of folic acid. The distribution is expressed as a percentage of the dosed folic acid radioactivity.

Group 1: Normal rats receiving 76 microgram/kg carbon-14 labelled folic acid and 8 microgram/kg tritium labelled folic acid (taken from table 1, chapter 5 of this thesis).

Group 2: Normal rats receiving same dose of dual labelled folic acid as rats of group 1, 24 hours after an oral dose of methotrexate (100 mg/kg), (taken from table 3, chapter 5 of this thesis).

Group 3: Normal rats receiving 76 microgram/kg carbon-14 labelled folic acid and 8.8 microgram/kg tritium labelled folic acid, 24 hours after an oral dose of 6-mercaptopurine (25 mg/kg), (taken from table 1 of this chapter)

The figures in parentheses give number of animals in each group.

	Rat group	Metabolites A		B		C		D		E	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
0-6 hr urine	1 (4)	71.1	51.7	16.9	12.3	7.7	13.0	4.9	10.6	-	-
	2 (6)	90.1	77.4	-	-	5.3	6.9	-	-	-	11.1
	3 (5)	62.3	49.1	12.3	16.0	16.6*	19.0*	-	-	-	-
6-24 hr urine	1 (4)	45.6	30.5	19.2	16.9	13.2	13.2	18.0	11.0	-	10.0
	2 (6)	84.2	58.5	5.0	6.1	6.5	7.5	-	-	-	15.0
	3 (5)	38.9	32.3	24.2	21.8	18.7	15.3	6.9	5.8	-	20.1

Table 4 Average distribution of radioactivity in urinary metabolites of various groups of rats following an oral dose of folic acid. The distribution is expressed as a percentage of chromatographed radioactivity on each column.

Group 1: Normal rats receiving 76 microgram/kg carbon-14 labelled folic acid and 8 microgram/kg tritium labelled folic acid (taken from table 4, chapter 5 of this thesis).

Group 2: Normal rats receiving same dose of dual labelled folic acid as rats of group 1, 24 hours after an oral dose of methotrexate (100 mg/kg), (taken from table 4, chapter 5 of this thesis)

Group 3: Normal rats receiving 76 microgram/kg carbon-14 labelled folic acid and 8.8 microgram/kg tritium labelled folic acid, 24 hours after an oral dose of 6-mercaptopurine (25 mg/kg), (taken from table 2 of this chapter).

Figures in parentheses give number of animals in each group.

Metabolites A to E appear at the calibrated sites of folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate, 4a-hydroxy-5-methyltetrahydrofolate and p-aminobenzoyl-L-glutamate respectively on DE52 ion-exchange column.

* These figures may not be due to 10-formyltetrahydrofolate only, since other metabolites 4a-hydroxy-5-methyltetrahydrofolate and p-aminobenzoyl-L-glutamate do not appear separately in DE52 ion-exchange column chromatogram of 0-6 hours urine sample (figure 8.1).

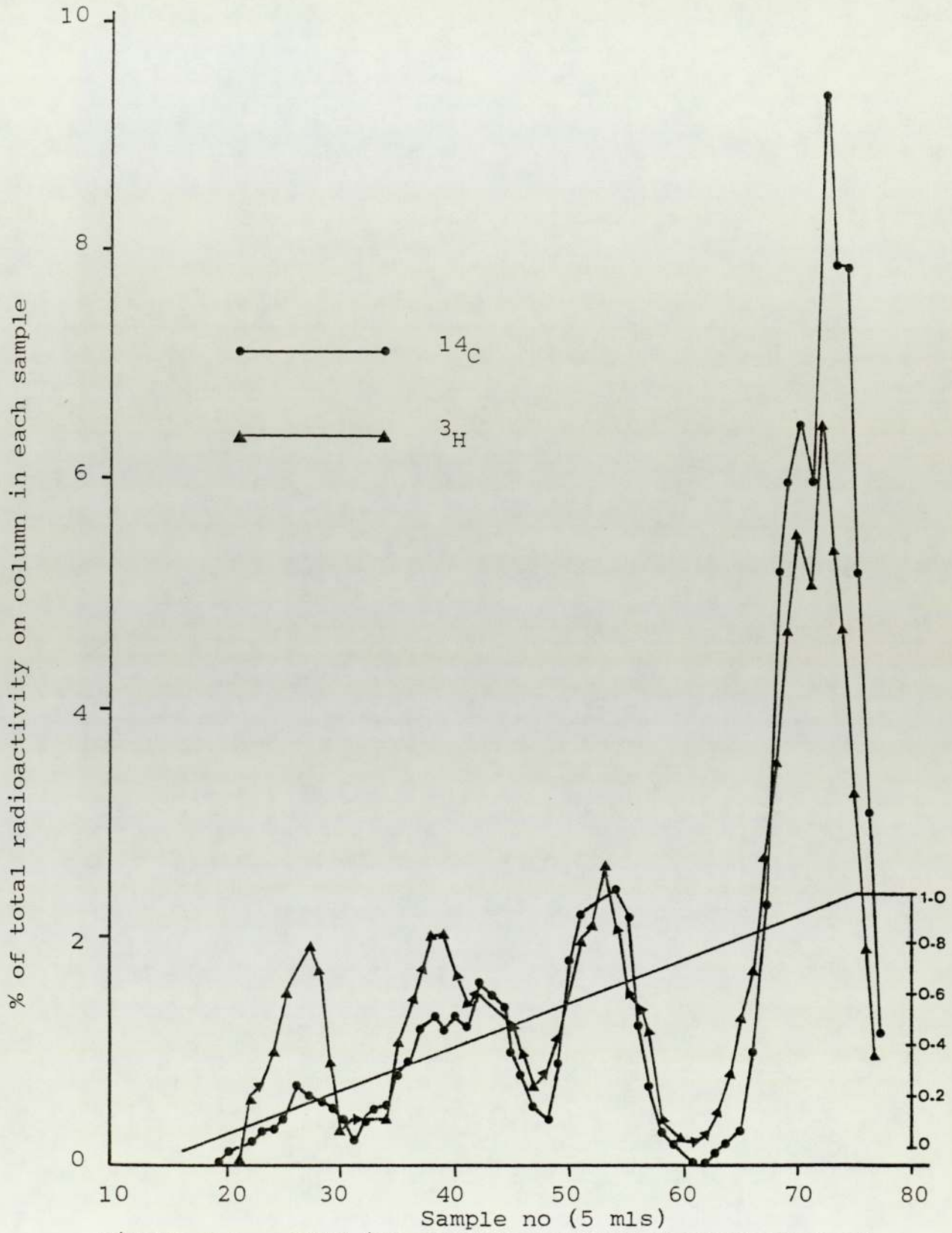


Figure 8.1 DEAE ion-exchange column chromatogram of 0-6 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after 6-MP administration.

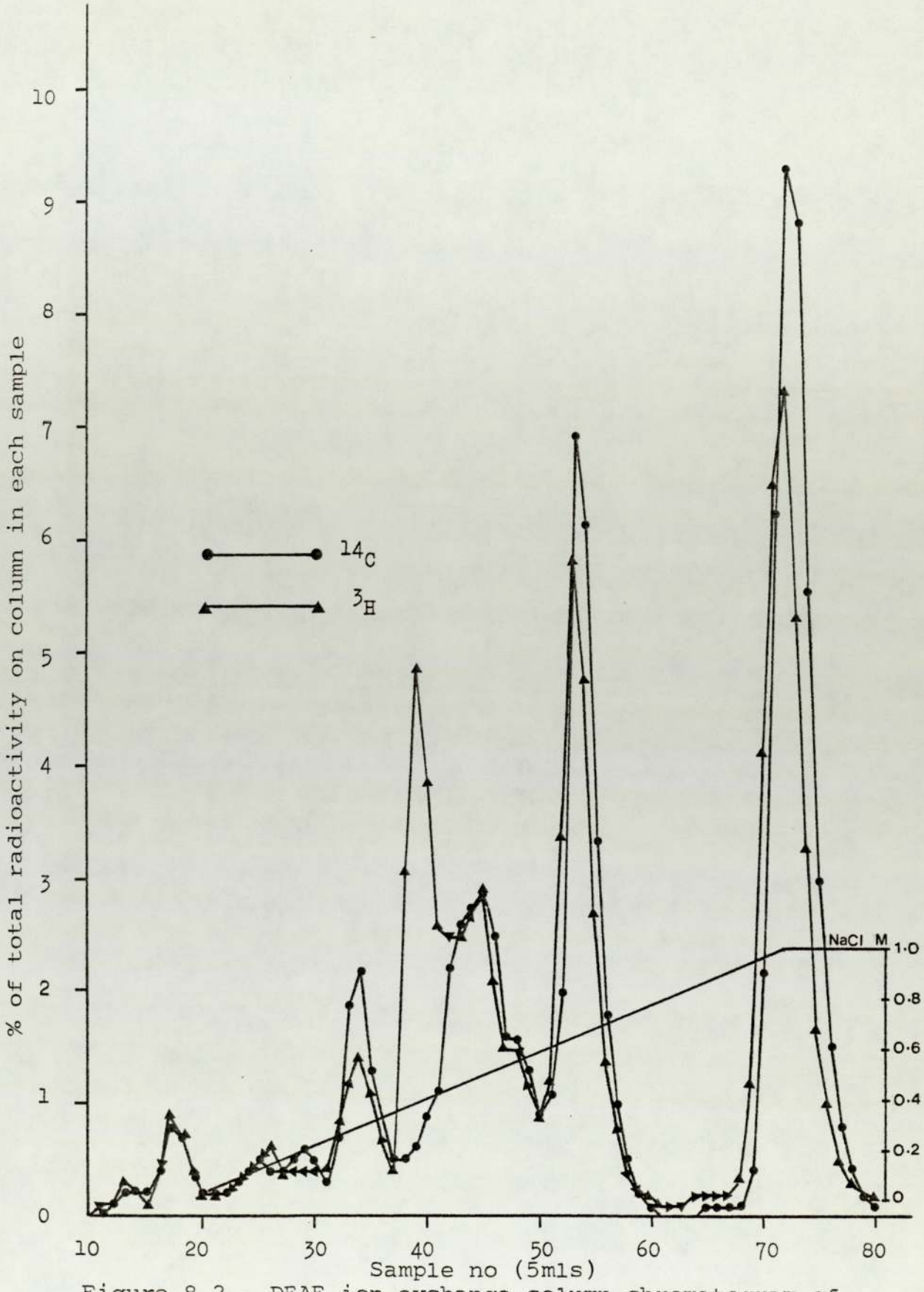


Figure 8.2 DEAE ion-exchange column chromatogram of 6-24hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after 6-MP administration.

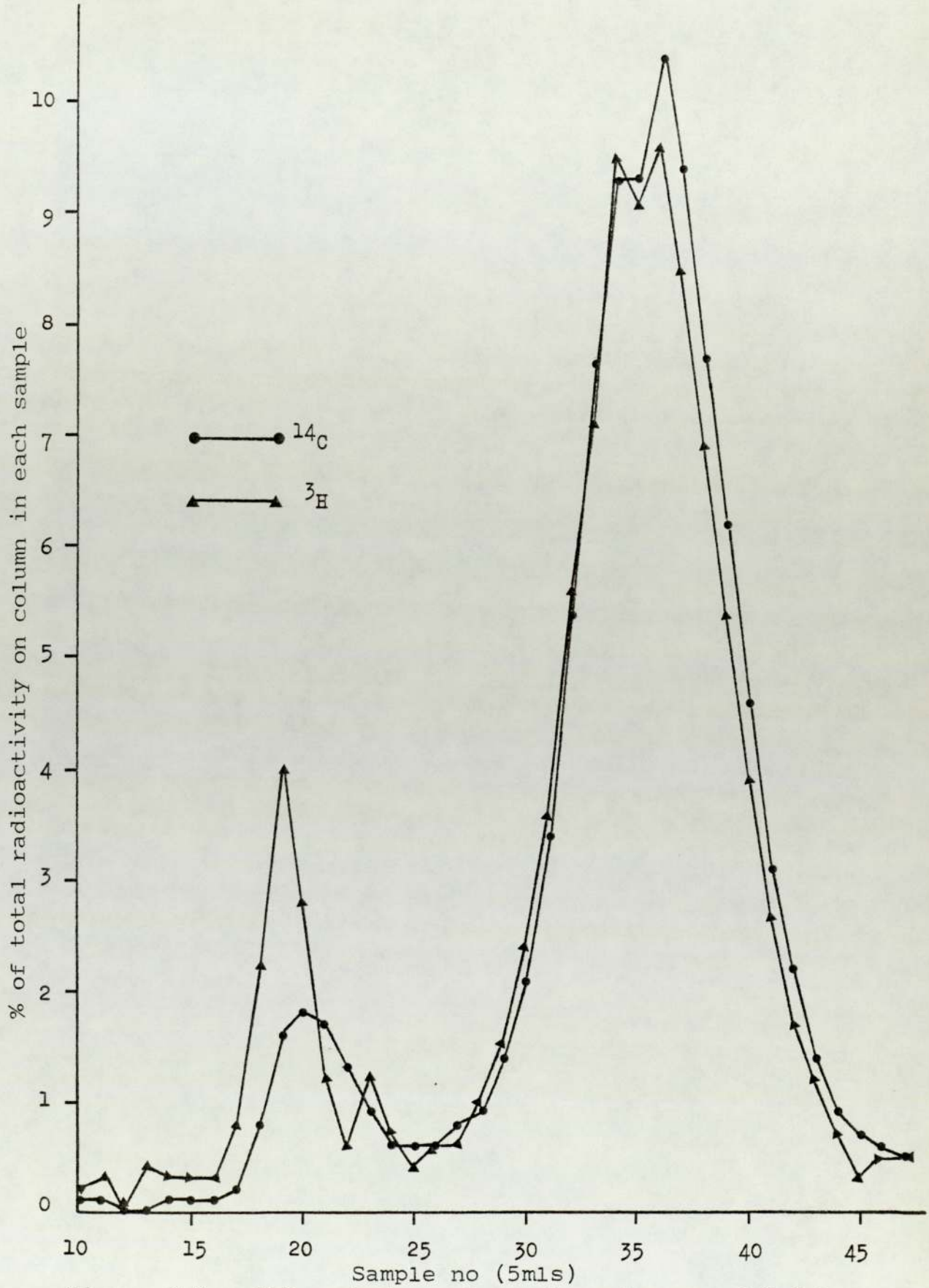


Figure 8.3 G15 sephadex column chromatogram of 0-6 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after 6-MP administration.

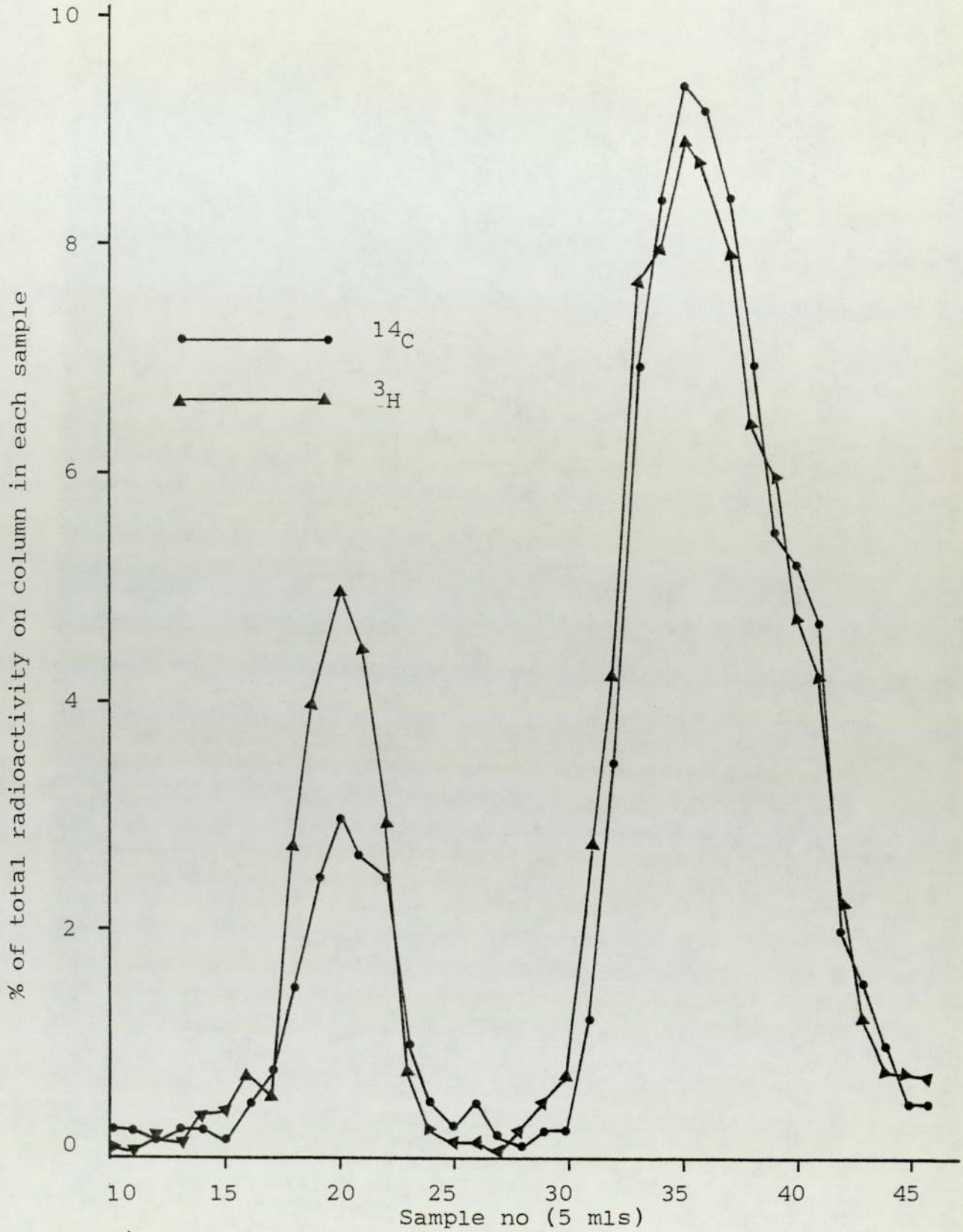


Figure 8.4 G15 sephadex column chromatogram of 6-24 hr urine of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after 6-MP administration

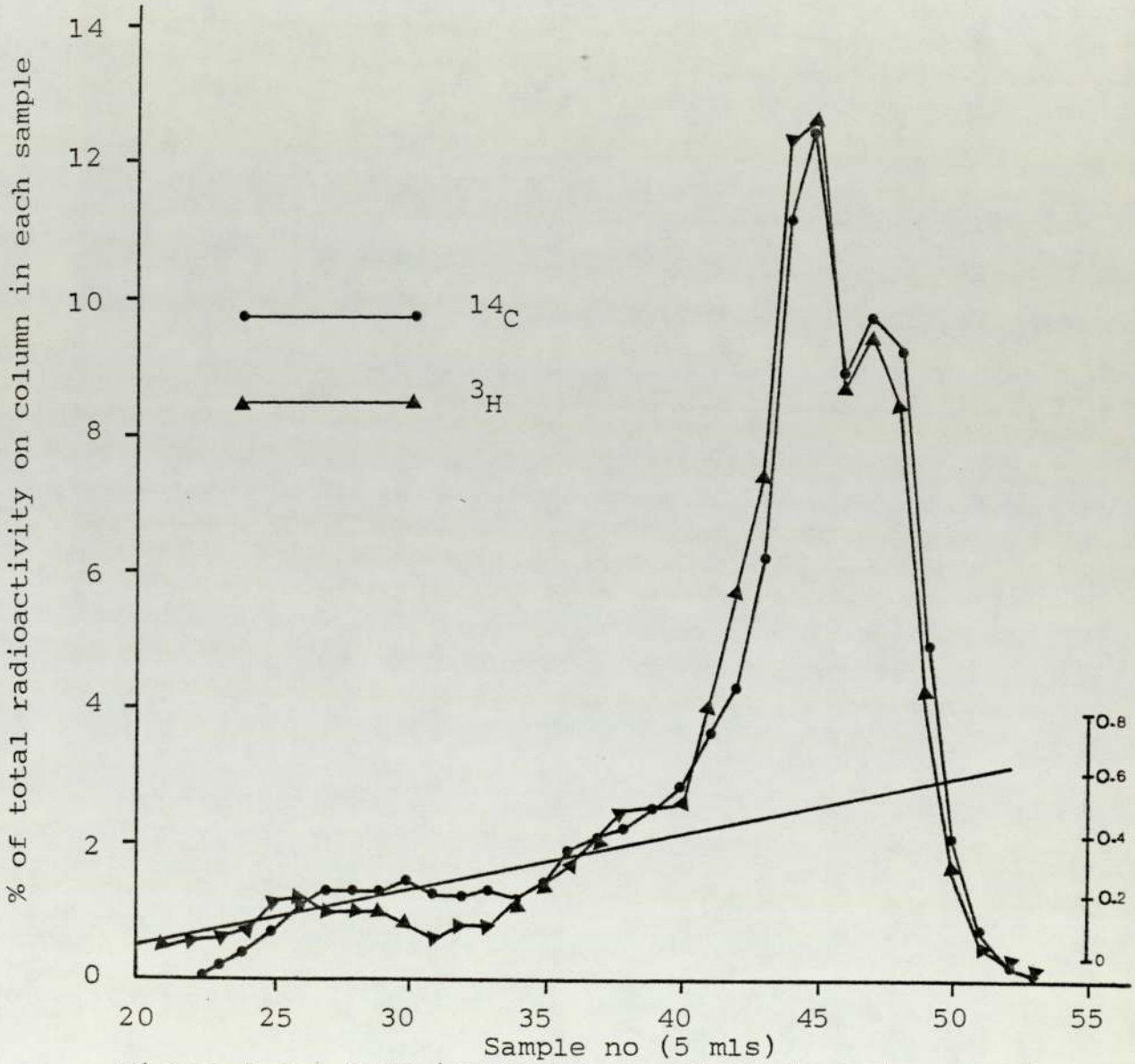


Figure 8.5 DEAE ion-exchange column chromatogram of 'boiled' liver extract of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after 6-MP administration.

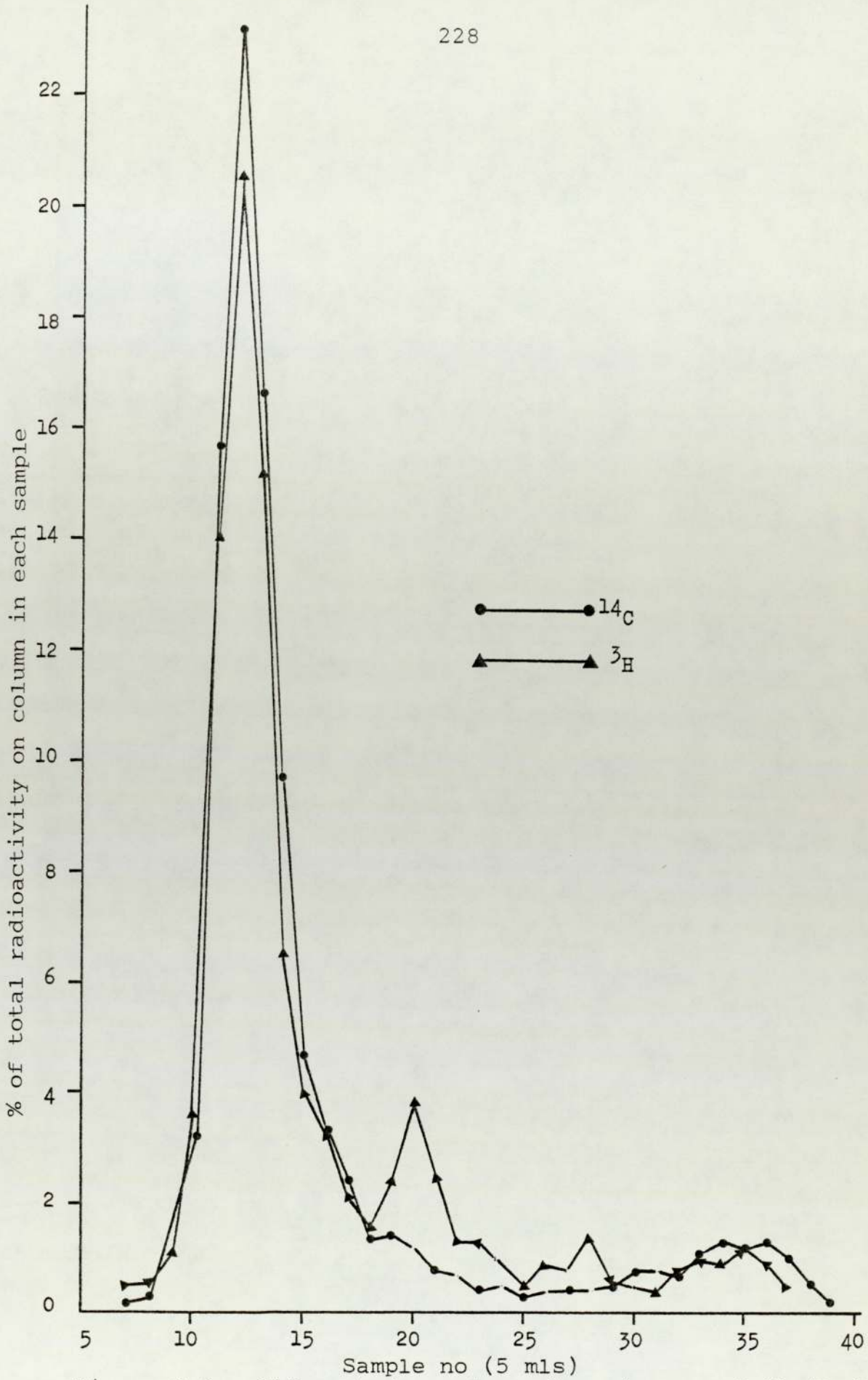


Figure 8.6 G15 sephadex column chromatogram of 'boiled' liver extract of rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after 6-MP administration.

Discussion

A Distribution of radioactivity

Table 1 gives the distribution of radioactivity, following an oral dose of a mixture of 2-^{14}C folic acid (76 microgram/kg) and 3'-5'-9 ^{3}H folic acid (8.8 microgram/kg), in urine, liver and gut of rats receiving 6-mercaptopurine (25 mg/kg) 24 hours before folic acid administration. The average distribution of dosed radioactivity in urine, liver and gut is given in table 3. Table 3 also includes the average distribution of radioactivity in urine, faeces, liver and gut of rats receiving same dose of dual labelled folic acid only, and rats receiving same dose of dual labelled folic acid 24 hours after methotrexate (100 mg/kg) administration.

Table 3 shows that rats receiving 6-mercaptopurine (rat group 3) did not have any faeces in 24 hours after folic acid administration. This is reflected in low total radioactivity recovery (^{14}C-47.8%, ^{3}H-57.6%) as compared to rats receiving folic acid after methotrexate administration (^{14}C-90.7%, ^{3}H-98.4%) and rats receiving folic acid only (^{14}C-71.2%, ^{3}H-78.3%). It is also evident from table 3 that in 0-6 hours urine sample, rats treated with methotrexate excreted excessive amounts of radioactivity (^{14}C-21.2%, ^{3}H-22.0%) as compared to rats receiving no methotrexate (^{14}C-7.9%, ^{3}H-8.4%). Rats receiving 6-mercaptopurine, however, excreted 16.6% of ^{14}C and 20.2% of ^{3}H radioactivity in 0-6 hours urine, which is comparable to amount of radioactivity excreted in 0-6 hours urine of rats receiving

methotrexate.

Radioactivity present in 6-24 hours urine samples of three groups of rats (see table 3) shows that rats receiving 6-mercaptopurine (group 3 rats) have excreted highest amounts (^{14}C-15.5%, ^{3}H-19.7%) as compared to methotrexate treated rats (group 2 rats; ^{14}C-11.8%, ^{3}H-13.2%) and rats receiving folic acid only (group 1 rats; ^{14}C-12.4%, ^{3}H-14.2%).

The radioactivity retained by the liver was highest in rats receiving no drug (^{14}C-11.0%, ^{3}H-14.2%) and lowest in rats receiving methotrexate (^{14}C-2.7%, ^{3}H-4.4%). Rats receiving 6-mercaptopurine, on the other hand, retained 9.5% of ^{14}C and 11.8% of ^{3}H radioactivity in the liver, which is comparable to amount of radioactivity retained in the liver of rats receiving folic acid only. Thus it is apparent that 6-mercaptopurine does not affect the uptake and retention of folate in the liver unlike methotrexate.

Amounts of radioactivity recovered in the gut of 6-mercaptopurine treated, methotrexate treated and rats receiving folic acid only are, however, comparable.

B Urinary metabolites

Table 2 shows distribution of urinary radioactivity in the metabolites resolved by DE52 ion-exchange column chromatograms (figures 8.1 and 8.2). The distribution has been expressed as a percentage of the total radioactivity chromatographed on each column.

Table 4 gives the average distribution of radioactivity in urinary metabolites of rats receiving dual

labelled folic acid (group 1 rats), rats receiving dual labelled folic acid 24 hours after methotrexate administration (group 2 rats) and rats receiving dual labelled folic acid 24 hours after 6-mercaptopurine administration (group 3 rats). From table 4 the following points may be deduced:

(1) Group of rats receiving 100 mg/kg methotrexate have excreted large amount of unchanged folic acid in 0-6 hours and 6-24 hours urines. This is reflected by high percentage of urinary radioactivity associated with unmetabolised folic acid appearing in the urine (90.1% of ^{14}C and 77.4% of ^{3}H radioactivity in 0-6 hours urine; 84.2% of ^{14}C and 58.5% of ^{3}H radioactivity in 6-24 hours urine).

The other two groups of rats (i.e. group receiving dual labelled folic acid only and the group receiving 6-mercaptopurine prior to dual labelled folic acid administration) have excreted similar amounts of unmetabolised folic acid in 0-6 hours and 6-24 hours urines.

Thus it appears that, unlike methotrexate, 6-mercaptopurine does not increase the excretion of unchanged folic acid in the urine of rats, if given 24 hours before folic acid administration.

(2) The amounts of 5-methyltetrahydrofolate in the urine of three groups of rats differ substantially. The 0-6 hours of 6-mercaptopurine treated animals and the animals receiving no drug show similar amounts of 5-methyltetrahydrofolate. The 0-6 hours urine sample of 6-mercaptopurine treated rats contains 12.3% of ^{14}C and 16.0% of ^{3}H urinary radioactivity as 5-methyltetrahydrofolate. Similarly 0-6 hours urine of rats receiving dual labelled folic acid only contains

16.9% of ^{14}C and 12.3% of ^{3}H urinary radioactivity as 5-methyltetrahydrofolate. However, methotrexate treated animals show no 5-methyltetrahydrofolate in 0-6 hours urine.

The amount of urinary radioactivity associated with 5-methyltetrahydrofolate in 6-24 hours urine of rats treated with 6-mercaptopurine is higher (^{14}C-24.2%. ^{3}H-21.8%) than the amounts of urinary radioactivity present in 6-24 hours urine of rats receiving dual labelled folic acid only (^{14}C-19.2%. ^{3}H-16.9%) and in 6-24 hours urine of rats re receiving methotrexate prior to folic acid administration. (^{14}C-5%, ^{3}H-6.1%).

Thus it seems that administration of 6-mercaptopurine to rats receiving folic acid has increased the excretion of 5-methyltetrahydrofolate in the urine. This suggests that 6-mercaptopurine interferes with the utilization of 5-methyltetrahydrofolate in the animal system.

(3) Amounts of urinary radioactivity associated with 10-formyltetrahydrofolate in 0-6 hours urines of three groups of rats cannot be compared, since 10-formyltetrahydrofolate peak in DE52 ion-exchange column chromatogram (figure 8.1) is not clear of metabolite 4a-hydroxy-5-methyltetrahydrofolate. However, urinary radioactivity associated with 10-formyltetrahydrofolate in 6-24 hours urines of three groups of rats show that 6-mercaptopurine treated animals excrete highest amount (^{14}C-18.7%, ^{3}H-15.3%) as compared to methotrexate treated rats (^{14}C-6.5%, ^{3}H-7.5%) and rats receiving folic acid only (^{14}C-13.2%, ^{3}H-13.2%).

This suggests that 6-mercaptopurine also inhibits

utilization of 10-formyltetrahydrofolate.

(4) Distribution of p-aminobenzoyl-L-glutamate in the urines of three groups of rats suggests that 6-mercaptopurine increases the breakdown of folate molecule. This is evident from higher percentage of urinary radioactivity associated with this metabolite in 6-24 hours urine of rats treated with 6-mercaptopurine.

CHAPTER 9

METABOLISM OF 3'-5'-9 ^{3}H-2-^{14}C FOLIC ACID IN RAT BEARING WALKER 256 CARCINOSARCOMA AFTER ORAL METHOTREXATE

Introduction

Little is known about the effects of methotrexate on folate metabolism in the rats with transplanted tumours. The studies reported in this chapter elucidate some aspects of effects of methotrexate on folate metabolism in the rats bearing Walker 256 carcinosarcoma. This was thought tobe of considerable importance and interest to investigate the influence of of presence of a tumour on methotrexate-folate interaction and folate metabolism in the living mammalian system.

The folate metabolism in normal adult Wistar rats in the presence and absence of methotrexate has been described in some of the previous chapters. Barford and Blair (145) have studied the metabolism of 2-^{14}C folic acid in the normal rats and the rats bearing Walker 256 carcino-sarcoma. The authors reported that after oral administration of 2-^{14}C folic acid (78 microgram/kg), the total recovery of the dosed radioactivity in the urine in 48 hours was 13.6% in the normal and 22% in the urine of tumour bearing rats, the majority of the radioactivity was excreted in the first 24 hours. Thus presence of tumour in the rat increased the excretion of orally administered folic acid. The authors also reported that in both 0-6 hours and 6-24 hours urine samples of tumour bearing rats the majority of urinary metabolite was 10-formyltetrahydrofolate and the amount of this metabolite increased with increasing time. This observation shows remarkable similarity with the urinary metabolites of 0-6 and 6-24 hour samples of the rats dosed with methotrexate

24 hours before folic acid administration.

Previous results in this thesis demonstrate that oral administration of methotrexate (10 mg/kg or 100 mg/kg), 24 hours before folic acid administration, decreases the uptake of folate in the liver and accelerates the urinary excretion of administered folate in rat. It was observed that methotrexate supresses the formation of folate co-enzymes considerably, though 10-formyltetrahydrofolate was supressed to a much lesser degree compared to rest of the reduced folates. This was reflected by higher levels of 10-formyltetrahydrofolate in the urine of methotrexate treated animals. This observation suggests that methotrexate inhibits purine biosynthesis in addition to inhibition of dihydrofolate reductase. However, the observation that 10-formyltetrahydrofolate was the major urinary metabolite of folic acid in Wistar rats bearing Walker 256 carcinosarcoma (145), indicates altered metabolism of folic acid in the animal due to the presence of tumor. Though the reason for such a change remains to be established.

It has been documented that the transport of naturally occuring folates and low doses of methotrexate in the methotrexate resistant Walker 256 carcinosarcoma was reduced (168). However, the authors claim that dietary depletion of folate had marked effect on the growth of Walker 256 carcinosarcoma. The rats maintained on a diet deficient in folate,for two weeks before or from the time of transplantation of Walker 256 carcinosarcoma resulted in inhibition of tumor growth by more than 95% on a weight

basis on 28th day after implantation. They also claimed that the inhibition of tumor growth on animals was not effected by adequate stores of folate in the liver and that Walker 256 carcinosarcoma is not capable of parasitizing the host or of competing with tissues for folate in achieving optimum growth.

Cell death is thought to be a consequence of inhibition of dihydrofolate reductase by methotrexate, followed by depletion of intra-cellular stores of the reduced folates, necessary for the synthesis of DNA, RNA and some amino acids (87). The difference in response to methotrexate, in fastly growing and resting population of cells indicates that methotrexate is a phase specific drug, killing cells undergoing S-phase of cell cycle with little effect on cells not in the S-phase of the cell cycle (169). It is therefore expected that sensitivity of various normal or neoplastic tissues to methotrexate will vary from one to another (74,87), depending upon the difference in optimum conditions i.e. uptake,levels of dihydrofolate reductase and cell phase offered by specific tissue. On the other hand, observation that methotrexate infusion results in acute elevation of hepatic enzymes (121,170) apparently seems to have no relation with the property of the drug to inhibit dihydrofolate reductase and cell phase specificity. Resistance of neoplastic tissue to methotrexate, however, cannot be explained at present on the basis of any one of the mechanism of methotrexate action.

McCullough et.al (165) have isolated a folate

cleaving enzyme carboxypeptidase G1 from Pseudomonas stutzeri, which was found to have anti-tumor activity against methotrexate resistant Walker 256 carcinosarcoma (166). The enzyme hydrolyses the C-terminal glutamate residue from folates and is also capable of cleaving circulating methotrexate (167) in the animal system. The folate depleting activity was studied in mice and was found to decrease the plasma level of folate to 20% of control levels, it also decreased the hepatic folate stores.

Thus foregoing studies suggest that the Walker 256 carcinosarcoma is sensitive to the levels of folate in the animal.

Materials and Methods

Five male Wistar rats, weighing 150-200 grams, transplanted with Walker 256 carcinosarcoma were dosed with methotrexate 100 mg/kg orally 16 days after transplantation. Twenty four hours later animals were dosed orally with a mixture of 3'-5'-9 ^{3}H folic acid and 2-^{14}C folic acid in water, each animal receiving 8.2 microgram/kg body weight of 3'-5'-9' ^{3}H folic acid and 76 microgram/kg body weight of 2-^{14}C folic acid in a mixture of the labelled materials in water. The rats were housed singly in metabowls and were killed 24 hours after folic acid administration.

Collection of Biological Samples

The urine samples were collected between 0-6 and 6-24 hours after folic acid administration, in flasks containing 5 mls of 0.05 molar phosphate buffer pH 7 containing 5 mg/100 mls dithiothreitol and 5 gm/100 mls ascorbic acid. The faeces were collected in empty flasks between 0-24 hours.

The livers, gut and tumours of the animals were taken out immediately after killing. Livers of two rats were freeze dried together with faeces, gut and tumours for the estimation of retained radioactivity. The livers of three rats were extracted by hot extraction procedure as described in chapter 2.

The tumours of the rats were prominent and palpable at the time of methotrexate dosing. While the tumours of two rats were invisible and inpalpable 24 hours after methotrexate administration. The tumours of rest of the three animals were reduced remarkably in size.

Column Chromatography of Urine Samples and Liver Extract

The 0-6, 6-24 hours urine samples and liver extract were separately chromatographed on DE52 ion-exchange and G15 sephadex columns. The columns were packed and eluted in the same fashion as described in chapter two.

RESULTS

Table 1 gives the distribution of radioactivity in urine, liver, gut, tumor and faeces of the animals, expressed as a percentage of the dosed folic acid radioactivity.

Column chromatography of urine samples and liver extract

Figure 9.1 is a DE52 ion-exchange column chromatogram of 0-6 hours urine sample. Figure 9.2 is a DE52 ion-exchange column chromatogram of 6-24 hours urine sample. Table 2 gives the distribution of radioactivity in the metabolites expressed as a percentage of the total radioactivity chromatographed on each column (figures 9.1 and 9.2). The major compound present in the urine was identified as unchanged dosed 2-^{14}C folic acid and 3'-5'-9 ^{3}H folic acid. The peak corresponding to this compound appeared between 0.9 and 1.0 molar sodium chloride concentration on the gradient in the two chromatograms (figures 9.1 and 9.2). The above mentioned DE52 ion-exchange column chromatograms are comparable to the corresponding chromatograms of the urine of normal rats (see chapter 5).

The second peak appears at about 0.6 molar sodium chloride concentration on the gradient and contains both ^{14}C and ^{3}H radioactivity. This peak was identified as 5-methyl-tetrahydrofolate on the basis of its elution position on DE52 ion-exchange columns. This peak,which is smallest of the peaks appearing, is present in both 0-6 hours and 6-24 hours urine chromatograms (figures 9.1 and 9.2).

The third peak appears at about 0.4 molar sodium chloride concentration on the gradient in both 0-6 hours and

6-24 hours urine chromatograms (figures 9.1 and 9.2) and contains both ^{14}C and ^{3}H activity. This peak eluted at the calibrated site of 10-formyltetrahydrofolate on DE52 ion-exchange column.

The 6-24 hours urine chromatogram (figure 9.2) shows a somewhat prominent fourth peak containing radio-activity due to tritium only. It appears just before the 10-formyltetrahydrofolate peak on DE52 ion-exchange column. This peak was identified as p-aminobenzoyl-L-glutamate on the basis of its elution position. The DE52 ion-exchange column chromatograms of the urine of normal rats treated with methotrexate showed the same peak, which was identified by isotope dilution procedure (see chapter 5).

Figure 9.3 and 9.4 are G15 sephadex column chromatograms of 0-6 hours and 6-24 hours urine samples respectively.

Figure 9.5 is a G15 sephadex column chromatogram of liver extract, which shows a big characteristic peak of some polyglutamate form of folate.

Rat No	Urine				Liver		Faeces		Gut		Tumour		Total	
	0-6 hrs		6-24 hrs											
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1	17.3	18.0	6.5	10.6	5.3	8.3	24.7	28.2	2.9	3.0	0.1	0.1	56.8	68.2
2	17.7	20.4	6.7	10.9	2.1	8.4	15.3	19.5	3.0	3.1	0.1	0.1	44.9	62.4
3	18.1	19.2	10.0	13.7	-	-	23.9	40.0	6.6	7.8	-	-	58.6	80.7
4	18.4	23.0	10.2	11.2	-	-	22.5	26.0	2.2	1.9	-	-	53.3	62.1
5	32.4	34.4	11.1	13.1	-	-	30.1	32.0	4.9	3.9	-	-	78.5	83.4
Average	20.8	23.0	8.9	11.9	3.7	8.3	23.3	29.1	3.9	3.9	0.1	0.1	58.4	71.4

Table 1 Distribution of radioactivity in urine, liver, gut, tumour and faeces of rats bearing Walker 256 carcinosarcoma, expressed as percentage of an oral dose of a mixture of carbon-14 labelled folic acid (76 microgram/kg) and tritium labelled folic acid (8.2 microgram/kg). The rats received an oral dose of methotrexate (100 mg/kg) 24 hours before folic acid administration.

Note: Weight of dry tumour approximately 1 gm.

Chromatogram	Metabolites							
	A		B		C		E	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Figure 9.1	81.5	79.8	1.9	1.9	6.5	6.0	-	1.8
Figure 9.2	76.9	75.3	4.2	4.2	12.6	10.5	-	8.3

Table 2 Distribution of radioactivity in various urinary metabolites as resolved by DE52 ion-exchange column, expressed as a percentage of total radioactivity chromatographed on each column.

Metabolites A, B, C and E appear at the calibrated sites of folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and p-aminobenzoyl-L-glutamate on DE52 ion-exchange column respectively.

Rat group	Urine 0-6 hrs		Urine 6-24 hrs		Liver		Faeces		Gut		Tumour		Total	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1 (4)	7.9	8.4	12.4	14.2	11.0	13.9	33.8	38.8	6.1	3.0	-	-	71.2	78.3
2 (6)	21.2	21.8	11.7	13.2	2.7	4.4	48.4	51.9	6.6	6.8	-	-	90.7	98.4
3 (13)	6.2	-	11.2	-	14.7*	-	37.7*	-	-	-	4.0*	-	74.0	-
4 (5)	20.8	23.0	8.9	11.9	3.7	8.3	23.3	29.1	3.9	3.9	0.1	0.1	60.7	76.3

Table 3 Average distribution of radioactivity in biological samples of various groups of rats following an oral dose of folic acid.

Group 1: Normal rats receiving 76 microgram/kg of carbon-14 labelled folic acid and 8 microgram/kg of tritium labelled folic acid (taken from table 1, chapter 5 of this thesis).

Group 2: Normal rats receiving same dose of dual labelled folic acid as rats of group 1, 24 hours after an oral dose of methotrexate (100 mg/kg), (taken from table 3, chapter 5 of this thesis).

Group 3: Walker 256 carcinoma bearing rats receiving 78 microgram/kg carbon-14 labelled folic acid (taken from Barford and Blair (145)).

Group 4: Walker 256 carcinoma bearing rats receiving 76 microgram/kg carbon-14 labelled folic acid and 8.2 microgram/kg tritium labelled folic acid, 24 hours after an oral dose of methotrexate (100 mg/kg), (taken from table 1 of this chapter).

Figures in parentheses give number of animals in each group.

* The figures represent radioactivity recovered 48 hours after folic acid administration.

Note: Distribution in the above table is expressed as a percentage of the total dosed folic acid radioactivity.

	Rat group	Metabolites A		B		C		D		E	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
0-6hr urine	1 (4)	71.1	51.7	16.9	12.3	7.7	13.0	4.9	10.6	-	-
	2 (6)	90.1	77.4	-	-	5.3	6.9	-	-	-	11.1
	3 (13)	7.5	-	22.0	-	46.2	-	21.7	-	-	-
	4 (5)	81.5	79.8	1.9	1.9	6.5	6.0	-	-	-	1.8
6-24hr urine	1 (4)	45.6	30.5	19.2	16.9	13.2	13.2	18.0	11.0	-	10.0
	2 (6)	84.2	58.5	5.0	6.1	6.5	7.5	-	-	-	15.0
	3 (13)	3.1	-	7.7	-	56.0	-	15.0	-	-	-
	4 (5)	76.9	75.3	4.2	4.2	12.6	10.5	-	-	-	8.3

Table 4 Average distribution of radioactivity in urinary metabolites of various groups of rats following an oral dose of folic acid.

Group 1: Normal rats receiving 76 microgram/kg carbon-14 labelled folic acid and 8 microgram/kg tritium labelled folic acid (taken from table 4,chapter 5 of this thesis).

Group 2: Normal rats receiving same dose of dual labelled folic acid as rats of group 1, 24 hours after an oral dose of methotrexate (100 mg/kg), (taken from table 4,chapter 5 of this thesis).

Group 3: Walker 256 carcinoma bearing rats receiving 78 microgram/kg carbon-14 labelled folic acid (taken from Barford and Blair (145)).

Group 4: Walker 256 carcinoma bearing rats receiving 76 microgram/kg carbon-14 labelled folic acid and 8.2 microgram/kg tritium labelled folic acid, 24 hours after an oral dose of methotrexate (100 mg/kg), (taken from table 2 of this chapter).

Figures in parentheses give number of animals in each group.

Metabolites A to E appear at the calibrated sites of folic acid, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate, 4a-hydroxy-5-methyltetrahydrofolate and p-aminobenzoyl-L-glutamate respectively on DE52 ion-exchange column.

Note: Distribution in the above table is expressed as a percentage of total chromatographed radioactivity on DE52 ion-exchange column.

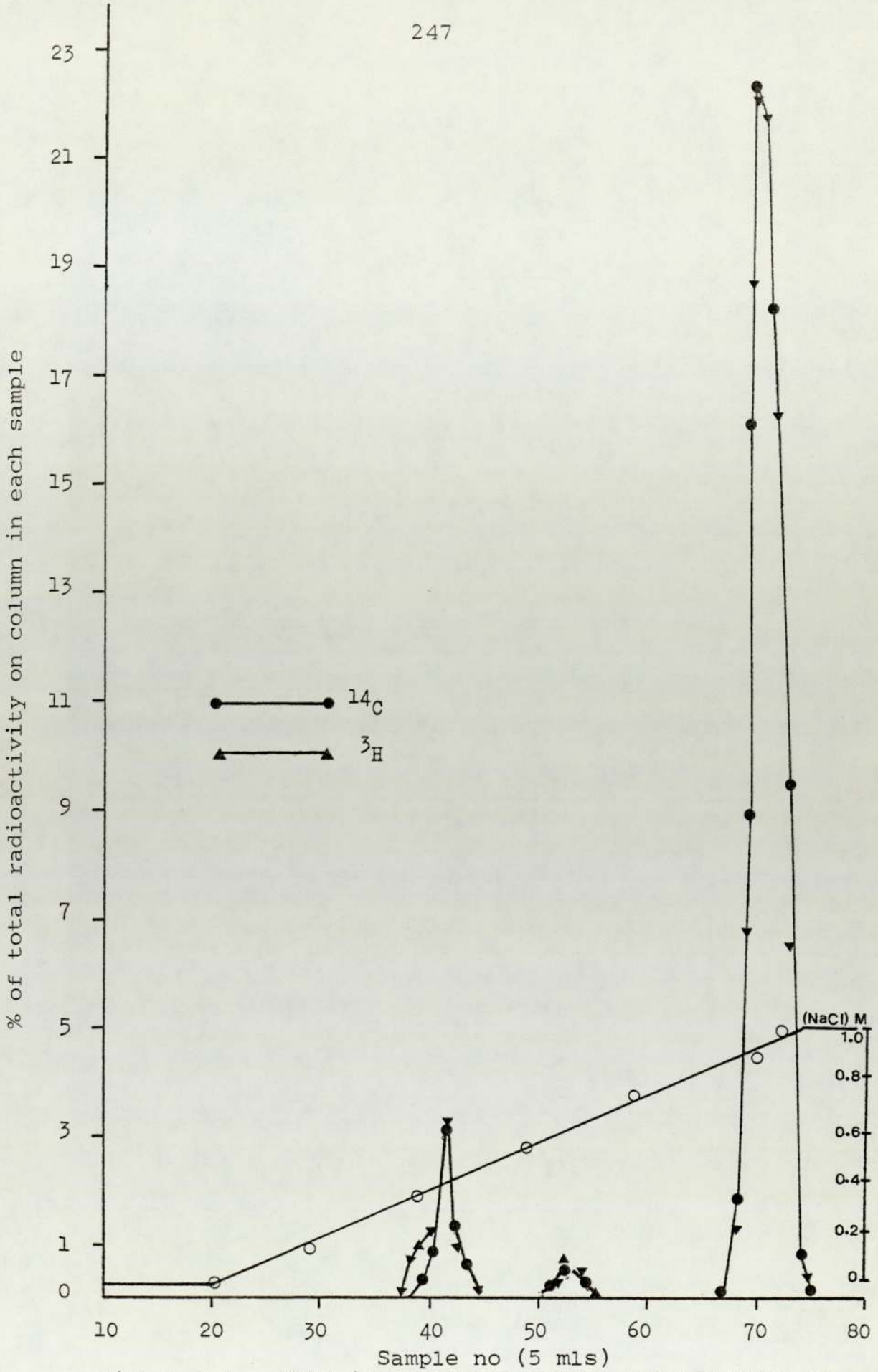


Figure 9.1 DEAE ion-exchange column chromatogram of 0-6 hr urine of Walker 256 carcinosarcoma bearing rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate administration.

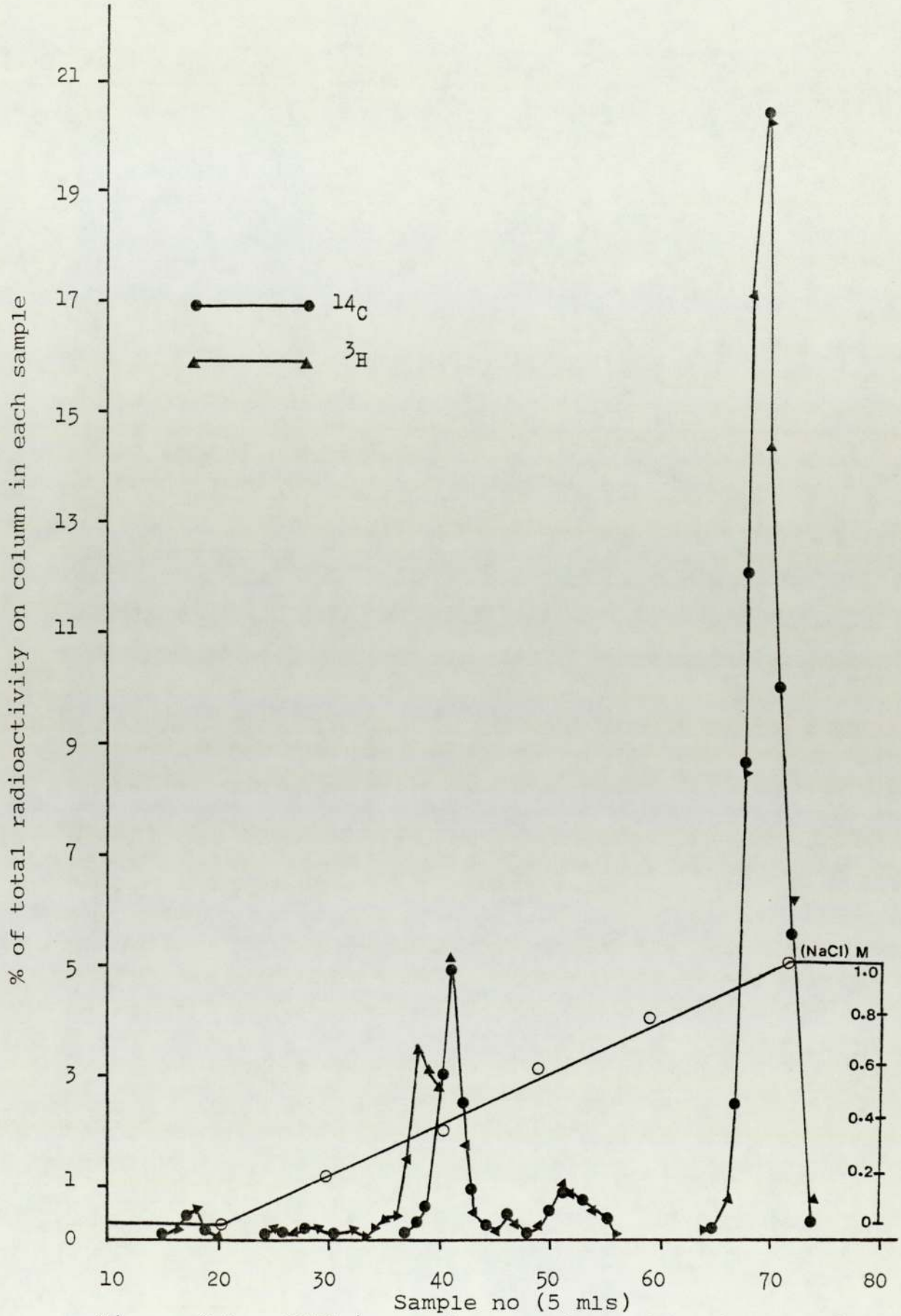


Figure 9.2 DEAE ion-exchange column chromatogram of 6-24 hr urine of Walker 256 carcinosarcoma bearing rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate administration.

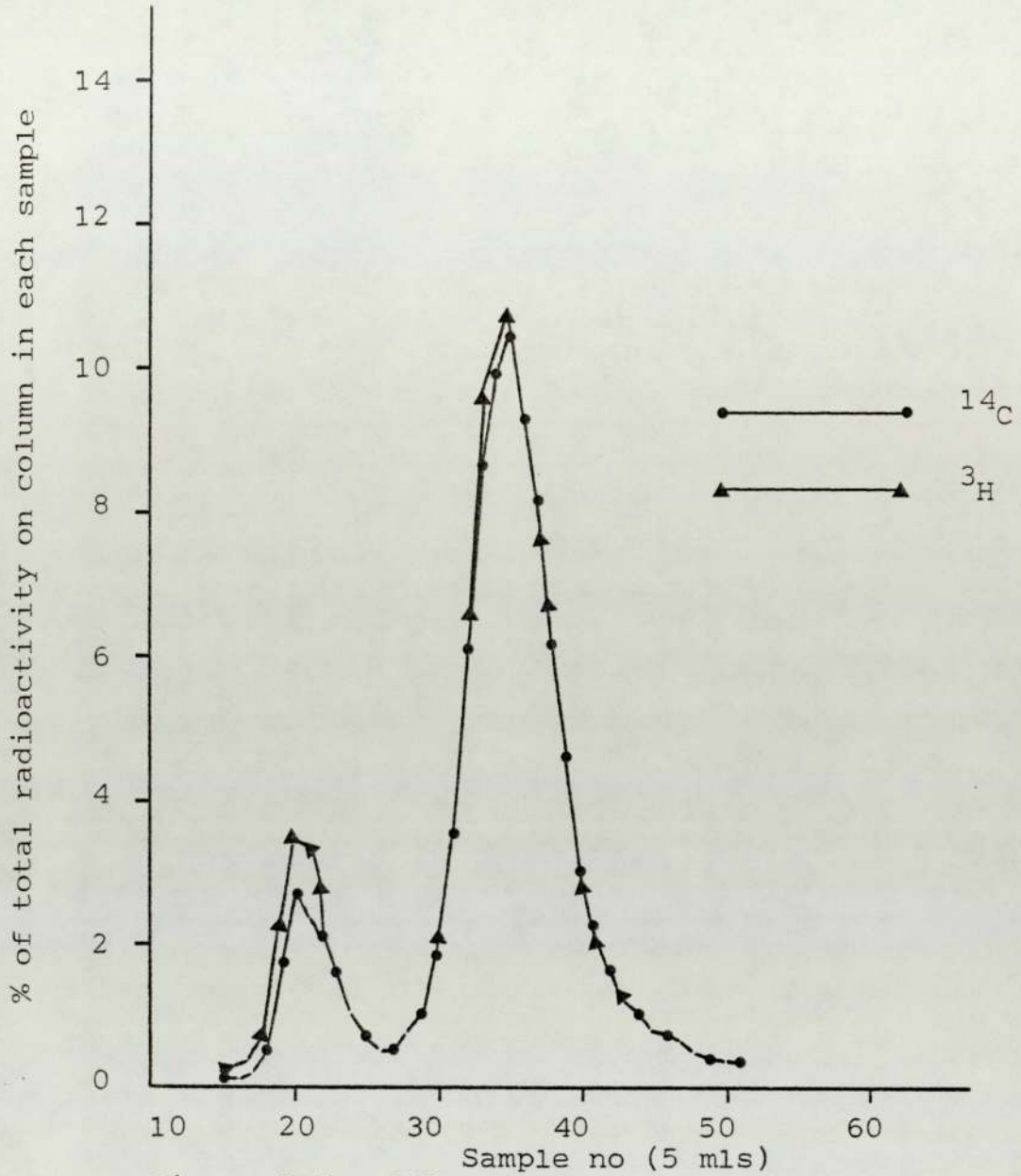


Figure 9.3 G15 sephadex column chromatogram of 0-6 hr urine of Walker 256 carcinosarcoma bearing rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate administration.

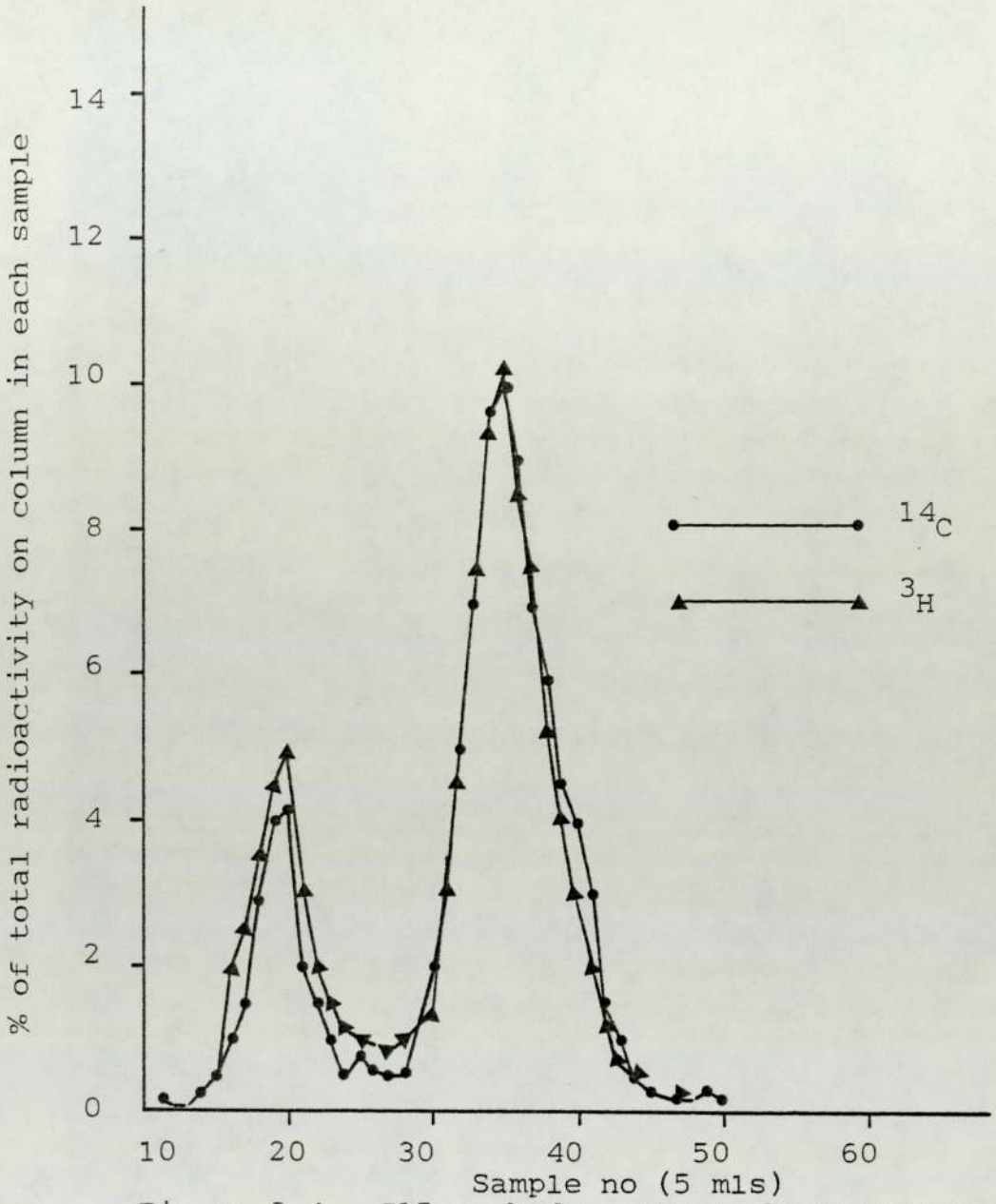


Figure 9.4 G15 sephadex column chromatogram of 6-24 hr urine of Walker 256 carcinosarcoma bearing rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate administration.

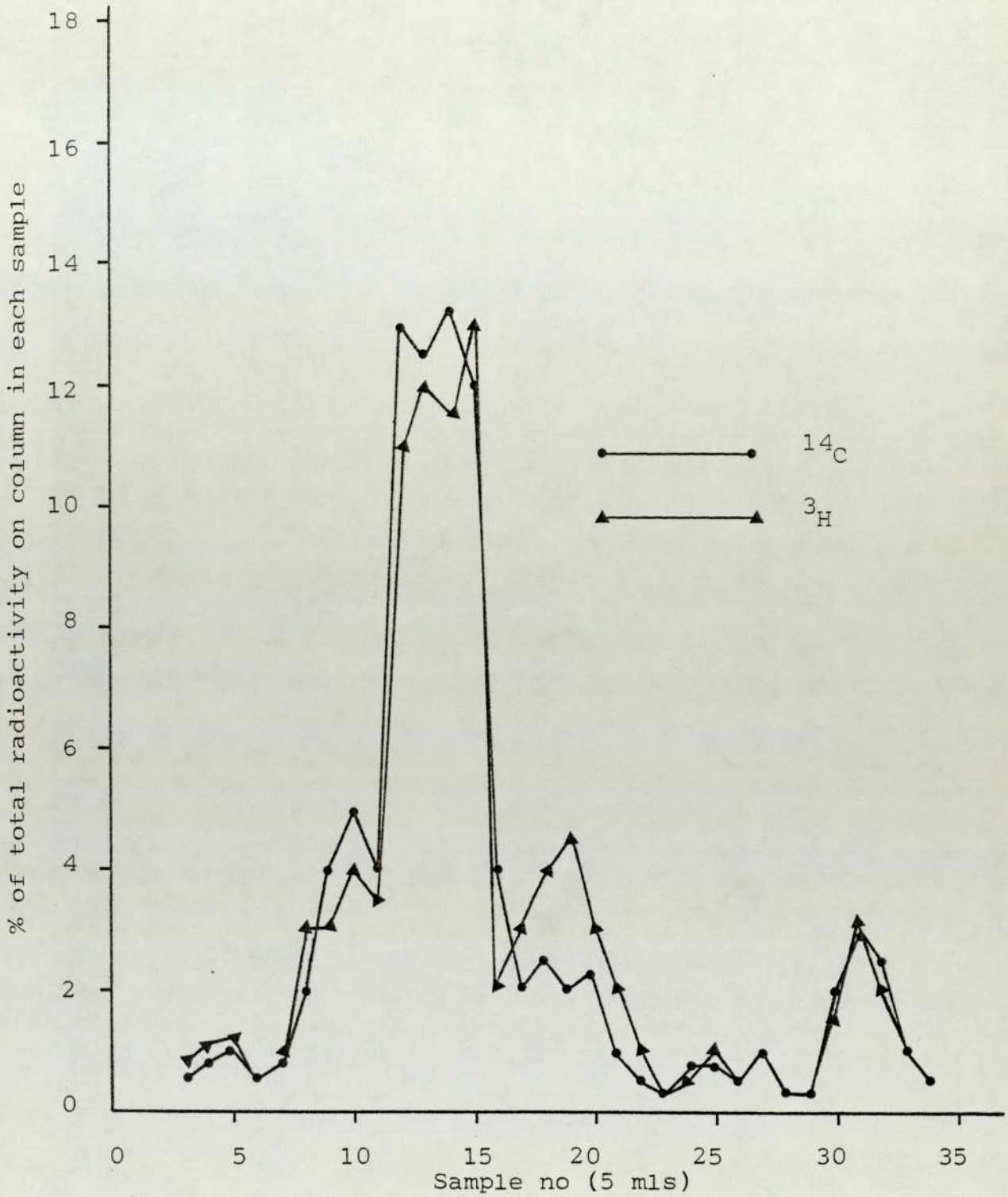


Figure 9.5 G15 sephadex column chromatogram of 'boiled' liver extract of Walker 256 carcinosarcoma bearing rats receiving a mixture of tritium and carbon-14 labelled folic acid 24 hours after methotrexate administration.

Discussion

A Distribution of radioactivity

Table 1 gives distribution of radioactivity of an oral dose of dual labelled folic acid (2-^{14}C folic acid 76 microgram/kg, 3'-5'-9 ^{3}H folic acid 8.2 microgram/kg) in urine, liver, gut, tumour and faeces of rats bearing Walker 256 carcinoma. Rats were dosed with 100 mg/kg methotrexate 24 hours before folic acid administration. Table 3 gives average distribution of folic acid radioactivity in biological samples of normal and tumour bearing rats. The rats in table 3 have been classified into four groups. Group 1 represents normal rats receiving dual labelled folic acid only, group 2 represents normal rats receiving dual labelled folic acid 24 hours after an oral dose of 100 mg/kg methotrexate, group 3 represents Walker 256 carcinoma bearing rats receiving 2-^{14}C folic acid only (taken from Barford and Blair (145)), and group 4 represents Walker 256 carcinoma bearing rats receiving dual labelled folic acid 24 hours after an oral dose of 100 mg/kg methotrexate. In the following discussion group numbers characterise specific rats dosed according to above mentioned classification.

Comparable amounts of radioactivity were found in 0-6 hours urines of rats of groups 2 and 4 (group 2 ^{14}C-21.2%, ^{3}H-22%; group 4 ^{14}C-20.8%, ^{3}H-23%). This means that methotrexate effects the excretion of dosed folic acid radioactivity in 0-6 hours urine equally in normal and tumour bearing rats. Rats of groups 1 and 3 excreted lesser amounts of radioactivity as compared to rats of groups 2 and 4 in 0-6 hours urine.

However, amounts of radioactivity present in 0-6 hours urines of rats of groups 1 and 3 were comparable (group 1 ^{14}C-7.9%, ^{3}H-8.4% ; group 3 ^{14}C-6.2%). This indicates that methotrexate increases the excretion of radioactivity in 0-6 hours urine of both normal and tumour bearing rats. The 6-24 hours urine samples of all four groups of rats, however, contain comparable amounts of radioactivity. This suggests that administration of methotrexate predominantly increases the excretion of dosed folic acid radioactivity in 0-6 hours urine of both normal and tumour bearing rats.

The radioactivity retained by the liver of group 1 animals (^{14}C-11.0%, ^{3}H-13.9%) is comparable to that retained by the liver of group 3 animals (14.7% of 2-^{14}C folic acid radioactivity). However, considering that group 3 rats were killed 48 hours after 2-^{14}C folic acid administration and group 1 rats were killed 24 hours after dual labelled folic acid administration, it appears that group 3 rats retained slightly higher amounts of radioactivity in the liver. Rats of group 4 retained 3.7% of ^{14}C and 8.3% of ^{3}H radioactivity in the liver, which is comparable to the amount of radioactivity retained in the liver of rats of group 2 (^{14}C-2.7%, ^{3}H-4.4%). The difference appears in the amounts of ^{3}H radioactivity, which is higher in group 4 liver. This could be due to higher rates of breakdown of folic acid molecule.

Group 4 animals excreted lowest amount of radioactivity in the faeces (^{14}C-23.3%, ^{3}H-29.1%), while highest amount of radioactivity was found in the faeces of group 2 rats (^{14}C-48.4%, ^{3}H-51.9%). Thus presence of tumour in rat

increases the absorption of folic acid from the gastro-intestinal tract if methotrexate is administered 24 hours before folic acid.

Gut of the rats of group 4 retained less radio-activity (^{14}C-3.9%, ^{3}H-3.9%) as compared to the gut of rats of groups 1 and 2. Thus presence of methotrexate in tumour bearing rat decreases the retention of folates in the gut.

Striking difference was observed in the amounts of radioactivity associated with tumours of group 4 (^{14}C-0.1%, ^{3}H-0.1%) and group 3 rats (^{14}C-4.0%). This suggests that methotrexate has substantially reduced the uptake and retention of folic acid in tumour.

B Column chromatography of urine samples

Figures 9.1 and 9.2 are DE52 ion-exchange column chromatograms of 0-6 and 6-24 hours urine samples respectively of Walker 256 carcinoma bearing rats dosed orally with dual labelled folic acid, 24 hours after 100 mg/kg methotrexate administration. Table 2 gives the distribution of radio-activity in the metabolites. The distribution in table 2 have been included in table 4 for comparison, as table 4 also includes the distribution of radioactivity in urinary metabolites of 0-6 and 6-24 hours urine samples of three other groups of rats mentioned earlier. Some of the observations from table 4 are summarised below.

(1) Much higher amounts of unchanged folic acid are associated with 0-6 and 6-24 hours urines of Walker 256 carcinoma bearing rats (0-6 hours urine contained ^{14}C-81.5%, ^{3}H-79.8% ; 6-24 hours urine contained ^{14}C-76.9%, ^{3}H-75.3%)

treated with 100 mg/kg methotrexate 24 hours before folic acid administration, which is comparable to the amounts of radioactivity associated with unchanged folic acid in the 0-6 and 6-24 hours urines of rats of group 2. Previous studies (145) show that Walker 256 carcinoma bearing rats receiving 2-^{14}C folic acid only,excreted very small amounts of unchanged folic acid in the urine (see group 3, table 4).

(ii) The urinary radioactivity associated with 5-methyl-tetrahydrofolate in 0-6 and 6-24 hours samples is much higher in rats of group 1 (0-6 hours urine ^{14}C-16.9%, ^{3}H-12.3%; 6-24 hours ^{14}C-19.2%, ^{3}H-16.9%) as compared to rats of groups 2 and 4. Rats of group 2, however, did not excrete 5-methyl-tetrahydrofolate in 0-6 hours urine. This suggests that methotrexate reduces the excretion of 5-methyltetrahydro-folate in urine of tumour bearing rats as it does in normal rats.

(iii) It has been observed that 5-methyltetrahydrofolate is the major metabolite in the normal rat after oral folic acid. However, table 4 shows that the major metabolite in the urine of tumour bearing rats (group 3) is 10-formyltetra-hydrofolate. The significance of this observation is not clear. On the other hand, urine of normal rats treated with methotrexate contained comparatively higher amounts of 10-formyltetrahydrofolate. In fact it was the only major folate beside unchanged folic acid in the urine of metho-trexate treated animals. Comparable amounts of 10-formyltetra-hydrofolate were found in the urine of group 4 rats.

CHAPTER 10

GENERAL CONCLUSIONS

The discussion in this chapter follows the order of the experimental work described in previous chapters.

In chapter 3 the effects of increased cellular proliferation accompanying faster normal growth in young weanling rats on 2-^{14}C folic acid metabolism were evaluated and compared with mature rats in a similar study. Tables 1, 2 and 3 in this chapter show the results of study of two groups in this thesis. The recoveries in urine, faeces and liver in the two groups (table 1) show no significant difference. Chromatographic analysis of pooled urine samples from each group of rats show that the ratio of 5-methyltetrahydrofolate to 10-formyltetrahydrofolate is higher in the urine of mature rats (tables 2 and 3). Since 4a-hydroxy-5-methyltetrahydrofolate may be an analytical artefact formed by oxidation of 5-methyltetrahydrofolate, it is summed with 5-methyltetrahydrofolate in these tables (209). Moreover, relatively more folic acid is excreted in the urine of mature rats. Since the amount excreted in urine and faeces is the same for both groups these changes probably reflect changes in cellular metabolism. The effect of abnormal faster proliferation on folate metabolism when a tumour is present in the animal and effect of administration of a drug e.g. methotrexate is discussed later in this chapter.

Chapter 4 details the results of effects of an oral dose of 10 mg/kg methotrexate when administered 24 hours before 2-^{14}C folic acid on the metabolism of the latter. Similarly chapter 5 deals with effects of 10 mg/kg and 100 mg/kg methotrexate on the metabolism of 3'-5'-9-^{3}H-2^{14}C-

folic acid. The recovery of radioactivity in urine, liver and gut of rats treated as mentioned above is compared with control rats in tables 4 and 5. Significant differences in the amounts of recovered radioactivity are observed in 0-6 hours urine and liver of control and methotrexate treated animals. Tables 4 and 5 show that methotrexate treated animals excrete very high amounts of radioactivity in the first 6 hours after folic acid administration and their livers contain significantly less radioactivity. Tables 6 and 7 detail the quantities of some of the identified urinary metabolites and the ratio of major co-enzymes in the urine. These tables indicate that excess of radioactivity in 0-6 hours urine samples of methotrexate treated rats is mainly due to excretion of higher amounts of unmetabolised folic acid in these samples. The ratio of 5-methyltetrahydrofolate to 10-formyltetrahydrofolate in the urine of methotrexate treated animals is very low as compared to controls e.g. the amount of 10-formyltetrahydrofolate in the urine of methotrexate treated animals is comparatively higher. The results of these findings are summerized below.

(i) The amount of 2-^{14}C folic acid or 3'-5'-9 ^{3}H folic acid radioactivity in the liver after 24 hours is significantly reduced by methotrexate (tables 4 and 5)

(ii) Methotrexate treated animals excrete significantly higher amount of radioactivity in the 0-6 hours urine mainly due to unmetabolised folic acid (tables 4,5 and 6).

(iii) No significant difference is observed in the radioactivity of 6-24 hours urine samples of methotrexate treated and control animals (tables 4 and 5).
(iv) Low and high dose of methotrexate do not prevent the formation of 10-formyltetrahydrofolate while 5-methyltetrahydrofolate levels are significantly low e.g. the ratio of 5-methyltetrahydrofolate to 10-formyltetrahydrofolate is low (tables 6 and 7).

In addition small amounts of polyglutamates were found in the liver of methotrexate treated animals. Dihydrofolate or its anticipated breakdown products were not found in the urine after methotrexate administration. Thus inhibition of dihydrofolate reductase with concurrent build up of dihydrofolate is not found at these concentrations of methotrexate.

Chapter 7 details the effects of methotrexate on 2-^{14}C 5-methyltetrahydrofolate metabolism. 2-^{14}C 5-Methyltetrahydrofolate was isolated from the urine of rats dosed with 2-^{14}C folic acid. Being a reduced folate it should by-pass the effect of methotrexate as inhibitor of dihydrofolate reductase. If methotrexate inhibits or reduces the retention of folates in liver by virtue of its inhibition effects on dihydrofolate reductase only, then a compound which is a product of the reaction rather than a substrate would enter the folate pools at the same rate as in untreated animals. Table 8 shows that retention of radioactivity in the liver of rats orally dosed with 7 microgram/kg 2-^{14}C 5-methyltetrahydrofolate 24 hours after 100 mg/kg

oral methotrexate is significantly reduced as compared to controls. Moreover retention of radioactivity in the gut of methotrexate treated animals is also significantly reduced.

These observations cannot be explained by the hypothesis that methotrexate completely inhibits dihydrofolate reductase. Goldman et.al 1978 (210) suggest that methotrexate is a very rapidly reversible inhibitor of dihydrofolate reductase and hence much greater levels of free intracellular methotrexate are required to abolish synthesis of reduced folates within cells, and that only very small amounts (< 5% of total enzyme present) of dihydrofolate reductase are enough for the synthesis of reduced folates. The conversion of dihydrofolate to tetrahydrofolate has been shown to be extremely fast at very low levels of dihydrofolate reductase. These observations support the results of experiments in this thesis suggesting that oral methotrexate 100 mg/kg or less does not inhibit the formation of reduced folates.

Again the observations of the metabolism of 5-^{14}C methyltetrahydrofolate in presence and absence of methotrexate (chapter 6), show that methotrexate does not effect the transfer of the methyl group e.g. creatine and the general pattern of metabolites in the urine of two groups of animals is identical. Table 9 shows that radioactivity recovered in faeces and liver of the two groups varies significantly. The methotrexate treated animals excrete larger amounts of dosed 5-^{14}C methyltetrahydrofolate radioactivity in the faeces as compared to controls and retain

less in the liver.

From these data it seems most likely that the retention of reduced folate in liver in the presence of methotrexate is not due to its effect on dihydrofolate reductase. The effects of methotrexate 24 hours after oral administration would appear to be adequately explained by reduction in the rate of transport of folic acid and reduced folates into the cell. It has been observed (238) that methotrexate 10 mg/kg given orally to the rat 24 hours before, markedly reduces the rate of folic acid transport across the rat intestine and that this reduction is correlated with a less acid surface pH on the intestinal surface. A similar explanation could be valid for the reduced retention in liver cells. By analogy with intestinal absorption methotrexate should reduce the reabsorption of folic acid by the kidney tubules and this may explain part of the increased urinary radioactivity. That the effect of methotrexate 24 hours after dosing may be due to reduced folate transport into the cell could explain why the toxic effects of methotrexate can be simply reversed by increasingly large doses of 5-methyltetrahydrofolate (211).

Table 10 shows that an oral dose of 25 mg/kg 6-mercaptopurine given 24 hours before an oral dose of folic acid mixture does not significantly effect the uptake and retention of folic acid both in liver and gut. This observation is in contrast to what is observed in case of methotrexate administration. But no significant difference is observed in the radioactivity of 0-6 hours urine of methotrexate treated

animals and 6-mercaptopurine treated animals. Thus both drugs increase the excretion of folate radioactivity in the first 6 hours of folate administration.

Tables 11 and 12 show the analysis of 0-6 and 6-24 hours urine samples of 6-mercaptopurine treated rats (group A), control rats (group B), methotrexate treated rats (group C) and Walker 256 carcinosarcoma bearing rats treated with methotrexate (group D). The highest levels of 10-formyl-tetrahydrofolate are found in the urines of rats treated with 6-mercaptopurine. The normal rats treated with methotrexate show only 10-formyltetrahydrofolate and no 5-methyltetrahydrofolate in the urine. The ratio of 5-methyltetrahydrofolate to 10-formyltetrahydrofolate is least in methotrexate treated and 6-mercaptopurine treated rats and highest in controls in 0-6 hours urine samples. This ratio increases in 6-24 hours urine samples of 6-mercaptopurine treated animals significantly. Thus looking at the tables 10, 11 and 12 the effect of 6-mercaptopurine on folate metabolism can be summarized as follows:

(i) The uptake and retention of folate in the liver and gut is not reduced by 6-mercaptopurine (table 10).

(ii) Increase in the excretion of radioactivity in 0-6 hours urine samples is observed.

(iii) Analysis of urine shows that levels of 10-formyltetrahydrofolate in the urine are increased (table 11).

(iv) Significant increase in the levels of 5-methyltetrahydrofolate are observed in 6-24 hours urine samples as compared to 0-6 hours urine samples (table 12).

These observations can be explained as follows.

It has been documented that inosinic acid or adenylic acid acts as the feedback regulator of the enzymic synthesis of amino-imidazole carboxamide ribotide, which serves as an enzyme regulator (212) in normal cell conditions. Similarly when 6-mercaptopurine is dosed, it is converted to 6-methyl-mercaptopurine ribotide which acts as a false feedback regulator, tightly binding the amino-imidazole carboxamide ribotide synthesizing enzymes, preventing its formation. Therefore, high levels of 10-formyltetrahydrofolate in the urine of 6-mercaptopurine treated animals are observed. This effect is greater in the 0-6 hours urine samples.

So far it is clear that like methotrexate 6-mercapto-purine also shows increased levels of 10-formyltetrahydro-folate in the urine. It may be noted that methotrexate may inhibit purine synthesis by a mechanism different from 6-mercaptopurine.

The final experiments demonstrate the effects of 100 mg/kg oral methotrexate on the folate metabolism in the rats bearing Walker 256 carcinosarcoma. The major effects derived from tables 11, 12, 13 and 14 are summarized below:
(i) Transport of folate from the gut in the tumour bearing rats is significantly increased which is reflected by less radioactivity in the faeces (tables 13 and 14).
(ii) Significant increase in the radioactivity of 0-6 hours urine is observed in methotrexate treated animals, both in normal and tumour bearing rats.

In addition to above mentioned observations the uptake of folate radioactivity in the tumour of methotrexate

treated animals was found to be considerably reduced which was reflected by very low amount of radioactivity recovered in the tumour (table 15) as compared to the amount of radioactivity in the tumour of rats not treated with methotrexate (145). Moreover the retention and uptake of folate radioactivity in the liver of the methotrexate treated tumour bearing animals is also significantly reduced (table 13).

The chromatography of pooled urine samples of normal rats treated with methotrexate and similarly treated tumour bearing rats show that there is no significant difference in the 10-formyltetrahydrofolate levels in 0-6 hours urine samples (table 11), while significant increase of 10-formyltetrahydrofolate is observed in 6-24 hours urine samples of tumour bearing rats (table 12). Moreover tumour bearing rats show some 5-methyltetrahydrofolate in 0-6 hours urine samples while normal rats treated with methotrexate do not show any 5-methyltetrahydrofolate.

It may be noted that increase in the levels of 10-formyltetrahydrofolate may be due to two completely different reasons.

(i) Faster proliferation and its effect on folate metabolism. This was demonstrated in chapter 3 of this thesis. cf. p 74

(ii) Effects of methotrexate and 6-mercaptopurine on the utilization of 10-formyltetrahydrofolate.

Thus in methotrexate and 6-mercaptopurine treated animals the effect on the utilization of 10-formyltetrahydrofolate is observed, while in tumour bearing rats the first effect is observed.

Group of Rats		Urine 0-6 hours	Urine 6-24 hours	Liver	Faeces
Young rats	A (15)	8.7±1.3	5.4±0.7	11.9±2.3	37.1±2.3
Mature rats	B (8)	8.6±1.4 NS	8.6±3.3 NS	13.1±2.1 NS	36.3±2.7 NS
Mature rats	C (8)	6.3±2.0 NS	4.8±1.4 NS	15.3±1.2 NS	44.8±3.6 NS

Table 1 Radioactivity recovered in urine, liver and faeces of young and mature rats, expressed as a percentage of an oral dose of 2-^{14}C folic acid. Amounts are given as mean ± SEM. Number of rats in each group is given in parentheses.

Group A Three week old male Wistar rats orally dosed with 76 microgram/kg 2-^{14}C folic acid and killed 48 hours after this dose.

Group B Adult male Wistar rats orally dosed with 76 microgram/kg 2-^{14}C folic acid and killed 24 hours after this dose.

Group C Adult male Wistar rats orally dosed with 78 microgram/kg 2-^{14}C folic acid and killed 48 hours after this dose. Barford and Blair (145).

Student's t test applied as follows:

Group A vs Group B

Group A vs Group C

Group of rats	Number of rats	Folic acid	5-Methyltetra-hydrofolate	10-Formyltetra-hydrofolate	5-Methyltetrahydrofolate / 10-Formyltetrahydrofolate Ratio
Young rats	15	33.6	30.0	25.4	1.2
Mature rats	8	42.6	34.1	15.5	2.2

Table 2 Analysis of 0-6 hours urine samples of young and mature rats. All the rats received an oral dose of carbon-14 labelled folic acid (76 microgram/kg each animal). The distribution of radioactivity in the metabolites is expressed as a percentage of total radioactivity of pooled urine sample (10 mls) applied to DE52 ion-exchange column. The amounts under 5-methyltetrahydrofolate give the total of 5-methyltetrahydrofolate and the oxidation product 4a-hydroxy-5-methyltetrahydrofolate.

Group of rats	Number of rats	Folic acid	5-Methyltetrahydrofolate	10-Formyltetrahydrofolate	5-Methyltetrahydrofolate / 10-Formyltetrahydrofolate Ratio
Young rats	15	8.5	30.0	11.5	2.6
Mature rats	8	12.7	64.8	16.1	4.0

Table 3 Analysis of 6-24 hours urine samples of young and mature rats. All the rats received an oral dose of carbon-14 labelled folic acid (76 microgram/kg each animal). The distribution of radioactivity in the metabolites is expressed as a percentage of total radioactivity of pooled urine sample (10 mls) applied to DE52 ion-exchange column. The amounts under 5-methyltetrahydrofolate give the total of 5-methyltetrahydrofolate and the oxidation product 4a-hydroxy-5-methyltetrahydrofolate.

Group of rats	Number of rats	Urine		Liver	Gut
		0-6 hours	6-24 hours		
A	8	8.6 ± 1.4	8.6 ± 3.3	13.1 ± 2.1	4.8 ± 0.6
B	12	13.5 + 2.4	8.2 + 1.7	5.3 + 0.6	2.8 + 0.8
		NS	NS	$p < 0.02$	$p < 0.05$
C	6	25.4 ± 2.4	6.6 ± 1.8	3.8 ± 0.4	3.0 ± 0.6
		$p < 0.001$	NS	$p < 0.001$	$p < 0.1$
D	4	17.4 ± 2.1	11.0 ± 1.6	3.2 ± 0.3	2.3 ± 0.3
		$p < 0.01$	NS	$p < 0.01$	$p < 0.05$

Table 4 Radioactivity recovered in urine, liver and gut of control and methotrexate treated rats. The distribution is expressed as a percentage of dosed carbon-14 folic acid (76 microgram/kg) radioactivity (mean ± SEM).

Group A Control rats received carbon-14 folic acid only and were killed 24 hours after folic acid dosing.

Group B Received carbon-14 folic acid 24 hours after an oral dose of 10 mg/kg methotrexate and were killed 24 hours after folic acid dosing.

Group C Treated same as group B but killed 48 hours after folic acid dosing.

Group D Received a mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (8 microgram/kg) after an oral dose of 10 mg/kg methotrexate and were killed 24 hours after folic acid dosing.

Student's t test is applied as: Group A vs Groups B, C and D. NS= not significant.

Group of rats	Number of rats	Urine				Liver		Gut	
		0-6 hours		6-24 hours					
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
A	4	7.9±1.6	8.4±1.9	12.4±2.1	14.2±2.8	11.0±0.8	13.9±0.5	6.1±0.9	3.0±0.9
B	4	17.4±2.1	20.7±3.6	11.0±1.6	14.4±1.6	3.2±0.3	6.4±1.0	2.3±0.3	2.7±0.6
		$p < 0.02$	$p < 0.05$	NS	NS	$p < 0.001$	$p < 0.001$	$p < 0.01$	NS
C	6	21.2±2.8	21.8±2.2	11.7±2.1	13.2±3.5	2.7±0.4	4.4±0.9	6.6±1.3	6.8±1.7
		$p < 0.01$	$p < 0.01$	NS	NS	$p < 0.001$	$p < 0.001$	NS	NS

Table 5 Radioactivity recovered in urine, liver and gut of control and methotrexate treated rats. The distribution is expressed as a percentage of carbon-14 and tritium radioactivity dosed (mean ± SEM). Rats were killed 24 hours after folic acid dosing.

Group A Control rats received a mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (9 microgram/kg) only.

Group B Received same dose of carbon-14 and tritium labelled folic acid as in group A 24 hours after an oral dose of 10 mg/kg methotrexate.

Group C Received same dose of carbon-14 and tritium labelled folic acid as in group A 24 hours after an oral dose of 100 mg/kg methotrexate.

Student's t test applied as follows: Group A vs Group B; Group A vs Group C.

NS = not significant.

Group of rats	Number of rats	Folic acid	5-Methyltetra-hydrofolate	10-Formyltetra-hydrofolate	5-Methyltetrahydrofolate / 10-Formyltetrahydrofolate Ratio
A	4	71.1	21.8	7.7	2.83
B	4	83.6	2.5	15.3	0.16
C	6	90.1	-	5.3	0.0

Table 6 Analysis of 0-6 hours urine of control and methotrexate treated rats. The distribution of radioactivity in the metabolites is expressed as a percentage of total radioactivity (carbon-14 only) of pooled urine sample (10 mls) applied to DE52 ion-exchange column. The amounts under 5-methyltetrahydrofolate give the total of 5-methyltetrahydrofolate and the oxidation product 4a-hydroxy-5-methyltetrahydrofolate if present.

Group A Control rats received only a mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (9 microgram/kg) orally.

Group B Received an oral dose of 10 mg/kg methotrexate 24 hours before an oral dose of folic acid mixture.

Group C Received an oral dose of 100 mg/kg methotrexate 24 hours before an oral dose of folic acid mixture.

Group of rats	Number of rats	Folic acid	5-Methyltetra-hydrofolate	10-Formyltetra-hydrofolate	5-Methyltetrahydrofolate / 10-Formyltetrahydrofolate Ratio
A	4	45.6	37.2	13.2	2.81
B	4	45.7	3.0	18.2	0.16
C	6	84.2	5.0	6.5	0.76

Table 7 Analysis of 6-24 hours urine of control and methotrexate treated rats. The distribution of radioactivity in the metabolites is expressed as a percentage of total radioactivity (carbon-14 only) of pooled urine sample (10 mls) applied to DE52 ion-exchange column. The amounts under 5-methyltetrahydrofolate give the total of 5-methyltetrahydrofolate and the oxidation product 4a-hydroxy-5-methyltetrahydrofolate if present.

Group A Control rats received a mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (9 microgram/kg) orally.

Group B Received an oral dose of 10 mg/kg methotrexate 24 hours before an oral dose of folic acid mixture.

Group C Received an oral dose of 100 mg/kg methotrexate 24 hours before an oral dose of folic acid mixture.

Group of rats	Number of rats	Urine	Gut	Liver	Faeces
		0-24 hours			
A	4	17.4 ± 1.3	5.6 ± 1.4	13.5 ± 1.0	34.4 ± 3.1
B	6	19.8 + 0.4	3.5 + 0.5	5.5 + 1.0	38.4 + 1.3
		NS	$p < 0.05$	$p < 0.05$	NS

Table 8 Radioactivity recovered in urine, gut, liver and faeces of control and methotrexate treated rats. The distribution is expressed as a percentage of carbon-14 radioactivity dosed (mean ± SEM).

Group A Received 2-^{14}C 5-methyltetrahydrofolate (7 microgram/kg) orally.

Group B Received 2-^{14}C 5-methyltetrahydrofolate (7 microgram/kg) orally 24 hours after an oral dose of 100 mg/kg methotrexate.

Student's t test applied as: Group A vs Group B.

NS = not significant.

Group of rats	Number of rats	Urine		Liver	Faeces
		0-6 hours	6-24 hours		
A	4	13.7 ± 4.7	17.4 ± 1.1	9.7 ± 1.8	23.9 ± 1.8
B	4	10.5 ± 3.4	10.9 ± 2.9	4.2 ± 0.4	33.0 ± 1.0
		NS	NS	$p < 0.05$	$p < 0.02$

Table 9 Radioactivity recovered in urine, liver and faeces of control and methotrexate treated rats. The distribution is expressed as a percentage of carbon-14 radioactivity dosed (mean ± SEM).

Group A Received 5-^{14}C methyltetrahydrofolate (70 microgram /kg) orally.

Group B Received 5-^{14}C methyltetrahydrofolate (70 microgram /kg) orally 24 hours after an oral dose of 100 mg/kg methotrexate.

Student's t test applied as follows: Group A vs Group B.

NS = not significant.

Group of rats	Number of rats	Urine				Gut		Liver	
		0-6 hours		6-24 hours					
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
A	4	7.9±1.6	8.4±1.9	12.4±2.1	14.2±2.8	6.1±0.9	3.0±0.9	11.0±0.8	13.9±0.5
B	5	16.6±3.5	20.2±4.0	15.5±2.5	19.7±3.5	6.2±1.4	5.9±0.9	9.5±2.1	11.8±2.5
		NS	$p < 0.05$	NS	NS	NS	NS	NS	NS
C	6	21.2±2.8	21.8±2.2	11.7±2.1	13.2±3.5	6.6±1.3	6.8±1.7	2.7±0.4	4.4±0.9
B		16.6±3.5	20.2±4.0	15.5±2.5	19.7±3.5	6.2±1.4	5.9±0.9	9.5±2.1	11.8±2.5
		NS	NS	NS	NS	NS	NS	$p < 0.01$	$p < 0.02$

Table 10 Radioactivity recovered in urine, liver and gut of control, methotrexate treated and 6-mercaptopurine treated rats. The distribution is expressed as a percentage of carbon-14 and tritium radioactivity dosed (mean ± SEM).

Group A Control rats received a mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (8.8 microgram/kg) only and killed 24 hours after folic acid dosing.

Group B Received same dose of carbon-14 and tritium labelled folic acid as in group A 24 hours after an oral dose of 25 mg/kg 6-mercaptopurine.

Group C Received same dose of carbon-14 and tritium labelled folic acid as in group A 24 hours after an oral dose of 100 mg/kg methotrexate.

Student's t test applied as: Group A vs Group B; Group C vs Group B. NS = not significant.

Group of rats	Number of rats	Folic acid		5-Methyltetrahydrofolate		10-Formyltetrahydrofolate		5-Methyltetrahydrofolate / 10-Formyltetrahydrofolate Ratio
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
A	5	62.3	49.1	12.3	16.0	16.6	19.0	0.74
B	4	71.1	51.7	21.8	22.9	7.7	13.0	2.83
C	6	90.1	77.4	-	-	5.3	6.9	0.0
D	5	81.5	79.8	1.9	1.9	6.5	6.0	0.29

Table 11 Analysis of 0-6 hours urine samples of rats treated with 6-mercaptopurine, rats treated with methotrexate, control rats and tumour bearing rats treated with methotrexate. The distribution of radioactivity in metabolites is expressed as a percentage of carbon-14 and tritium radioactivity of pooled urine samples (10 mls) applied to DE52 ion-exchange column. The amounts under 5-methyltetrahydrofolate give the total of 5-methyltetrahydrofolate and the oxidation product 4a-hydroxy-5-methyltetrahydrofolate if present.

Group A Received a mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (8 microgram/kg) 24 hours after an oral dose of 25 mg/kg 6-mercaptopurine.

Group B Control rats receiving same dose of folic acid as in group A, without prior drug treatment.

Group C Received same dose of folic acid as in group A 24 hours after an oral dose of 100 mg/kg methotrexate.

Group D Walker 256 carcinosarcoma bearing rats treated as rats in group C

Note: Rats in groups A, B and C were normal rats.

Group of rats	Number of rats	Folic acid		5-Methyltetra-hydrofolate		10-Formyltetra-hydrofolate		5-Methyltetrahydrofolate / 10-Formyltetrahydrofolate Ratio	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H		^{14}C
A	5	38.9	32.3	31.1	27.6	18.7	15.3		1.66
B	4	45.6	30.5	37.2	27.9	13.2	13.2		2.81
C	6	84.2	58.5	5.0	6.1	6.5	7.5		0.66
D	5	76.9	75.3	4.2	4.2	12.6	10.5		0.33

Table 12 Analysis of 6-24 hours urine samples of rats treated with 6-mercaptopurine, rats treated with methotrexate, control rats and tumour bearing rats treated with methotrexate. The distribution of radioactivity in metabolites is expressed as a percentage of carbon-14 and tritium radioactivity of pooled urine samples (10 mls) applied to DE52 ion-exchange column. The amounts under 5-methyltetrahydrofolate give the total of 5-methyltetrahydrofolate and the oxidation product 4a-hydroxy-5-methyltetrahydrofolate if present.

Group A Received a mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (8.8 microgram/kg) 24 hours after an oral dose of 25 mg/kg 6-mercaptopurine.

Group B Control rats receiving same dose of folic acid as in group A, without prior drug treatment.

Group C Received same dose of folic acid as in group A 24 hours after an oral dose of 100 mg/kg methotrexate.

Group D Walker 256 carcinosarcoma bearing rats treated as rats in group C.

Note: Rats in groups A, B and C were normal rats.

Group of rats	Number of rats	Urine 0-6 hours ^{14}C	Urine 0-6 hours ^{3}H	Urine 6-24 hours ^{14}C	Urine 6-24 hours ^{3}H	Gut ^{14}C	Gut ^{3}H	Liver ^{14}C	Liver ^{3}H	Faeces ^{14}C	Faeces ^{3}H
A	6	21.2±2.8	21.8±2.2	11.7±2.1	13.2±3.5	6.6±1.3	6.8±1.7	2.7±0.4	4.4±0.9	48.4±2.7	51.9±1.9
B	5	20.8±2.9	23.0±2.9	8.9±0.9	11.9±0.6	3.9±0.8	3.9±1.0	3.7±1.6	8.3±0.1	23.3±2.4	29.1±3.4
		NS	NS	NS	NS	NS	NS	NS	$p < 0.05$	$p < 0.001$	$p < 0.01$
C	4	7.9±1.5	8.4±1.9	12.4±1.1	14.2±2.8	6.1±0.9	3.0±0.9	11.0±0.8	13.9±0.5	33.8±1.8	38.8±6.6
B	5	20.8±2.9	23.0±2.9	8.9±0.9	11.9±0.6	3.9±0.8	3.9±1.0	3.7±1.6	8.3±0.1	23.3±2.4	29.1±3.4
		$p < 0.01$	$p < 0.01$	NS	NS	NS	NS	$p < 0.01$	$p < 0.01$	$p < 0.05$	NS

Table 13 Radioactivity recovered in urine, gut, liver and faeces of tumour (Walker 256 carcinosarcoma) bearing rats treated with 100 mg/kg methotrexate orally 24 hours before an oral dose of mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (8.2 microgram/kg), compared with similarly treated normal rats and normal rats receiving similar dose of folic acid mixture only. The distribution is expressed as a percentage of carbon-14 and tritium radioactivity dosed (mean ± SEM).

Group A Normal rats received 100 mg/kg methotrexate orally 24 hours before folic acid dosing.

Group B Walker 256 carcinosarcoma bearing rats treated as rats of group A.

Group C Normal rats received a mixture of carbon-14 and tritium labelled folic acid only without prior treatment with methotrexate.

Student's t test applied as follows: Group B vs Group A; Group B vs Group C. NS= not significant.

Group of rats	Number of rats	Urine		Faeces	Liver
		0-6 hours	6-24 hours		
A	13	6.5 ± 1.1	11.3 ± 1.6	37.8 ± 2.7	14.8 ± 0.7
B	5	20.8 ± 2.9	8.9 ± 0.9	23.3 ± 2.4	3.7 ± 1.6
		$p < 0.001$	NS	$p < 0.01$	$p < 0.001$

Table 14 Radioactivity recovered in urine, faeces and liver of Walker 256 carcinosarcoma bearing rats. The distribution is expressed as a percentage of carbon-14 radioactivity dosed (mean ± SEM).

Group A Walker 256 carcinosarcoma bearing rats received only carbon-14 labelled folic acid (78 microgram/kg) orally.

Group B Walker 256 carcinosarcoma bearing rats received a mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (8.2 microgram/kg) orally 24 hours after an oral dose of 100 mg/kg methotrexate.

Group of rats	Number of rats	Weight of tumour (grams)	Amount of radioactivity recovered from the tumour ^{14}C
A	5	10	0.1
B	13	10	4.0

Table 15 Radioactivity recovered from Walker 256 carcinosarcoma of rats orally dosed with carbon-14 folic acid or mixture of carbon-14 folic acid and tritium labelled folic acid. The amounts are expressed as a percentage of carbon-14 folic acid radioactivity dosed.

Group A Received mixture of carbon-14 folic acid (76 microgram/kg) and tritium labelled folic acid (8.2 microgram/kg) orally, 24 hours after 100 mg/kg oral methotrexate. The rats were killed 24 hours after folic acid administration.

Group B Received carbon-14 folic acid (78 microgram/kg) orally without prior drug treatment and were killed 48 hours after folic acid administration.

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