BIOSYNTHESIS OF FOLATE POLYGLUTAMATES IN THE RAT

A thesis

submitted for the degree of

DOCTOR OF PHILOSOPHY

by

JOHN BATES

in the Department of Chemistry

UNIVERSITY OF ASTON IN BIRMINGHAM

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SUMMARY

The biosynthesis of folate polyglutamates and the disposition of the folate monoglutamate pool have been investigated in the tissues of normal and tumour-bearing rats following the oral administration of radiolabelled folic acid.

The principal folate monoglutamates detected were identified as 5-methyltetrahydrofolate ($5CH_3THF$) and unmetabolised folic acid (FA). These species were found in the liver and intestine of normal and host rats and in the tumour tissue, showed maximal levels between 2-6 hours after folic acid administration and were not detected at 24 or 48 hours.

A progressive synthesis of folate polyglutamates was demonstrated in all tissues; these derivatives representing $85 \pm 10\%$ (mean $\pm$ SD) (n=20) of the total radiolabelled folate at 24 hours after administration.

DEAE-cellulose chromatography revealed three principal folate polyglutamates; folate polyglutamates A, B and C. Preliminary identification indicated these to be 10-formylfolic acid tetraglutamate ($10CHOFA(glu)_4$), 5-methyltetrahydrofolate triglutamate ($5CH_3THF(glu)_3$) and a tetrahydrofolate polyglutamate ($THF(glu)_n$) respectively.

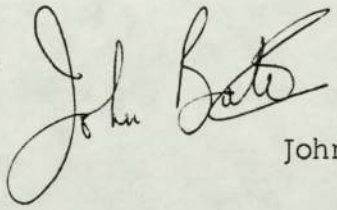
Tumour induced effects on the disposition of the hepatic folate pool were suggested. Between 2-6 hours after folic acid administration, the cumulative levels of $5CH_3THF$ were higher in rats bearing the sarcoma Mc103B or the Walker 256 carcosarcoma, than the corresponding normal rats. The biosynthesis of folate polyglutamates in the host liver was unaffected in rats bearing the sarcoma Mc103B, but reduced in rats bearing the Walker 256 carcosarcoma, compared to the normal rats.

High performance liquid chromatography (HPLC) has been developed for the analysis of folate polyglutamates. Under suitable conditions, folate polyglutamates were rapidly resolved from folate monoglutamates and in most cases, from each other. This procedure offers considerable advantages over more conventional methods of folate analysis. Under conditions of gradient elution, a good linear relationship was observed between the retention time and the number of glutamate residues of three classes of folate polyglutamates. This observation may allow an effective theoretical approach to the identification of these species. HPLC analysis of tissue extracts revealed certain difficulties.

KEY WORDS: folate polyglutamate biosynthesis, $5CH_3THF$, folic acid, tumour tissue.

This work was carried out from October 1978 to September 1981 in the Department of Chemistry in the University of Aston in Birmingham.

It was undertaken independently and has not been submitted for any other degree.



John Bates

ACKNOWIEDGEMENTS

I would like to thank Professor John A. Blair, Dr. Anne E. Pheasant and Dr. Michael J. Connor for their supervision of this work. I would also like to thank Helen Whitby for her encouragement and Linda Perkins for her excellent typing.

To my Mother and Father

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CHAPTER 1

INTRODUCTION

1.1. The biochemistry of folate coenzymes

The folates are an important class of coenzymes essential to a number of one-carbon unit transfer reactions in many areas of metabolism (Rowe, 1978). The active coenzymes are derivatives of folic acid (FA) (Fig. 1.1) but differ in three respects. The pyrazine ring of the pterin moiety (Fig. 1.2a) is reduced to either the 7,8-dihydro (Fig. 1.3) or the 5,6,7,8-tetrahydro (Fig. 1.4) levels. A substituent group may be present at N5 (Fig. 1.5), N10 (Fig. 1.6) or bridged between N5 and N10 (Fig. 1.7). Finally, additional glutamate residues may be added to the terminal glutamate forming the folate polyglutamates (Fig. 1.10).

5-Methyltetrahydrofolate (5-CH_3THF) (Fig. 1.5) and 10-formyltetrahydrofolate (10-CHOTHF) (Fig. 1.6) are the predominant plasma and urinary folates and represent transport forms. Folate polyglutamates form the bulk of intracellular folate and are considered the principal coenzymes. One-carbon unit transfer reactions dependent on the folate coenzymes are involved in the synthesis of purines and pyrimidines and in the metabolism of certain amino acids. The important metabolic pathways and the associated enzymes are given in Fig. 1.13 and Table 1.1. respectively. With the exception of dihydrofolate (DHF) (Fig. 1.3) the normal substrates for the folate-dependent enzymes are the fully reduced tetrahydro derivatives. The structures of the folate coenzymes (Fig.s 1.1-1.10) are presented as the folate monoglutamates. However, it should be emphasised that the coenzymes are predominantly folate polyglutamates in the cell.

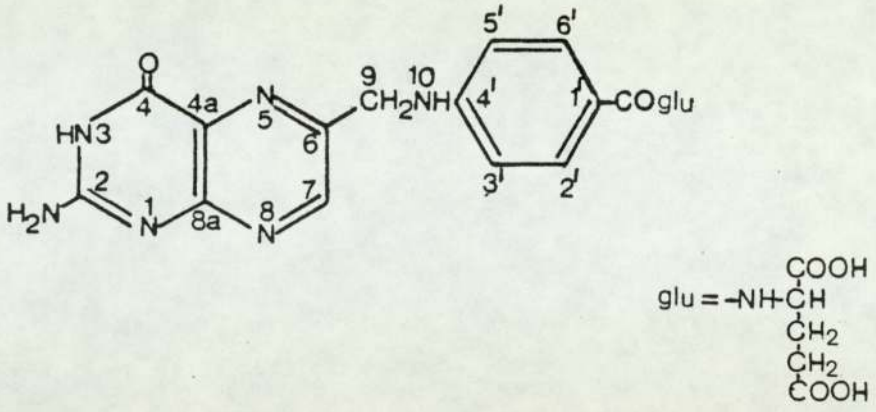


Fig. 1.1. Folic acid (FA)

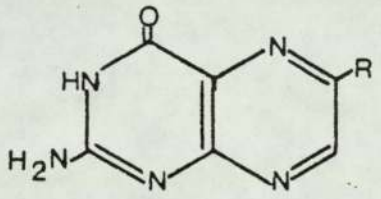


Fig. 1.2. a) R=H, Pterin
b) R=OH, Xanthopterin

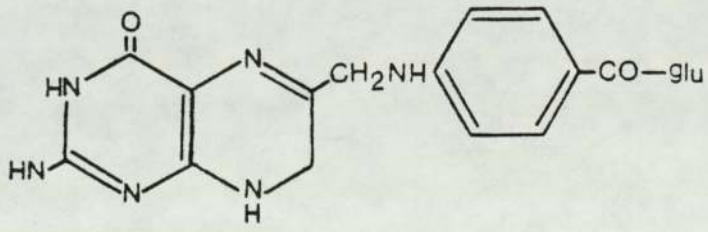


Fig. 1.3. Dihydrofolate (DHF)

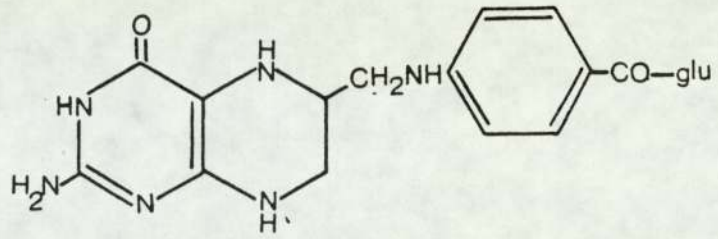


Fig. 1.4. Tetrahydrofolate (THF)

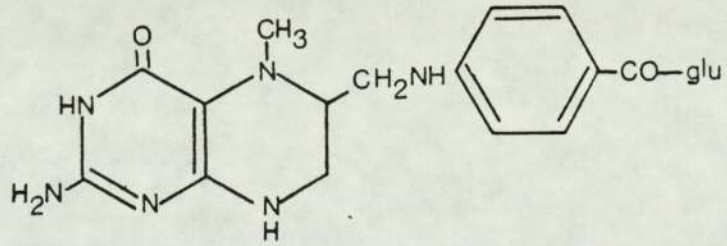


Fig. 1.5. 5-methyltetrahydrofolate (5-CH_3THF)

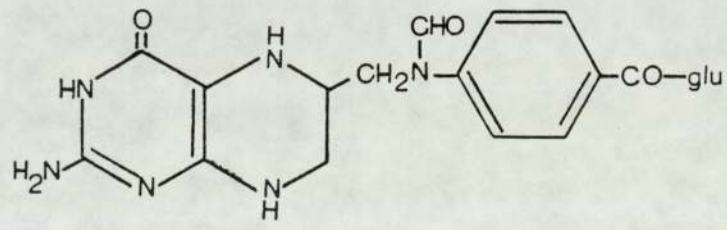


Fig. 1.6. 10-formyltetrahydrofolate (10-CHOTHF)

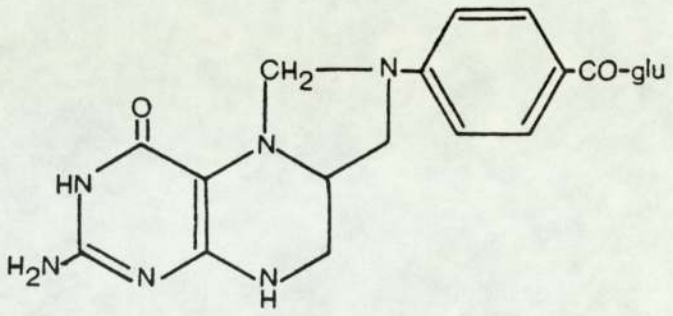


Fig. 1.7. 5,10-methylenetetrahydrofolate (5,10-CH_2THF)

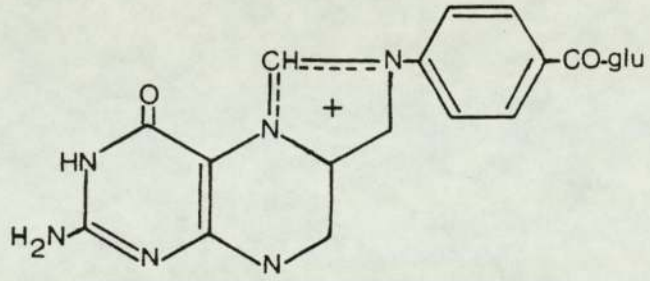


Fig. 1.8. 5,10-methenyltetrahydrofolate (5,10-CH=THF)

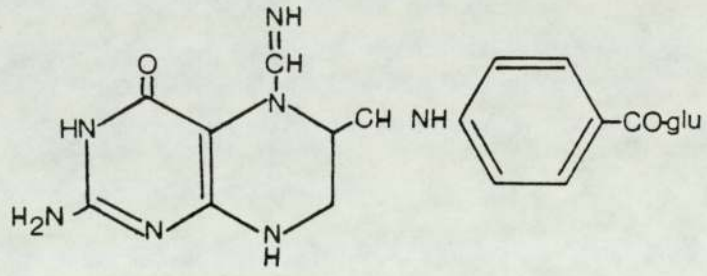


Fig. 1.9. 5-formiminotetrahydrofolate (5-HN=CHTHF)

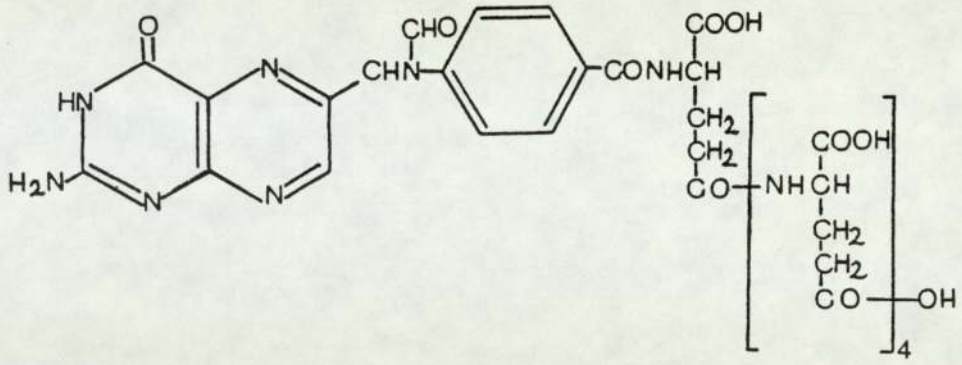


Fig. 1.10. 10-formylfolatetetraglutamate (10CHOFA$(glu)_4$)

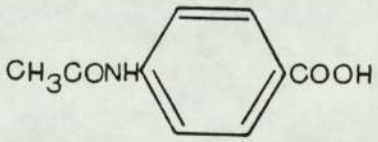


Fig. 1.11. p-acetamidobenzoic acid (p-AcB)

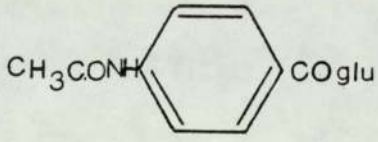


Fig. 1.12 p-acetamidobenzoyl-L-glutamate (p-AcBG)

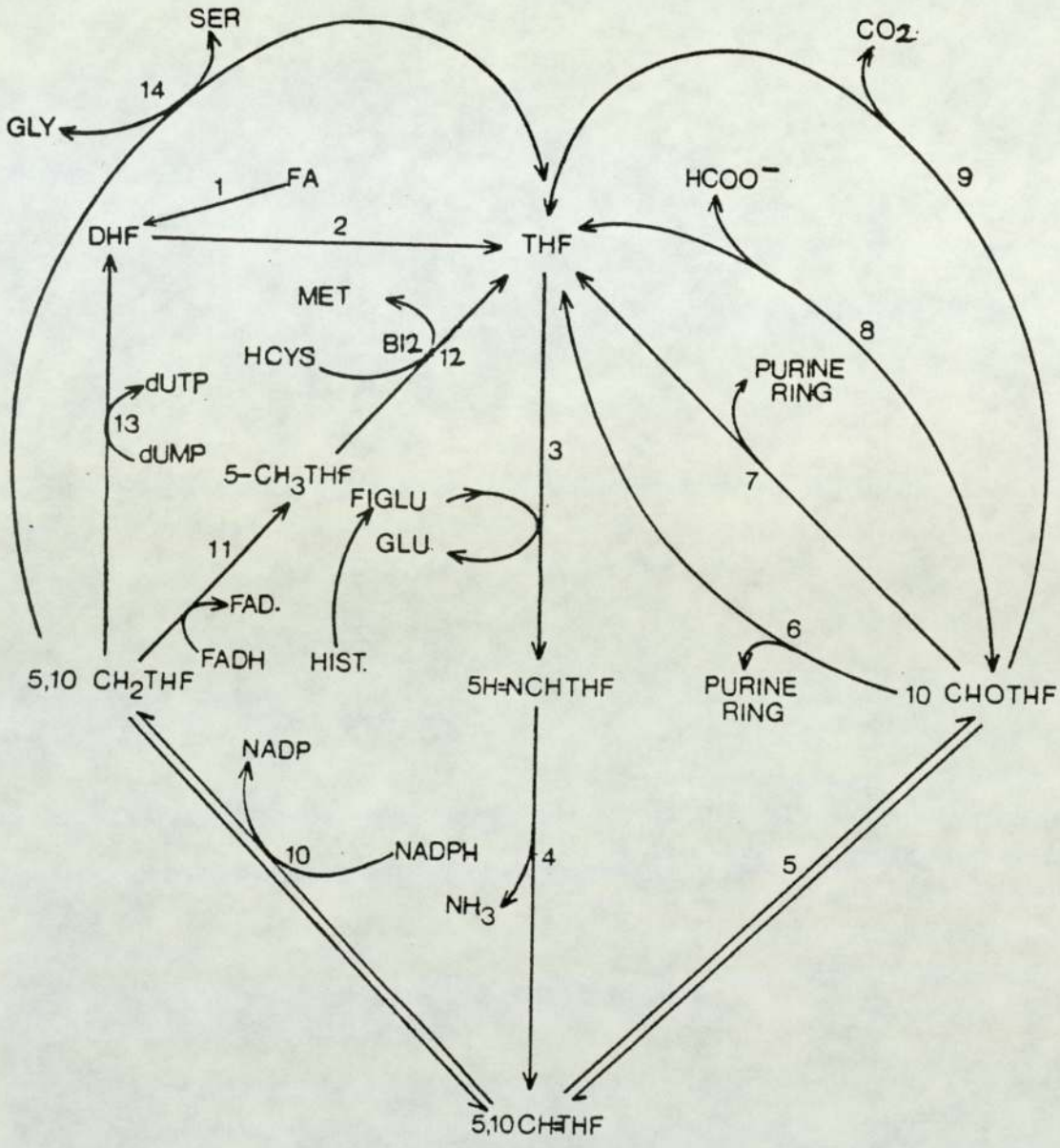


Fig. 1.13. The major metabolic pathways in the folate pool.

Abbreviations. Figlu: Formiminoglutamate. HCys: Homocysteine. glu: Glutamate. Met: Methionine. dUMP: deoxyuridylate. dTMP: deoxythymidylate. The enzymes represented by number 1 - 14 are given in Table 1.1.

	Name of enzyme	Enzyme Commission Class No.
1 & 2	Dihydrofolate reductase	E.C. 1.5.1.3.
3	Formiminotransferase	E.C. 2.1.2.5.
4	Cyclodeaminase	E.C. 4.3.1.4.
5	5,10CH=THF cyclohydrolase	E.C. 3.5.4.9.
6	Phosphoribosylglycinamide formyltransferase	E.C. 2.1.2.2.
7	Phosphoribosylaminoimidazole-carboxamide formyltransferase	E.C.2.1.2.3.
8	10CHOTHF synthetase	E.C. 6.3.4.3.
9	10CHOTHF dehydrogenase	E.C. 1.5.1.6.
10	5,10CH_2THF dehydrogenase	E.C. 1.5.1.5.
11	5,10CH_2THF reductase	E.C. 1.1.1.68.
12	Methionine synthetase	E.C. 2.1.1.13
13	Thymidylate synthetase	E.C. 2.1.1.b.
14	Serine hydroxymethyltransferase	E.C. 2.1.2.1.

Table 1.1. The major folate-dependent enzymes catalysing steps (1-14) represented in Fig. 1.13.

The synthesis of the pyrimidine ring requires 5,10-methylenetetrahydrofolate (5,10CH_2THF) (Fig. 1.7) which functions in the reductive methylation of 2'-deoxyuridylate to form 2'-deoxythymidylate (Fig. 1.14). In the synthesis of the purine ring, both carbon atoms 8 and 2 are derived from 10CHOTHF (Dev and Harvey, 1978). These two reactions are presented in Fig. 1.15.

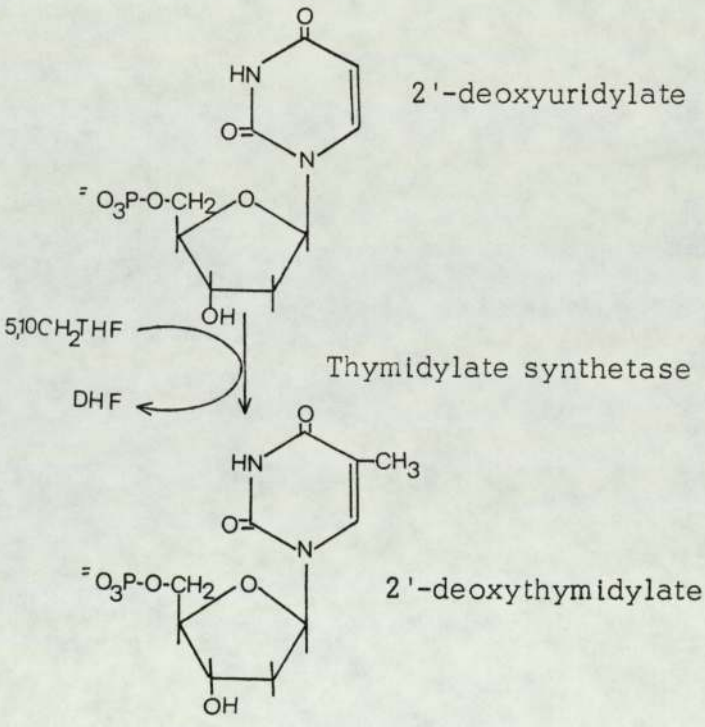


Fig. 1.14. The folate-dependent conversion of 2'-deoxyuridylate to form 2'-deoxythymidylate

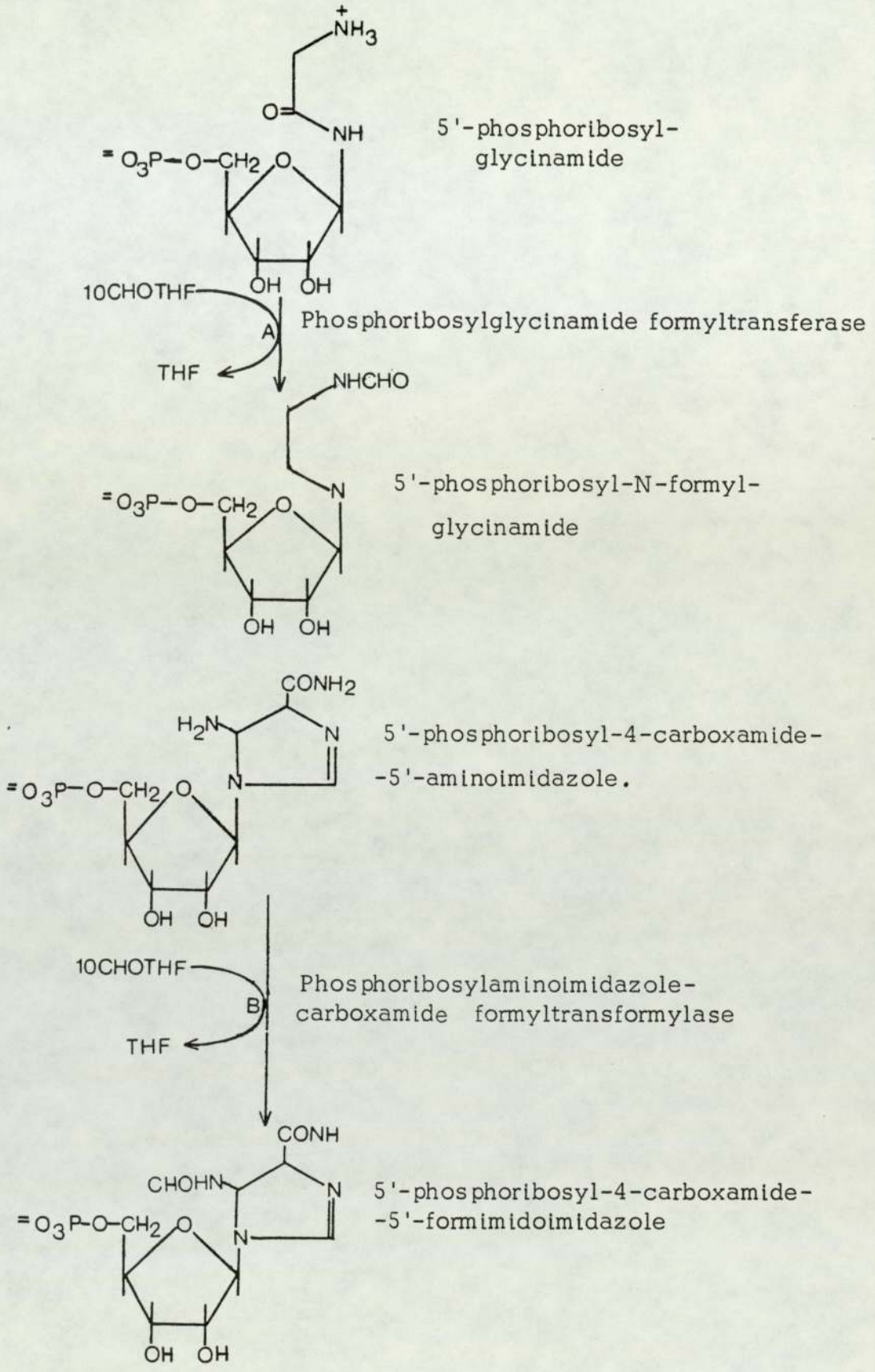


Fig. 1.15 The folate-dependent introduction of carbon atoms 8 (step A) and 2 (step B) into the purine ring.

Several reactions in amino acid metabolism are dependent on folate coenzymes. A folate-dependent equilibrium exists between serine and glycine (Schirch and Diller, 1971) (Fig. 1.16) and is catalysed by serine hydroxymethyltransferase. This reaction is an important point of entry of one-carbon units into the folate pool and proceeds by the conversion of THF to $5,10CH_2THF$.

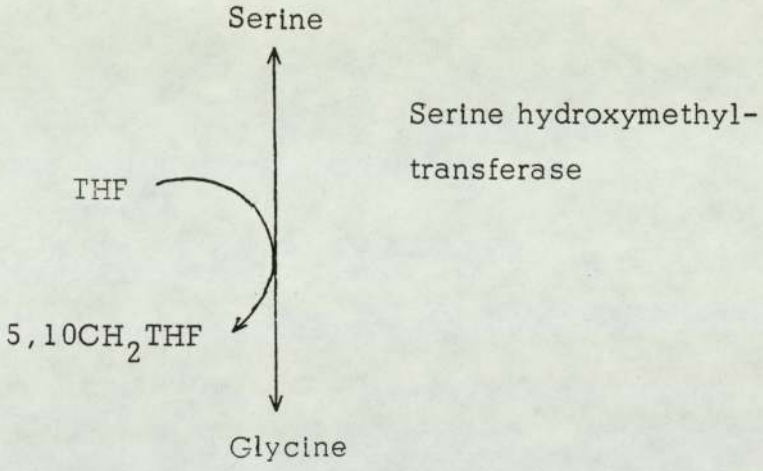


Fig. 1.16 The folate-dependent equilibrium between serine and glycine.

An important route of methionine synthesis is dependent on $5CH_3THF$ and is catalysed by the cobalamin requiring enzyme methionine synthetase (Taylor and Weissbach, 1973). This reaction provides the only route by which folate derived from $5CH_3THF$ can re-enter the folate pool (Fig. 1.17).

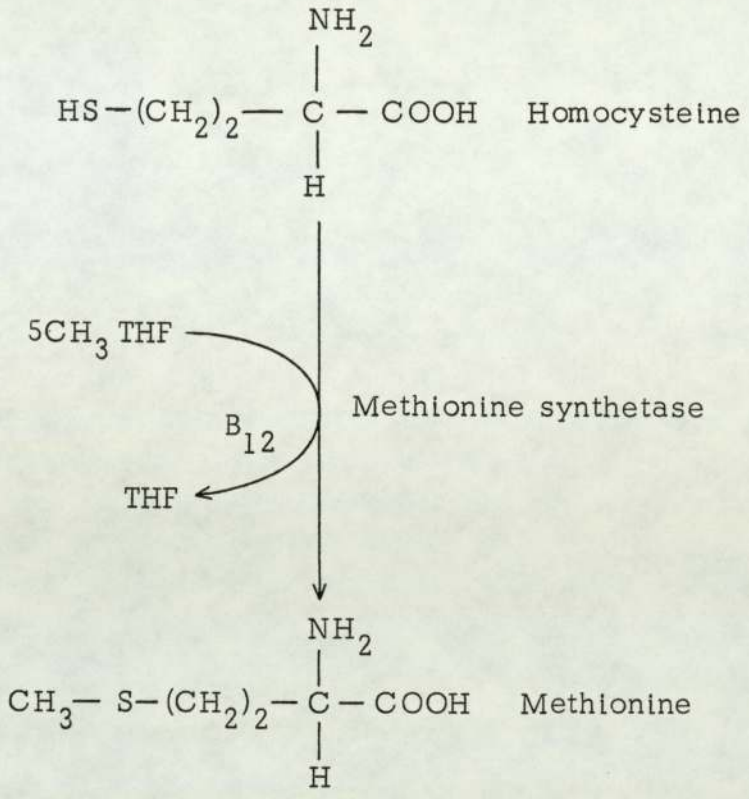


Fig.1.17 The folate-dependent synthesis of methionine from homocysteine.

Deficiency of cobalamin, as is found in pernicious anaemia, results in the trapping of folate as $5CH_3THF$ with the concomitant depletion of the other folate coenzymes. The so called "methyl trap" hypothesis (represented in Fig. 1.18) was invoked to explain folate deficiency in pernicious anaemia and is well supported experimentally (Taylor et al., 1974).

A further involvement of folate coenzymes in amino acid metabolism is found at the level of formiminoglutamic acid (Figlu) (Fig. 1.19). Figlu is derived from histidine metabolism and requires THF for conversion to glutamic acid. This generates 5-formiminotetrahydrofolate (5HN=CH-THF)

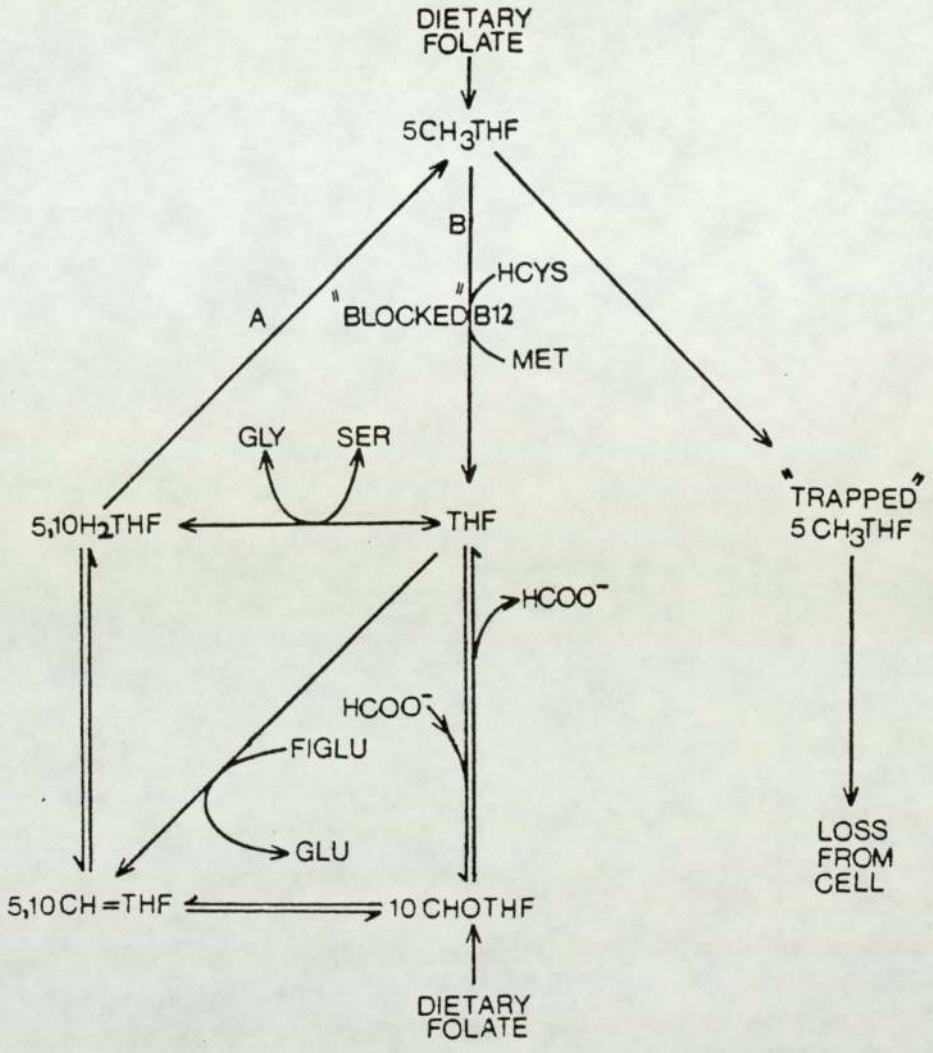


Fig. 1.18. Schematic representation of the "methyl-trap" hypothesis. The conversion of 5,10-CH_2THF to 5CH_3THF (step A) is irreversible in the cell. Cobalamin deficiency results in the "trapping" (step B) and subsequent loss of folate as 5CH_3THF.

Abbreviations. HCys: Homocysteine. Met: Methionine.

Gly: Glycine. Ser: Serine. glu: Glutamate. Figlu: Formiminoglutamate.

(Fig. 1.19) and is also a route of entry of one-carbon units into the folate pool (Tabor and Wyngarden, 1959).

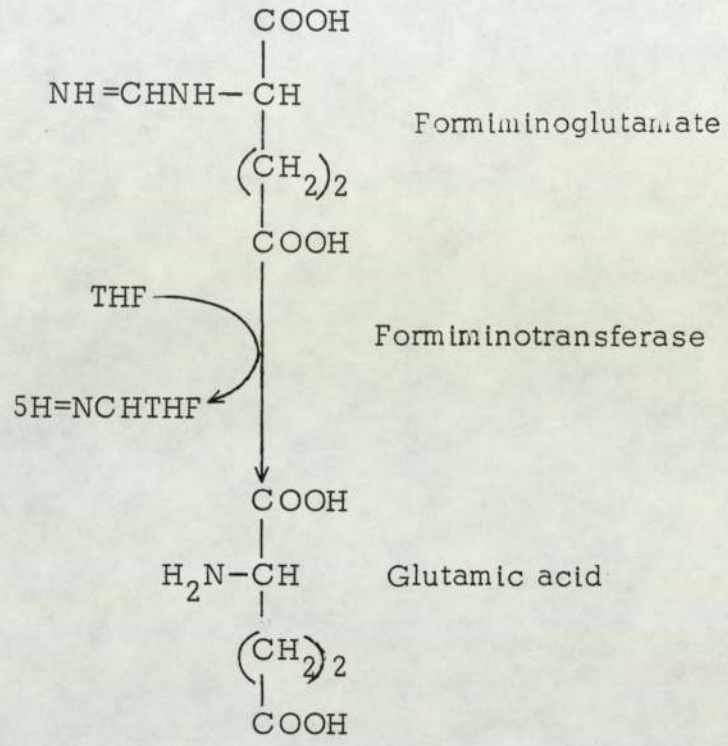


Fig. 1.19 The folate-dependent conversion of formiminoglutamic acid to glutamic acid

In addition to their coenzyme role in the cell, the folates also undergo catabolic reactions considered to involve cleavage of the central C_9-N_{10} bond (Connor et al., 1979). This reaction produces pteridine and p-aminobenzoyl derivatives, which are excreted in the urine. Folate catabolites detected include pterin (Fig. 1.2a) and xanthopterin (Fig. 1.2b) in human urine (Krumdieck et al., 1978), p-acetamidobenzoate (Fig. 1.11) in rat urine (Connor et al., 1979) and p-acetamidobenzoyl-L-glutamate (Fig. 1.12) in human urine. (Saleh et al., 1980).

Certain folates have also been detected in modified forms of the coenzyme. These include 5CHOTHF and 10CHOFA, both of which have been detected in rat urine (Connor, 1979). 5CHOTHF has no known biological function and is considered to be derived by isomerisation of 10CHOTHF (Blakley, 1969), whereas 10CHOFA was thought to result from the oxidation of 10CHOTHF. It has also been suggested that oxidation of the corresponding 10CHOTHF polyglutamate had occurred following the isolation of $10CHOFA(glu)_4$ from rat liver (Connor and Blair, 1980).

There have been many reports of multifunctional enzymes involved in folate metabolism. A trifunctional single protein containing $5,10CH_2THF$ dehydrogenase, 5,10CH=THF cyclohydrolase and 10CHOTHF synthetase activities and catalysing the following reactions (Fig. 1.20) has been isolated from sheep (Paukert et al., 1976) rabbit (Schirch, 1978) and porcine liver (Tan et al., 1977) and from yeast (Paukert et al., 1977).

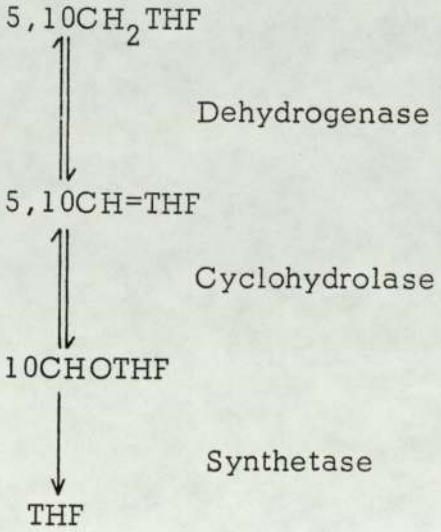


Fig. 1.20. Three folate-dependent enzyme activities of a widely distributed trifunctional single protein.

Independent assay of the dehydrogenase and cyclohydrolase active sites (Cohen and Mackenzie, 1978) revealed that the rate of 10CHOTHF production was greater than anticipated for a pathway involving diffusion of released 5,10CH=THF to cyclohydrolase active site. This observation suggests either proximal dehydrogenase and cyclohydrolase active sites or that "channelling" is possible on a single site. Either situation would allow more efficient processing of labile coenzymes.

Recently in studies with chicken liver (Caperelli et al., 1979), the trifunctional enzyme previously described was found associated with serine hydroxymethyltransferase, phosphoribosylaminoimidazolecarboxamide formyltransferase and phosphoribosylglycinamide formyltransferase to form a complex of four proteins with six enzyme activities. This complex was shown to produce 5'-phosphoribosyl-N-formylglycinamide from 5'-phosphoribosylglycinamide and $5,10CH_2THF$. This demonstrates sequential use of the dehydrogenase, cyclohydrolase and transformyltransferase activities.

Of critical importance to the functional state of these multifunctional folate enzymes is their specificity with respect to folate polyglutamates as these are considered the active coenzymes. In studies of the trifunctional enzyme and of a bifunctional enzyme formiminotransferase: cyclodeaminase catalysing the reactions below (Fig. 1.21), the folate polyglutamate derivatives were shown to be preferentially utilized (Mackenzie, 1979; Mackenzie and Baugh, 1980).

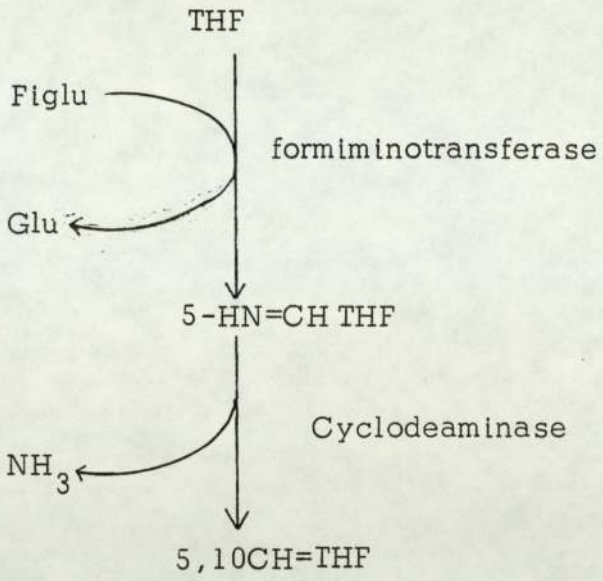


Fig. 1.21. Reactions catalysed by the bifunctional enzyme formimino-transferase: cyclodeaminase.

The mammal is unable to synthesise the molecular skeleton of the folate molecule de novo and therefore, careful regulation of folate metabolism is probably essential to ensure efficiency of utilisation. In this respect, a number of features of folate metabolism with possible regulatory function have been reported. Following investigation into gluconeogenesis from histidine (Krebs et al., 1976) it was observed that the isolated perfused rat liver and suspensions of rat hepatocytes were unable to metabolise histidine further than Figlu. The addition of methionine in physiological concentration however promoted the catabolism of Figlu and increased glucose production. These observations suggest a relationship in the metabolism of methionine, histidine and the folate coenzymes. Since it has been demonstrated that S-adenosyl methionine (synthesised from methionine) inhibits the enzyme $5,10CH_2THF$ reductase (Kutchbach

and Stokstad, 1967) this was suggested as an explanation for these observations. The inhibition of 5,10CH_2THF reductase would result in the accumulation of 5,10CH_2THF and those species in equilibrium with 5,10CH_2THF, namely 5,10CH=THF and 10CHOTHF. Since the intracellular level of 10CHOTHF is considered to be equal to or below the K_m for 10CHOTHF dehydrogenase, excess 10CHOTHF would be deformylated to give THF.

These observations suggest a careful regulation of one-carbon units in the folate pool. Thus, when methionine and hence S-adenosyl methionine levels are low, one-carbon units are conserved due to decreased activity of 10CHOTHF dehydrogenase. However, when methionine and therefore S-adenosyl methionine levels are high, feedback inhibition of 5,10CH_2THF reductase results in a shift of folate species towards 10CHOTHF and the eventual loss of one-carbon units by the deformylation reaction.

1.2. Folate polyglutamates

The earliest report of a folate polyglutamate came from Pfiffner and co-workers in 1945. This species was extracted from yeast and characterised as FA$(glu)_6$ (Fig. 1.22). Early attempts to isolate folate polyglutamates from mammalian tissues however, produced contradictory reports. Furthermore, the precise identity of folate polyglutamates has also been the subject of much controversy. This conflict has arisen due to the use of inappropriate techniques for both isolation and characterisation

and will be further discussed (Section 1.3).

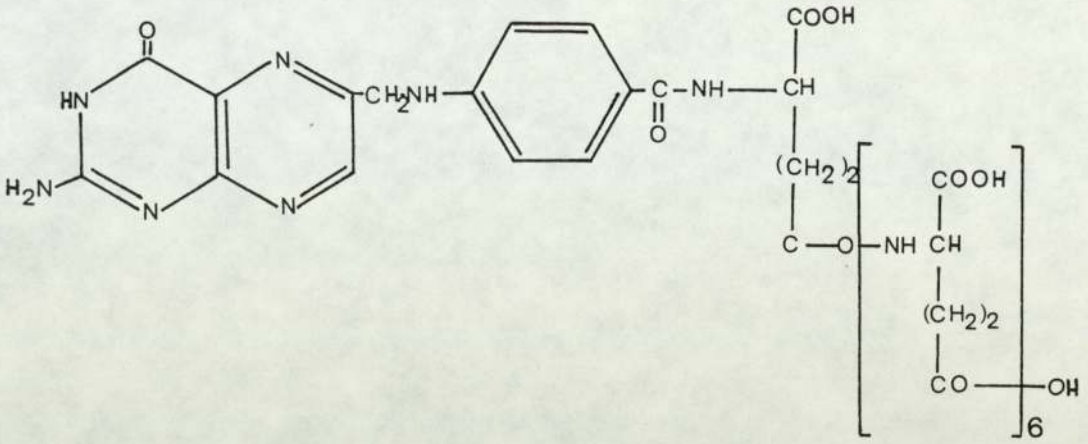


Fig. 1.22. FA hexaglutamate, isolated from yeast by Pfiffner and co-workers, 1945.

It is now well recognised that folate polyglutamates are the major derivatives in mammalian tissues (Corrocher et al., 1972; Whitehead, 1973; Lavoie et al., 1975; Richardson et al., 1979; Connor and Blair, 1980). Folate polyglutamates are better substrates than the respective folate monoglutamates for many of the folate-dependent enzymes. These include thymidylate synthetase (Dolnick and Cheng, 1978), methionine synthetase (Coward et al., 1975), DHF reductase (Coward et al., 1974) and phosphoribosylaminoimidazolecarboxamide formyltransferase (Baggott and Krumdieck, 1979). Widely distributed multi-functional folate-dependent enzymes (previously described) also show a preferential substrate

requirement for folate polyglutamates. The better substrate properties of folate polyglutamates, compared to the respective folate monoglutamates, are demonstrated by lower K_m values and increased values of V_{max}. These findings indicate that folate polyglutamates are the principal active coenzymes.

Several studies have shown that some folates exhibit inhibitory properties in certain folate-dependent reactions. Moreover, the folate polyglutamate forms of these coenzymes show markedly greater inhibitory properties when compared to the respective folate monoglutamates (Kisliuk and Gaumont, 1979; Matthews and Baugh, 1980). Thus, in addition to their substrate properties, folate polyglutamates may also serve as metabolic inhibitors in the regulation of folate metabolism. Kisliuk et al., (1974) reported that DHF polyglutamates inhibit thymidylate synthetase. Since DHF (presumably as DHF polyglutamate) is produced in the synthesis of thymidylate, inhibition of thymidylate synthetase by DHF polyglutamate may represent end-product inhibition and thereby regulate thymidylate synthesis.

Further indication of the importance of folate polyglutamates has come from studies of mutant chinese hamster ovary cells which failed to grow in the absence of exogenous glycine, thymidylate and adenosine (McBurney and Whitmore, 1974; Taylor and Hanna, 1977). Examination of the intracellular folate revealed markely reduced levels of coenzyme and the absence of folate polyglutamate derivatives. These findings

suggest either a direct requirement for folate polyglutamates or that these derivatives are essential for the intracellular retention of folate in order that the requirements for de novo synthesis of purines and pyrimidines can be met.

1.3. Experimental study of folates

Folate extraction from the tissues

Early attempts to isolate folates from mammalian tissues involved autolysis in the presence of reducing agents (e.g. ascorbate, mercaptoethanol). Reducing agents were employed to protect labile coenzymes. Using this procedure, several studies (Grossowicz et al., 1963; Silverman and Keresztesy, 1951) indicated that folate monoglutamates were the natural forms of folate and were thus concluded to be the active coenzymes.

The failure to isolate folate polyglutamates in these studies is considered to have resulted from the action of γ-glutamyl carboxypeptidase (E.C. 3.4.12.10) or "conjugase", a lysosomal enzymes (Shin et al., 1976) present in mammalian tissues. This enzyme hydrolyses the γ-peptide bond of folate polyglutamates thereby converting these derivatives to folate monoglutamates. Autolysis of mammalian tissues liberates lysosomal conjugase and is therefore inappropriate for the extraction of folate polyglutamates.

Efforts were later made to inactivate endogenous conjugase and

thereby prevent the breakdown of folate polyglutamates during extraction. The use of a rapid heating procedure (to denature conjugase) in the presence of ascorbate has been shown to be effective in this respect (Shin et al., 1972; Whitehead, 1973; Connor and Blair, 1980). Sotobayashi and co-workers (1966) failed to detect folate polyglutamates in rat tissue following a less rapid heating procedure during extraction. These and other studies (Connor, 1979) show the importance of rapidly deactivating conjugase in order to protect the folate polyglutamates. Cold extraction procedures employing either trichloroacetic acid (to precipitate protein) and anti-oxidants or sucrose (to stabilise lysosomes) and anti-oxidants are also effective for the isolation of folate polyglutamates (Connor, 1979).

Storage of folate extracts

Folate extracts are normally stored frozen in the presence of ascorbate prior to analysis. In certain cases (Marchetti et al., 1980; Barbiroli et al., 1980) whole tissues have been stored frozen and then thawed before extraction. However, few investigations have been undertaken to ascertain the effects of storage frozen and thawing on folates retained under these conditions. Studies indicate that storage frozen of folate extracts (in which endogenous conjugase has been deactivated by heating) in the presence of ascorbate, provides a suitable method. Using this procedure, Bird and co-workers (1965) reported no appreciable loss of folate in rat liver extract after four weeks, however the effects of more long-term storage were not investigated. Storage of whole tissues under these conditions prior to extract preparation however, results in the

considerable breakdown of folate polyglutamates.

Analysis of folate coenzymes

The folates are characterised by variable lability, complex properties and great diversity of chemical structure. In addition, these coenzymes are present in very low concentration in the tissues (0.1 - 9.0 ug g^{-1} wet weight) (Richardson et al., 1979). These features give rise to many difficulties in the identification of these species.

Following the early discovery that folates elicit a growth response in certain bacteria, microbiological assay was developed for the detection and measurement of these coenzymes and is still in routine clinical use. Three bacteria are employed: Lactobacillus casei, Streptococcus faecalis and Pediocroccus cerevisiae. Since these bacteria vary in their response to particular folate species, certain deductions can be made regarding the identity of the folates concerned (Blakley, 1969). However, these organisms give a poorer response to folates having more than three glutamate residues which therefore require prior conjugase treatment.

Broad differences in growth response of these organisms are observed. L.casei responds to all folates, S. faecalis to all folates except $5CH_3$THF and P. cerevisiae to formyltetrahydrofolates and THF. Despite these useful differences, misinterpretation and erroneous assumption are commonly found in the literatures. It is widely assumed that each organism responds to different folates to the same extent. However, Pollock and Kaufman (1978) reported that different folates produce a

different response in the same organism. Therefore, the general assumption that the levels of $5CH_3THF$ can be determined by subtracting the growth response of S. faecalis from L. casei, may be incorrect. Furthermore, the use of impure conjugase preparations (Reed et al., 1977) may cause interconversion of folate coenzymes due to the presence of contaminating folate-dependent enzymes. In this case, deductions regarding the specific identity of extracted folates would be invalid. In some cases, microbiological assay cannot distinguish between certain folates. Thus, 10CHOTHF 5CHOTHF and THF all produce a growth response for all three organisms (Blakley, 1969).

The most important technique for the detection of folates involves the use of radiolabelled tracers and is now commonly employed. Both ^{14}C and ^{3}H labelled tracers are available. The use of radiolabelled tracers is not without certain difficulties however. Commercial tritiated folic acid is normally supplied as the [3',5',7,9-^{3}H] derivative. It has been reported (Connor et al., 1979) that the folate molecule cleaves through the central C_9-N_{10} bond in vivo. This process therefore raises difficulties regarding the identity of the labelled species since C_9-N_{10} cleavage of the tritiated folates produces ^{3}H only labelled p-aminobenzoyl derivatives. This similarly applies to commercially available ^{14}C labelled folic acid ([2^{14}C] folic acid). In this case, C_9-N_{10} cleavage of the ^{14}C labelled folates would produce a ^{14}C labelled pterin derivative. However, the use of a mixture of [3',5',7,9-^{3}H] and [2^{14}C] folic acid and the subsequent demonstration of dual labelled derivatives provides

good evidence for the intact folate molecule.

Knowledge of the exact positioning of the radioactive atom or atoms is important for any considerations of their distribution in folate catabolites. The distribution of the ^{3}H atoms in commercial [3',5',7,9-^{3}H] folic acid (Amersham International, Bucks, U.K.) is approximately 20% at C7, 33% at C9 and 41% distributed between C3' and C5'. Therefore, determination of the amount of catabolic C_9-N_{10} cleavage by measurement of the levels of p-aminobenzoyl derivatives produced in this reaction, would necessitate correction according to the precise quantitative distribution of the isotope.

Identification of folate coenzymes is normally achieved by chromatographic methods in conjunction with specific techniques of detection. Microbiological assay provides useful supporting evidence of identity and is especially useful for the demonstration of folate polyglutamates having more than three glutamate residues which show enhanced microbiological activity after conjugase treatment. However, microbiological assay cannot distinguish between folate derived from an administered dose and that of the folate pool.

DEAE-cellulose chromatography is commonly employed in the purification and identification of folates. This technique allows separation of most folate monoglutamates (Moran et al., 1976; Connor, 1979). Investigations into the separation of folate polyglutamates on DEAE-cellulose however, have produced conflicting reports. Using FA poly-

glutamates, Baugh and Krumdieck (1971) found that these species eluted in order of increasing number of glutamate residues. In similar studies, Kisliuk et al., (1974) reported that DHF polyglutamate derivatives elute at a similar salt concentration regardless of the number of glutamate residues. Following studies of hepatic folates in the rat, Connor and Blair (1980) reported that the derivatives 10CHOFA $(glu)_4$ and 10CHOFA-$(glu)_3$ were just resolved on DEAE-cellulose. Improved separation was observed on DEAE-Sephadex however in both cases, 10CHOFA$(glu)_3$ eluted after 10CHOFA$(glu)_4$. DEAE-cellulose does not allow the separation of folate monoglutamates from all folate polyglutamates (Moran et al., 1976; Kisluik et al., 1974; Connor, 1979).

Gel filtration is very useful in the purification and identification of folate coenzymes. On Sephadex G15, folate polyglutamates elute near the void volume (V_o) and folate monoglutamates elute later (Shin et al., 1972; Connor and Blair, 1980). This procedure is both simple and effective for the separation of folate polyglutamates from folate monoglutamates. On Sephadex G75, protein elutes at the void volume and folate mono and polyglutamates elute later as a single peak (Connor, 1979). Thus, Sephadex G75 chromatography allows convenient determination of folate bound to protein, by measurement of the folate eluting at the void volume.

The availability of authentic folate monoglutamates, and more recently, folate polyglutamates, has allowed identification of folates by co-chromatography. As a single chromatographic system giving good

separation of all folates has not been developed, identification by co-chromatography should be demonstrated in two or more systems. Despite the important requirements for identification by co-chromatographic means, the literature contains many incorrect assumptions. Several reports (Shin et al., 1972; Barbiroli et al., 1980; Perry et al., 1980) have claimed to identify the glutamyl chain length of tissue folate polyglutamates following co-chromatography with authenic folate markers. In these studies it was assumed that the chromatographic properties of folate coenzymes are related solely to the number of glutamate residues, as no consideration was given to the state of reduction and substitution of the coenzymes under investigation. Kisluik et al., (1974) reported that the state of reduction and substitution of the folate molecule have a greater influence on chromatographic properties than the length of the poly-glutamyl chain. Other methods of folate analysis include thin layer chromatography (Scott, 1980; Brown et al., 1973) paper chromatography and electrophoresis (Connor and Blair, 1980).

Analysis of tissue folate has also been attempted by degradative methods based on oxidative or reductive cleavage of the C_9-N_{10} bond. These techniques were devised in an attempt to reduce the complexity of the naturally occurring folates which encompass differences in both the state of reduction and substitution of the pterin moiety. Theoretically, the cleavage process yields p-aminobenzoyl (glutamate)$_n$ derivatives which can be identified chromatographically. Oxidative cleavage (Houlihan and Scott, 1972; Brown et al., 1974; Reed et al., 1977) using

neutral or alkaline potassium permangamate was based on the established cleavage of folic acid (Zakrewski et al., 1970). Other studies (Maruyama et al., 1978) have shown that biologically active tetrahydrofolates do not cleave under these conditions. Reductive cleavage (Baugh et al., , 1974) employing Zn/HCl was again based on the specific reaction of folic acid (Hutchings et al., 1947) and does not occur for 10CHOTHF, $5CH_3THF$, $5,10\text{-}CH_2THF$ and THF (Baugh et al., 1979; Lewis and Rowe, 1979). Therefore, in the present form, oxidative or reductive cleavage methods are inappropriate for the analysis of folates.

Both fluorescence and ultra-violet spectroscopy are useful in the analysis of folates (Connor and Blair, 1980) particularly in the detection and identification of authentic folates used as chromatographic markers.

1.4. Biosynthesis of folate polyglutamates

In vivo studies

In vivo studies of folate polyglutamate biosynthesis have been carried out using the rat (Shin et al., 1976; Hillman et al., 1977), guinea pig (Corrocher et al., 1972) monkey (Brown et al., 1974) and sheep (Smith and Osborne-White, 1973). These studies have usually involved the use of folate tracers, however comparison and interpretation of the literature is made difficult by the absence of standardisation. These problems are compounded by the differing and often inappropriate methods employed for the subsequent analysis of folate derivatives.

Considerable variance is seen in the literature with respect to the quantity of folate precursor administered in these studies. Dose-dependent effects have been reported (Connor and Blair, 1979) and may explain the marked differences found for the tissue recovery of folate following administration. Thus, in studies with the rat, Houlihan and Scott (1972) dosing 4μg (Kg^{-1} body wt.) (intraperitoneal injection) and Leslie and Baugh (1974) dosing 700μg (Kg^{-1} body wt.) (intraperitoneal injection) reported hepatic recoveries of administered folate at 24 hours of 50% and 4% respectively. The metabolism of administered folate also differs according to the route of administration. Steinberg and co-workers (1979) found that the percentage ^{3}H FA appearing in the liver and bile after 1 hour was twice as high after intestinal loop injection as compared with right heart injection. Despite the widespread diversity found in the literature with regard to both dose and route as administered folate, the subsequent pattern of metabolism relating to these factors has been little investigated.

Few time-course studies of folate polyglutamate biosynthesis have been undertaken in the mammal. During an investigation of the folate enterohepatic cycle, Hillman and co-workers (1977) reported that after intravenous injection (5μg Kg^{-1} body wt.) of ^{3}H FA, 80% of administered folate was excreted in the bile within 6 hours, at which time 10-20% had become incorporated into the hepatic folate polyglutamate pool. Chromatographic studies revealed that the biliary radiolabelled folate was predominantly $5CH_3THF$ with lesser amounts of 10CHOTHF, 5CHOTHF and

unidentified folate monoglutamate. At 3 and 6 hours, the hepatic levels of unmetabolised ^{3}H FA and ^{3}H $5CH_3THF$ combined, represented 14 and 16% of the administered folate respectively. The hepatic folate polyglutamate derivative was reported to be $5CH_3THF(glu)_4$ however supportive evidence for this was not given. During this study it was observed that following intravenous injection of $5^{14}CH_3$ THF, greater than 90% was excreted unchanged into the bile within 6 hours, with less than 2% entering the hepatic folate polyglutamate pool. This observation indicates that $5CH_3THF$ is a poor substrate for the biosynthesis of folate polyglutamates.

More detailed studies on the time-course synthesis of folate polyglutamates was carried out by Shin et al., (1976). This group investigated the hepatic subcellular distribution of folate derivatives following intraperitoneal injection (160 μg Kg^{-1} body wt.) of ^{3}H FA. At 1 hour after injection, recovery of radioactivity was highest in the microsomal fraction however at 5,10 and 24 hours was highest in the soluble fraction. A progressive increase in the percentage of folate as folate polyglutamate in the soluble fraction was observed at 2,5 and 24 hours, these values representing 9, 39 and 60% respectively. It was found that the rate of reduction of folic acid was slowest in the microsomal fraction compared to other subcellular fractions. As the biosynthesis of folate polyglutamates was also found to be slowest in the microsomal fraction, this was considered to indicate the requirement of folate polyglutamate synthesis for reduced folate substrates, as has been previously reported (Masurekar

and Brown, 1975; Ritari et al., 1975).

Shin and co-workers (1972) investigated the hepatic distribution of folates in the rat 24 hours after the intravenous infection (1 μg Kg^{-1} body wt.) of ^{3}H FA. The major derivatives were shown to be folate polyglutamates and the identification of 10CHOFA$(glu)_4$ was reported following co-chromatography of the purified species with the authentic compound on both Sephadex G10 and G15. The chromatographic properties of other folate polyglutamates on these columns were not reported however. The detection of THF$(glu)_4$ was also claimed, identification being based on differential microbiological assay, chromatographic properties on Sephadex G15 and the lability of the species. Several other folate polyglutamates were also reported including 5CHO THF$(glu)_n$ (n = 1, 2 and 4), 5CH_3THF-$(glu)_n$ (n = 1, 2 and 4) and 10CHOTHF$(glu)_n$ (n =1 and 2). In these cases however, identification was established by microbiological assay and/or the "elution pattern" on Sephadex G15 and G25, independently of but in relation to, other folate markers and should therefore be interpreted with caution. The use of anti-oxidants during chromatography and re-chromatography of these species was not reported.

Recently, a more definitive investigation was undertaken to identify folate polyglutamates isolated from rat liver (Connor and Blair, 1980). Following oral administration (112 μg Kg^{-1} body wt.) of radiolabelled folic acid to rats, 70% of the radioactivity retained in the liver at 48 hours was incorporated into two folate polyglutamates. The major

species was extracted and then purified by Sephadex G15, DEAE-cellulose and DEAE-Sephadex ion-exchange chromatography and identified as 10CHOFA$(glu)_4$ by spectral and chromatographic analysis, microbiological and glutamate assay and by identification of the product (10CHOFA) of intestinal conjugase treatment. The minor derivative, though less well characterised, was identified as 10CHOFA$(glu)_3$.

Many studies of folate polyglutamate synthesis have been carried out using the oxidative or reductive cleavage procedure (Baugh et al., 1974; McGing et al., 1978; Brown et al., 1974; Lavoie et al., 1974). These studies are therefore subject to the criticisms previously described and will not be discussed in detail.

Information on the in vivo substrate requirements of folate polyglutamate synthesis has been obtained from studies of folate interrelationships in Vitamin B_{12} deficiency and Vitamin B_{12} deactivation. Vitamin B_{12} deactivation is achieved using nitrous oxide (McGing et al., 1978; Scott et al., 1979) and has been used as a convenient model for functional B_{12} deficiency. It is now well documented that dietary B_{12} deficiency (Davidson et al., 1975; Shane et al., 1977) and B_{12} deactivation (McGing et al., 1978; McGing and Scott, 1980) causes reduced hepatic folate incorporation in the mammal. More specifically, reduced folate incorporation in B_{12} deficiency has been reported to result from impaired synthesis of folate polyglutamates (Thenen and Stokstad, 1973; Chanarin et al., 1974; Scott et al., 1974). These studies indicate that Vitamin

B_{12} has a functional involvement in the synthesis of folate polyglutamates. Moreover, this wo uld appear to be associated with the demethylation of $5CH_3THF$ since this is the only known B_{12}-dependent step in mammalian folate metabolism.

Vitamin B_{12} deficiency is considered to result in the trapping of folate as $5CH_3THF$ and as previously described, this forms the basis of the "methyl trap" hypothesis. Thus, despite the apparent availability of $5CH_3THF$ under these conditions, albeit transiently, these findings suggest that $5CH_3THF$ is either not a substrate, or a poor substrate, for the synthesis of folate polyglutamates.

Methionine has been reported to reverse the decreased hepatic incorporation of folate in B_{12} deficient rats (Chiao & Stokstad, 1977) and B_{12} deactivated rats (McGing and Scott, 1980). Therefore, methionine appears able to alleviate the block in folate polyglutamate synthesis in these circumstances. As previously described, Krebs et al., (1976) proposed that methionine can modulate the conversion of $5,10CH_2THF$ to $5CH_3THF$ by the inhibition of $5,10CH_2THF$ reductase, via S-adenosyl-methionine. This was considered to result in the elevation of intra-cellular $5,10CH_2THF$ and those folates in equilibrium with this species, namely 5,10CH=THF and 10CHOTHF. Therefore, these findings suggests that the latter three folate derivatives are better substrates , either directly or indirectly, for the in vivo synthesis of folate polyglutamates.

In-vitro studies

The first in vitro study of the enzyme required for the synthesis of folate polyglutamates was described by Griffin and Brown (1964). This enzyme, isolated from E. coli, required THF (DHF and FA were inactive as substrates), L-glutamate and ATP for activity, and was shown to synthesise $THF(glu)_2$. Since this report, folylpolyglutamate synthetase has been investigated from a number of sources. In all cases, enzyme activity requires, in addition to the folate substrate, a purine nucleotide triphosphate (dATP and ATP being most effective), a mono and/or divalent cation (K^+ and/or Mg^{++}) and L-glutamate. Furthermore, this enzyme has been shown to have a broad specificity with respect to the folate substrate and in certain cases, the utilization of folate analogs (e.g. methotrexate) has been observed.

Sakami et al., (1973) reported that an extract of Neurospora could be separated with $(NH_4)_2SO_4$ into two enzyme fractions. The 45-60% fraction converted THF to $THF(glu)_1$, but only the 0-35% fraction converted $THF(glu)_1$ to higher THF polyglutamates; no details were published. Masurekar and Brown (1975) partially purified an enzyme from E. coli which converted 10CHOTHF to $10CHOTHF(glu)_1$. The specificity of this enzyme with respect to the folate substrate was 10CHO-THF > $5,10CH_2THF$ > THF. DHF, $5CH_3THF$, 5CHOTHF and FA were inactive. No higher folate polyglutamates were detected in these studies.

Recently, McGuire and co-workers (1980) reported a more detailed in vitro study of rat liver folylpolyglutamate synthetase following a 55 fold purification. The enzyme was shown to have a molecular weight of 69,000, was inactive in the absence of K^+ or other monovalent cations and had a rigid specificity for L-glutamate as the incoming amino acid. Substitution of the terminal acceptor glutamate of the folate substrate with D-glutamate caused a loss of substrate activity; the terminal L-glutamate being essential for enzyme binding. The addition of glutamate residues was considered to take place once at a time as γ-L-glutamyl-L-glutamate did not inhibit the reaction. A broad specificity with respect to folate substrate was observed and a number of folate analogs (including methotrexate) were also active, however this was dependent on the folate substrate concentration. At both 5 and 35 μM, THF, 10CHOTHF, 5,10CH_2THF and DHF were the most efficient substrates. 5CH_3THF was a poor substrate at 5 μM. However at 35 μM had markedly improved substrate properties.

In conclusion, despite the broad specificity of folypolyglutamate synthetase with respect to the folate substrate, this enzyme has a preferential requirement for THF, 10CHOTHF and 5,10-CH_2THF. Both in vivo and in vitro studies indicate that 5CH_3THF is a poor substrate for folypolyglutamate synthetase, however the substrate properties of 5CH_3-THF may be improved at increased intracellular concentrations of this coenzyme.

1.5. Folate metabolism in neoplastic disease

The central role of the folates in cellular growth and development has focussed attention on the possible involvement of these coenzymes in the proliferative processes commonly associated with neoplastic disease. Many *in vitro* studies of folate interrelationships in neoplasia have been carried out, however *in vivo* studies, particularly in relation to the synthesis and disposition of the folate polyglutamate pool, are far fewer.

Several disorders of folate metabolism associated with neoplasia have been described. Folate deficiency in humans suffering from cancerous lesions has been reported (Blakley, 1969). Evidence for this includes decreased plasma folate, abnormal rates of clearance of folic acid following injection and increased urinary excretion of Figlu after histidine loading. Poirier (1973) also reported increased urinary excretion of Figlu after histidine loading by tumour bearing rats. Increased hepatic folate incorporation and decreased urinary excretion of $5CH_3THF$, 48 hours after the oral administration of 10CHOFA to tumour bearing rats, has also been attributed to tumour induced folate deficiency (Connor and Blair, 1979). The primary cause of folate deficiency in neoplastic disease has not been identified, however it appears likely to result from the increased demand for folate by the proliferating neoplasm.

The metabolism of orally administered folic acid has been investigated in rats bearing the Novikoff hepatoma (Pheasant and Blair, 1979).

The quantitative pattern of urinary excreted folates was similar for both tumour bearing and control animals, except for a species chromatographing on DEAE-cellulose in the position of $5,10CH_2$-THF, which was increased in the urine of tumour bearing animals. Barford and Blair (1978) reported that rats bearing the Walker 256 carcosarcoma excreted more formyl folates and less $5CH_3$THF in the urine than control animals. Thus, the presence of tumour tissue appears to augment a shift towards the folates involved in the synthesis of thymidylate and the purines. Connor and Blair (1979) investigated the metabolism of 10CHOFA in rats bearing an implanted Walker 256 carcosarcoma. At 48 hours after administration, tumour tissue contained predominantly 10CHOTHF polyglutamates as shown by chromatographic and microbiological analysis. Folic acid is also metabolised to predominantly folate polyglutamates in the Novikoff hepatoma (Pheasant and Blair, 1979).

Neoplastic tissue also manifests changes in the activity of many folate dependent enzymes. Lepage et al., (1972) examined DHF reductase, serine hydroxymethyltransferase, $5,10CH_2$THF dehydrogenase, 10CHOTHF synthetase and formiminotransferase and found small to moderate decreases in activity in rat hepatomas compared to most livers. Jackson and Neithammer (1979) also reported reduced activity of these enzymes in rat hepatomas compared to the normal liver, with the notable exception of thymidylate synthetase, which was increased up to 123-fold greater than found in the normal liver.

The chemotherapy of neoplastic disease has been undertaken using folate analogs, which disrupt folate metabolism in the cell, and are otherwise known as antifolates. The antifolates exert their cytotoxicity through the inhibition of two major metabolic pathways; de novo thymidylate and purine synthesis. The 2,4-diamino folate analogs of which methotrexate (MTX) (Fig. 1.5.1) affords the most important example, are inhibitors of DHF reductase.

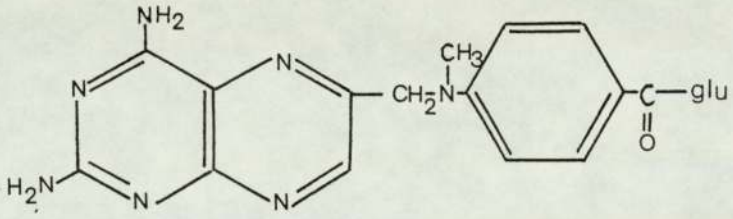


Fig. 1.23 Methotrexate (MTX)

Therefore, these analogs are able to block the essential formation of THF from DHF which leads ultimately to the intracellular depletion of functional tetrahydrofolates. Methotrexate has proved a successful anti-tumour agent for certain leukaemias and lymphomas (Bertino, 1979).

CHAPTER 2

EXPERIMENTAL

2. METHODS AND MATERIALS

2.1. Chemicals

The following chemicals were obtained commercially; folic acid, xanthopterin and pterin from Koch-Light Laboratories Limited, (Colnbrook, Bucks, U.K.); $5CH_3THF$ from Eprova Research Laboratories (Basle, Switzerland); Leucovorin (5CHOTHF) from Lederle Laboratories Division (Cyanamid of Gt. Britain, Limited, London); p-aminobenzoyl-L-glutamate and d,l-dithiothreitol from Sigma Chemical Company Limited (London, U.K); p-aminobenzoic acid and p-acetamidobenzoic acid from Aldrich Chemical Company Limited (Wembley, Middlesex, U.K.). 10 CHO-FA and p-acetamidobenzoyl-L-glutamate were prepared by Dr. M.J. Connor (in the Department of Chemistry, University of Aston in Birmingham, U.K.).

10CHOTHF (and 5,10-CH=THF)

5,10-CH=THF was prepared by acid isomerisation of 5CHO THF. 5CHO THF (5mg) was dissolved in 0.1M HCl (20 mls) containing mercapto-ethanol (1% w/v) and allowed to isomerise overnight in the dark. Ultra-violet spectroscopy revealed that isomerisation of 5CHO THF had taken place to give 5,10-CH=THF (λ_{max} 288 and 345 nm at pH 1.0). 10CHO THF was synthesised from 5,10-CH=THF by adjusting the solution to pH 9.0 (using dilute NaOH) and allowing to stand for 15 minutes. The solution was then adjusted to pH 7.0 using dilute HCl. Ultraviolet spectroscopy of the neutral solution revealed that 10CHOTHF had been synthesised (λ_{max} 258 nm). High-performance liquid chromatography

of this solution gave a single peak having the ultra-violet spectrum of 10CHOTHF.

Folate polyglutamates

Folate polyglutamate markers were synthesised by Dr. R. Nayyir Mazhir (in the Department of Chemistry, University of Aston in Birmingham, U.K). These were:

a) $FA(glu)_n$ (n = 3, 4 and 5), prepared using the method of Meienhofer et al., 1970.

b) $10CHOFA(glu)_n$ (n = 3, 4 and 5), prepared by formylation of the corresponding $FA(glu)_n$ according to the method of Blakley, (1959).

c) $5CH_3THF(glu)_n$ (n = 3, 4 and 5), prepared from the corresponding $FA(glu)_n$ according to Blair and Saunders, (1970).

Radiochemicals

[$2^{14}C$] folic acid (specific activity 50-60 mCi/mmol) and [3',5',7,9-^3H] folic acid (specific activity 500 mCi/mmol) were purchased from the Radiochemical Centre (now Amersham International Limited) (Bucks, U.K.). All other chemicals were of Analar grade.

2.2. Chromatography

i) Gel filtration

Sephadex G15 and G75 (Pharmacia Fine Chemicals, Uppsala, Sweden.) were prepared as a slurry using 0.05M phosphate buffer (pH 7.0)

containing dithiothreitol (5 mg%) and allowed to swell as described by supplier. After de-gassing under vacuum, slurries were packed under gravity into Perspex columns (2 x 60 cm) (Wright Scientific Limited, Surrey, U.K.). Following sample loading (1-15 mls), columns were eluted (1.0 ml min^{-1}) with 0.05M phosphate buffer (pH 7.0) containing dithiothreitol (5 mg%). The eluant passed through a U.V. monitor allowing detection of folate markers which were introduced into the sample before loading. Fractions were collected using an automatic fraction collector. In certain cases, fractions (5.0 mls) were collected into 1.0 ml 0.05M phosphate buffer containing sodium ascorbate (2% or 10% w/v) in order to reduce the oxidation of labile folates.

ii) Ion-exchange chromatography

Diethylaminoethyl (DEAE) cellulose (DE52, Whatman Limited, Maidstone, Kent, U.K.) was initially equilibriated with 0.05M phosphate buffer (pH7.0) containing dithiothreitol (5 mg %). After removal of fines by decanting, the slurry was de-gassed under vacuum and packed under gravity into glass columns (60 x 1.5 cm) plugged with glass wool. Following sample loading (5-120 mls) columns were eluted (1.0 ml min^{-1}) with a linear NaCl gradient (0-1.2M) in 0.05M phosphate buffer (pH 7.0) containing dithiothreitol (5 mg%). The eluent passed through a U.V. monitor allowing detection of folate markers which were introduced into the sample before loading. Fractions were collected using an LKB automatic fraction collector (LKB Instruments, Croydon, Surrey, U.K.). The gradient (8 hours in duration) was automated using an LKB Ultra Grad

attached to a peristaltic pump (LKB Instruments). When sample volume exceeded the void volume of the column, loading fractions were retained in order to prevent any loss of radiolabelled species eluting during loading. The gradient was determined by measuring the conductivity of every 10th fraction using a Mullard conductivity cell.

iii) High performance liquid chromatography (HPLC)

HPLC was carried out using an assembly comprising the following (a-f):

a) Gradient Master (LDC, Florida, U.S.A.)
(Model 1601) for the control of one or two consta Metric pumps, giving gradient control.

b) Two identical consta Metric pumps (LDC)
(Model 111) allowing operating pressures up to 5000 psi and an adjustable flow rate from 0.1 to 10.0 mls min^{-1}.

c) Spectro Monitor (LDC)
(Model 111) giving spectroscopic detection of folate markers.

d) HPLC Partisil anion-exchange ($-N(C_2H_5)_3^+$) column (M9, 10/50 SAX) (Whatman, USA).

e) Chart recorder (J J Instruments, Southampton, U.K.) (Model CR 652)
A two pen recorder allowing simultaneous recording of the spectroscopic absorbance (280 nm) of the eluent and the gradient.

f) Fraction collector (LKB Biocal, Stockholm, Sweden).

Prior to sample loading on to HPLC (sample loop injection, 2 mls) folate extracts were centrifuged to reduce protein content (35000 rpm for 45 min.) (Automatic Superspeed 50 Refrigerator Centrifuge) (MSE Limited, London.), filtered (0.22 μm paper filter) (Millipore Corporation, Bedford, U.S.A.), and adjusted to pH 4.5 using dilute phosphoric acid. Column elution was effected using a linear Na_2SO_4 gradient (0-0.5M) in 0.05M phosphate buffer (pH 4.5) containing dithiothreitol (5 mg %). When calibrating the system, authentic folate markers were treated similarly. During chromatographic runs, 60 fractions (2.0 mls) were collected.

2.3. Animals

Three groups of animals (A, B and C) were used :

Group A

Normal male Wistar rats (200-250 g) supplied by Scientific Products Farms Limited, Canterbury, U.K.

Group B

Normal male WAB/NOT rats (250-275 g) and WAB/NOT rats (225-250 bearing an implanted Sarcoma Mc 103B (supplied by The University of Nottingham).

Group C

Normal male Wistar rats (200-250 g) and male Wistar rats (200-225g bearing an implanted Walker 256 Carcosarcoma (supplied by the Institute of Cancer Research, Fulham, London.).

Before and during experimentation, animals were kept at 21°C in a closed room giving an automatic 12 hour light/12 hour dark cycle. Food (Breeding diet, Heygates Limited) and water were provided ad libitum.

2.4. Details of Tumour Lines

Sarcoma Mc 103B was induced (by supplier) in adult male WAB/NOT rat by subcutaneous injection of 3-methyl-cholanthrene (1 mg) (Sigma, London, U.K.) dissolved in trioctanoin (1 ml) (Eastman Kooak, New York, U.S.A). A tumour transplant line was established and maintained by subcutaneous trocar implantation of fragments of tumour tissue into the right flank. The tumour mass was allowed to grow for 2-3 weeks before experimentation.

Walker 256 carcosarcoma was passaged (by supplier) by subcutaneous implantation of 2×10^6 Walker-256-Carcosarcoma ascites into the right flank. The tumour mass was allowed to grow for one week before examination.

2.5. Dosing of animals

Rats were dosed orally by stomach intubation using a specially designed steel dosing needle. Each rat (lightly anaesthetised with ether) received 0.2 mls (0.05M phosphate buffer (pH 7.0) containing sodium ascorbate (2% w/v), [2^{14}C] folic acid (2 μCi) and [3',5', 7,9-^{3}H] folic acid (5 μCi). This dose provided 77-86 μg of folic acid per Kg body weight. After dosing, animals were kept in cages designed to prevent coprophagy.

2.6 Preparation of tissue extracts

Animals were killed by cervical dislocation, following a blow to the head which served to stun. Livers and intestines were rapidly excised by surgical opening of the body cavity. Intestinal contents were removed prior to extraction. Tumour tissue (restricted to a subcutaneous lump at the site of implantation) was removed *in toto* using a scalpel blade and scissors. Folates were extracted from the tissues using method A, B, C or D.

Method A

Tissues rapidly chopped and dropped into 5 volumes of 0.05M phosphate buffer (pH 7.0) containing sodium ascorbate (2% w/v), dithiothreitol (5 mg %) at 85-90^{o}C for 10 minutes. After cooling, tissue suspensions were rapidly homogenised (Potter-Elvehjem) and centrifuged (bench centrifuge, 10 minutes, 3000 g) and the supernatant (crude extract) retained for analysis. Using this procedure, 80-85% of tissue radioactivity was extracted.

Method B

As described for method A but with trichloroacetic acid (TCA) (10% w/v) added after the centrifugation, to precipitate protein. The supernatant was finally re-adjusted to pH 7.0 using NaOH.

Method C

Tissues were rapidly chopped and dropped into 5 volumes of ice-

cold 0.05M phosphate buffer (pH 7.0) containing sodium ascorbate (2% w/v), dithiothreitol (5 mg %) and 5 mls of saturated p-chloromercuribenzoic acid (dissolved in dilute NaOH) (to inhibit conjugase). TCA (10% w/v) was then added and the suspension homogenised, centrifuged and re-adjusted to pH 7.0 as previously described.

Method D

Tissues were rapidly chopped and dropped into ice-cold 0.05M phosphate buffer (pH 7.0) containing sodium ascorbate (2% w/v), dithiothreitol (5 mg %) and sucrose (0.25M). Suspensions were then homogenised at low speed (Potter-Elvehjem) centrifuged as described and the supernatant retained for analysis.

2.7. Measurement of radioactivity

Radioactivity was measured using either a Nuclear Enterprises (Type NE 8310) (Nuclear Enterprises, Edinburgh, U.K.) or Beckman (Model LS 7500, micro-processor controlled) (Beckman Instruments, Fullerton, U.S.A) liquid scintillation counter.

Samples were counted for 10 minutes (or 10,000 counts). When using NE 8310 counter, corrections for quenching were made using the external standard ratio method related to a quench curve prepared using increasing concentrations of 2,4-dichlorophenol-indophenol against a standard of each isotope. Both tritium and carbon-14 were counted simultaneously using specific window settings for each isotope. Corrections were also made for isotope overlap when using NE 8310 counter.

The Beckman counter was programmed to automatically correct for both quenching and isotope overlap. Samples were counted in glass vials after mixing with scintillation cocktail. Scintillation cocktail was prepared by thoroughly mixing toluene (1 litre) with Fisons emulsifier (Mix No. 1) (Fisons, Leics., U.K.) (500 mls), 2,5-diphenyloxazole (5 g) (PPO) (Fisons) and 1,4-bis-2-(5-phenyloxazole)-benzene (0.1g) (POPOP) (Fisons). Samples were prepared for counting using one of the following procedures (a-c) :

a) Column eluents from G15 and DE52 chromatography were counted as a clear homogeneous liquid by mixing 1.0 ml of sample or 1.0 ml sample / water with 10 mls of cocktail.

b) Column eluents from HPLC were counted either as a clear homogenous liquid or homogenous gel by mixing 0.5 mls sample/and 1.0 ml water or 2 mls of sample/and 8 mls water, with 10 mls of scintillant respectively.

c) Unless otherwise stated, radioactivity in tissues was determined by combustion using a Beckman Biological Materials Oxidiser. Carbon-14 was trapped as $^{14}CO_2$ in 15.0 mls of Fisons absorber P. Tritium was trapped as tritiated water and counted in 10 mls of Fisons absorber H.

2.8. Identification of folates

Species demonstrated as being labelled with both carbon -14

and tritium on both Sephadex G-15 and DEAE-cellulose were considered as intact folate derivatives. More specific information of folate identity was gained by showing co-chromatography with the authentic material. The distinctive chromatographic properties of $5CH_3THF$ and FA on Sephadex G15 and DEAE-cellulose allowed convenient identification of these species, and further information of identity was not pursued. 10CHOTHF was identified by its characteristic elution position from Sephadex G15.

The capacity of Sephadex G15 to separate folate polyglutamates from folate monoglutamates allowed general deductions in respect of folates identity and quantitation of these derivatives. In certain cases, differential microbiological assay was used as supportive evidence of folate identity and to demonstrate folate polyglutamates after conjugase treatment.

2.9. Other methods

Differential microbiological assay using L. casei, S.faecalis and P.cerevisiae was carried out in the Department of Haematology at the General Hospital, Birmingham (U.K.) under the supervision of Dr. R. Leeming. Ultra-violet spectroscopy was carried out using a Pye-Unicam S.P. 1700 Spectrometer.

CHAPTER 3

3.1. Extraction and partial characterisation of hepatic folate polyglutamates in the normal rat.

3.2. The biosynthesis of folate polyglutamates in the liver and intestine of the normal rat.

3.3. The chromatographic properties of authentic folates and folate catabolites.

INTRODUCTION

The present study is concerned with a preliminary investigation of hepatic folate extraction and identification, and the biosynthesis of folate polyglutamates in the rat. This was undertaken following the oral administration of radiolabelled folic acid to normal rats.

METHODS

EXPERIMENT 3.1. Extraction and partial characterisation of hepatic folate polyglutamates in the rat

10 normal male Wistar rats (group A) were dosed with a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. At 48 hours after administration, animals were killed and the livers (113 g of tissue in total) extracted (extraction A). Liver extracts were then pooled and freeze-dried to reduce the volume. The resulting residue was dissolved in 0.05M phosphate buffer (pH 7.0) containing dithiothreitol (5 mg %) and chromatographed in aliquots on Sephadex G15. The major peak resolved on Sephadex G15 (eluting close to V_o) was re-chromatographed on DEAE-cellulose. Differential microbiological assay, before and after conjugase treatment, was carried out at certain stages in the purification using L. casei, S. faecalis and P. cerevisiae. Analysis was undertaken between 0 - 3 months after extract preparation. The extract was stored frozen at -20°C.

EXPERIMENT 3.2. The biosynthesis of folate polyglutamates in the liver and intestine of the normal rat

10 normal Wistar rats (group A) were dosed with a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. At 2,4,6, 24 and 30 hours after administration, rats were killed in pairs and the livers and intestines extracted in pairs as shown below :

Rat 1	Liver	Extraction B
	Intestine	Extraction A
Rat 2	Liver	Extraction C
	Intestine	Extraction C

Crude tissue extracts were then chromatographed directly on Sephadex G15. In certain cases, peaks resolved on Sephadex G15 were re-chromatographed on DEAE-cellulose. Analysis was carried out between 0-3 months after extraction preparation. The extracts were stored frozen at -20°C.

EXPERIMENT 3.3. The chromatographic properties of authentic folates and folate catabolites on Sephadex G15 and DEAE-cellulose

Authentic folate monoglutamates, folate polyglutamates and folate catabolites were chromatographed on Sephadex G15 and DEAE-cellulose.

RESULTS

EXPERIMENT 3.1.

Hepatic recovery of radioactivity (measured from the tissue extract) was approx. 12% in the whole tissue (calculated as an average of 10 livers). As the extraction procedure employed was later shown to remove 85% of hepatic folate, the corrected value for hepatic recovery is approximately 14%.

Sephadex G15 chromatography of the 48h liver extract (Fig. 3.1) revealed a major dual labelled peak eluting near the void volume (V_o) (K_{av} = 0.18) and a minor species eluting later (K_{av} = 0.54). DEAE-cellulose chromatography resolved the major peak into 4 dual labelled derivatives (hereafter referred to as folate polyglutamates 1-4 in order of elution) (Fig. 3.2) eluting between 0.73 and 0.86M NaCl.

Differention microbiological assay revealed that folate present in the crude extract was active for all three organisms, showing in each case a 4 - 8 fold increase in microbiological activity after conjugase treatment. Total endogenous folate (L. casei activity after conjugase treatment) was 10.6 ug g^{-1} liver (wet weight). This amounts to 12.5 ug g^{-1} following correction for the extraction procedure.

The major dual labelled peak eluting close to V_o on Sephadex G15 was active for L. casei and S. faecalis (showing a 5-9 fold increase in activity after conjugase treatment) but inactive for P. cerevisiae. DEAE-cellulose resolved species 1-4 were active for L. casei and S. faecalis

(producing a 5-14 fold increase in activity after conjugase treatment) but inactive (i.e. < 2% of corresponding L. casei activity) for P. cerevisiae.

EXPERIMENT 3.2.

The recoveries of radioactivity in the liver and intestine (measured from tissue extracts) at 2,4,6,24 and 30 hours after administration are presented in Table 3.1. These values are corrected for the extraction procedure. In the liver, both extractions B and C revealed a progressive increase in retained radioactivity between 2-24 hours, becoming maximal at 30 hours. The pattern of intestinal radioactivity differed between extraction A and C. Extract A revealed highest levels at 2 hours followed by a progressive decline, however extraction C demonstrated variable levels at the different time periods.

Sephadex G15 chromatography of the liver and intestinal extracts showed one or more dual labelled derivatives in the tissues. These results are summarised in Tables 3.2 and 3.3 respectively.

Marked differences in both the qualitative pattern and the quantitative distribution of dual labelled derivatives were apparent between the different tissue extracts. Thus, at 24 hours in the liver, extraction B revealed three peaks eluting in fractions 10-16 ($K_{av} = 0.14$), 17-22 ($K_{av} = 0.29$) and 23-28 ($K_{av} = 0.54$) whereas extraction C demonstrated a broad dual labelled peak eluting in fractions 11-21 ($K_{av} = 0.14$). These chromatograms are presented in Fig. 3.3 and 3.4 respectively. Similarly, at 24 hours in the intestine, extraction A demonstrated a single species eluting

in fractions 11-18 (K_{av} = 0.11) whereas extraction C revealed a major species eluting considerably later, between fractions 23 - 28 (K_{av} = 0.54). These chromatograms are presented in Fig. 3.5 and 3.6 respectively.

In the intestine (extraction A) at 2,4 and 6 hours (but not 24 or 48 hours), a dual labelled peak eluting in fractions 28 - 48 was present, however a similar peak was not detected in the liver or intestine following extraction B and C. DEAE-cellulose chromatography of this peak at 2 hours demonstrated the presence of $5CH_3THF$ and FA, by co-chromatography with the authentic compounds.

EXPERIMENT 3.3

Results for the chromatographic study of authentic folate markers on Sephadex G15 and DEAE-cellulose are presented in Table.3.4. These show good resolution of $FA(glu)_4$, $10CHOFA(glu)_4$ and $5CH_3THF(glu)_4$ from the major folate monoglutamates on Sephadex G15, although not individually. In most cases, the major folate monoglutamates were also resolved from each other, however this was not the case for $5CH_3THF$ and FA.

DEAE-cellulose chromatography also allowed separation of the major folate monoglutamates, however in many cases these were not well resolved from the folate polyglutamates investigated. Notably, 10CHO-$FA(glu)_4$ was not well resolved from 10CHOFA however $5CH_3THF$ eluted earlier, and $FA(glu)_4$ eluted considerably earlier than the respective folate monoglutamates.

DISCUSSION

EXPERIMENT 3.1

This study shows that 48 hours after the oral administration of radiolabelled FA, the hepatic derivatives are predominantly folate polyglutamates. This was apparent from the chromatographic properties of these derivatives on Sephadex G15, and from the several fold increase in microbiological activity after conjugase treatment.

As the folates present in the initial crude extract were active for all three microorganisms, this indicates that folate polyglutamates are derivatives of 10CHOFA and/or 5CHOTHF and/or THF. The loss of P. cereviae activity following the initial Sephadex G15 chromatography indicates that a change in the identity of these species occurred during storage and/or chromatography. A possible explanation for this is that oxidation of labile 10CHOTHF polyglutamates (active for P. cerevisia) to 10CHOFA polyglutamates (inactive for P. cerevisiae) had taken place.

The microbiological activity of species 1-4 suggests their identity to be γ-glutamyl derivatives of 10CHOFA and/or FA and/or DHF. DHF folate readily undergoes oxidative breakdown to yield a pterin and a p-aminobenzoyl derivative (Blakley, 1969), therefore this is probably the least likely derivative.

EXPERIMENT 3.2

These studies show that the precise conditions of folate extraction from the tissueshave an important bearing on the identity of the folates isolated. The marked differences in the quantitative levels of folate polyglutamates (eluting from Sephadex G15 close to V_o) apparent between the extracts, indicates that breakdown of these derivatives occurred in certain cases.

The dual labelled derivative eluting from Sephadex G15 generally in fractions 23-28 (K_{av} = 0.54) in the liver and intestine following extraction B and/or C may have been formed by the use of TCA during extraction. In support of this, the intestinal extraction A (which did not incorporate the use of TCA) did not reveal the presence of this derivative at any time. This species has very similar chromatographic properties as 5CHOTHF and may have arisen due to acid isomerisation of 10CHOTHF due to the presence of TCA. The presence of 10CHOTHF may have resulted from the breakdown of 10CHOTHF polyglutamates in cases where folate polyglutamate breakdown was indicated.

As both extractions B and C have been successfully employed for the isolation of folate polyglutamates without breakdown (Connor 1979), this would suggest that the use of TCA was not responsible for the apparent breakdown of these derivatives in this study. More probable is that breakdown resulted from inadequate inactivation of conjugase during extraction.

Intestinal extraction A also revealed the presence of $5CH_3THF$ at 2 hours, however this was not the case for hepatic or intestinal extractions B and/or C. As other studies (Hillman et al., 1977; Steinberg et al., 1978) have shown that FA is metabolised to $5CH_3THF$ in vivo, extraction A appears the more appropriate procedure for the isolation of folate from the tissues.

Tissue	Time (hrs)	% of dose radioactivity in whole tissue			
		EXTRACTION B		EXTRACTION C	
		^{14}C	^{3}H	^{14}C	^{3}H
LIVER	2	7.5	9.5	5.1	7.2
	4	7.8	10.3	5.8	7.8
	6	10.3	12.9	11.0	13.2
	24	12.0	14.2	11.6	13.5
	30	12.8	16.2	12.9	16.0
		EXTRACTION A		EXTRACTION C	
		^{14}C	^{3}H	^{14}C	^{3}H
INTESTINE	2	5.5	4.0	3.5	5.2
	4	2.3	2.9	1.5	3.9
	6	3.6	4.3	2.7	4.6
	24	2.0	2.6	3.4	5.6
	30	2.0	2.7	2.1	4.2

Table 3.1. The recoveries of radioactivity in the liver and intestine at 2,4,6,24 and 30 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA to normal male Wistar rats (group A). All values (calculated from tissue extracts) have been corrected on the basis of an 85% extraction from the tissues.

Extraction B

Time (hours	Elution position on Sephadex G15 (K_{av}) Tube No.		% Hepatic radioactivity ^{14}C	^{3}H
2	10-17	(0.14)	30	44
	18-22	(0.36)	17	17
	23-28	(0.54)	23	25
4	10-17	(0.11)	47	55
	18-21	(0.30)	13	10
	22-27	(0.54)	21	13
6	10-18	(0.14)	73	70
	19-23	(0.36)	10	11
	24-28	(0.54)	11	17
24	10-16	(0.14)	25	29
	17-22	(0.29)	35	37
	23-28	(0.54)	29	31
30	10-21	(0.22)	56	69
	22-28	(0.54)	36	26
Extraction C				
2	11-20	(0.25)	57	64
	23-28	(0.54)	25	23
4	11-21	(0.22)	63	75
	22-28	(0.54)	21	18
6	11-21	(0.14)	61	59
	23-28	(0.54)	20	24
24	11-21	(0.14)	65	80
30	11-20	(0.14)	67	84

Table 3.2. The distribution of hepatic radiolabelled derivatives following Sephadex G15 chromatography of liver extracts (Extractions B and C) at 2,4,6,24 and 30 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA to normal male Wistar rats (group A). $K_{av} = V_e - V_o / V_t$ V_o, V_o = void volume; V_e = elution volume of solute; V_t = total volume of chromatography bed.

Extraction A

Time (hours)	Elution position on Sephadex G15 (K_{av}) Tube No.		% Intestinal radioactivity ^{14}C	% Intestinal radioactivity ^{3}H
2	11-17	(0.14)	11	16
	18-24	(0.36)	16	24
	28-48	(0.98)	69	55
4	11-17	(0.11)	34	39
	18-24	(0.32)	16	18
	28-48	(0.98)	37	34
6	17-17	(0.11)	36	41
	18-24	(0.36)	18	21
	28-48	(0.98)	39	32
24	11-18	(0.11)	67	74
30	11-18	(0.11)	69	80
Extraction C				
2	11-16	(0.18)	12	14
	17-22	(0.32)	18	22
	23-30	(0.54)	56	56
4	11-17	(0.11)	74	67
	23-28	(0.54)	8	21
6	11-17	(0.14)	42	81
	18-22	(0.32)	22	21
	23-28	(0.54)	31	32
24	11-17	(0.14)	20	23
	18-22	(0.32)	18	22
	23-28	(0.54)	49	48
30	11-18	(0.11)	76	78
	21-28	(0.54)	10	15

Table 3.3. The distribution of intestinal radiolabelled derivatives following Sephadex G15 chromatography of intestinal extracts (extraction A and C) at 2,4,6,24 and 30 hours after the oral administration of a mixture of [2-^{14}C] and [3',5,7,9-^{3}H] FA to normal rats (group A). $K_{av} = V_e - V_o / V_t - V_o$ V_o = void volume; V_e = elution volume of solute V_t = total volume of chromatography bed.

Folate derivative	Sephadex G15 Fraction (K_{av}) (5.0 mls)		DEAE-cellulose NaCl molarity
FA	37	(0.98)	0.96
10CHOFA	22	(0.43)	0.51
10CHOTHF	18	(0.28)	0.42
$5CH_3THF$	37	(0.98)	0.67
5CHOTHF	27	(0.61)	0.62
$5,10CH_2THF$*	25	(0.54)	0.61
$FA(glu)_4$	12	(0.07)	0.56
$10CHOFA(glu)_4$	12	(0.07)	0.52
$5CH_3THF(glu)_4$	12	(0.07)	0.54
Pterin	35	(0.90)	0.35
Xanthopterin*	57	(1.70)	0.54
p.AcBA*	36	(0.94)	0.41
p.Ac.BG*	19	(0.33)	0.41
^3H_2O*	21	(0.39)	0.00

Table 3.4. Chromatographic properties of several folates and folate catabolites on Sephadex G15 (60 x 2 cm) and DEAE-cellulose (40 x 2 cm). $K_{av} = V_e - V_o / V_t - V_o$
V_o = void volume; V_e = elution volume of solute.
V_t = total volume of chromatography bed. * Determined by A. Saleh, Chemistry Department, University of Aston in Birmingham.

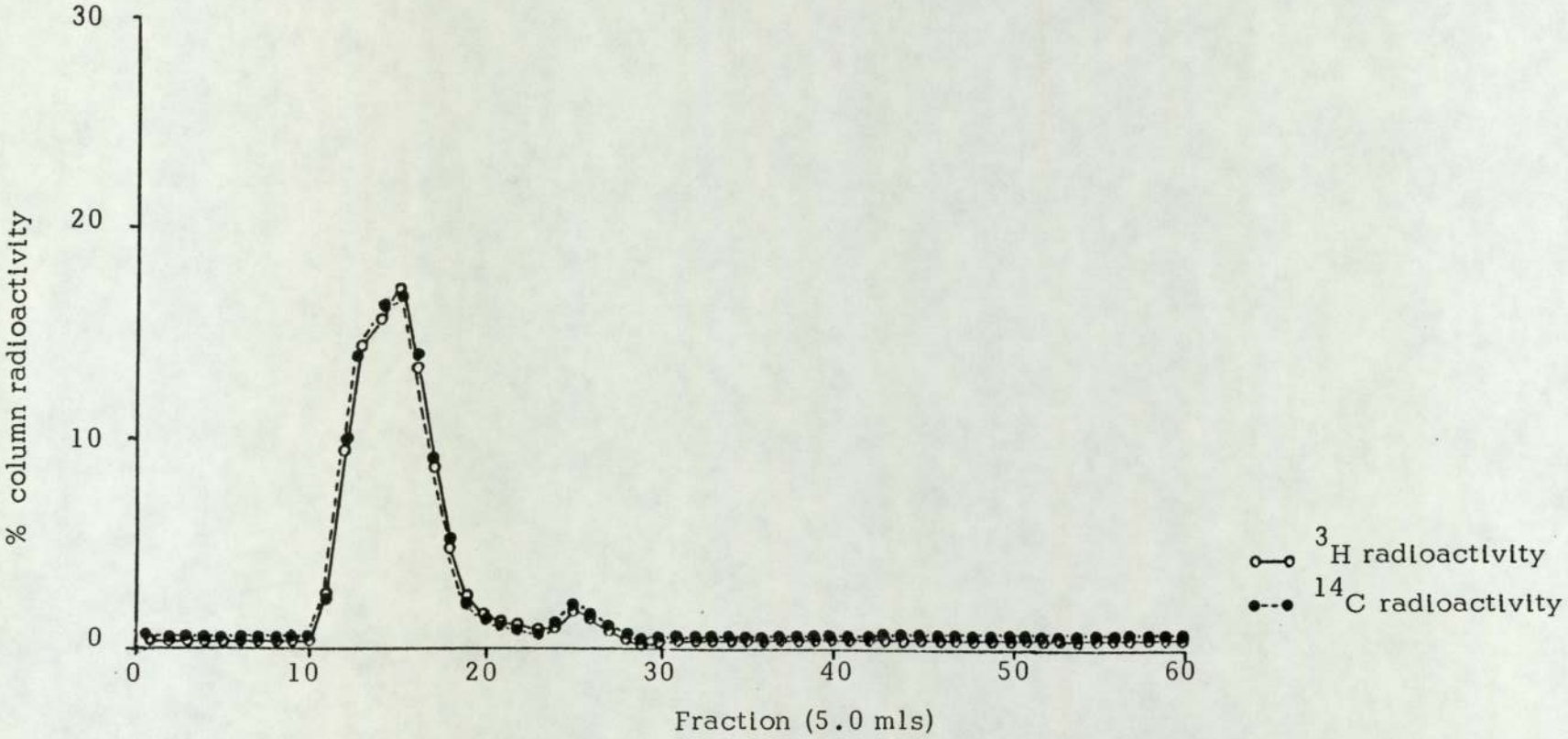


Fig. 3.1. Sephadex G15 chromatography of 48 hour liver extract (extraction A) (normal male Wistar rats).

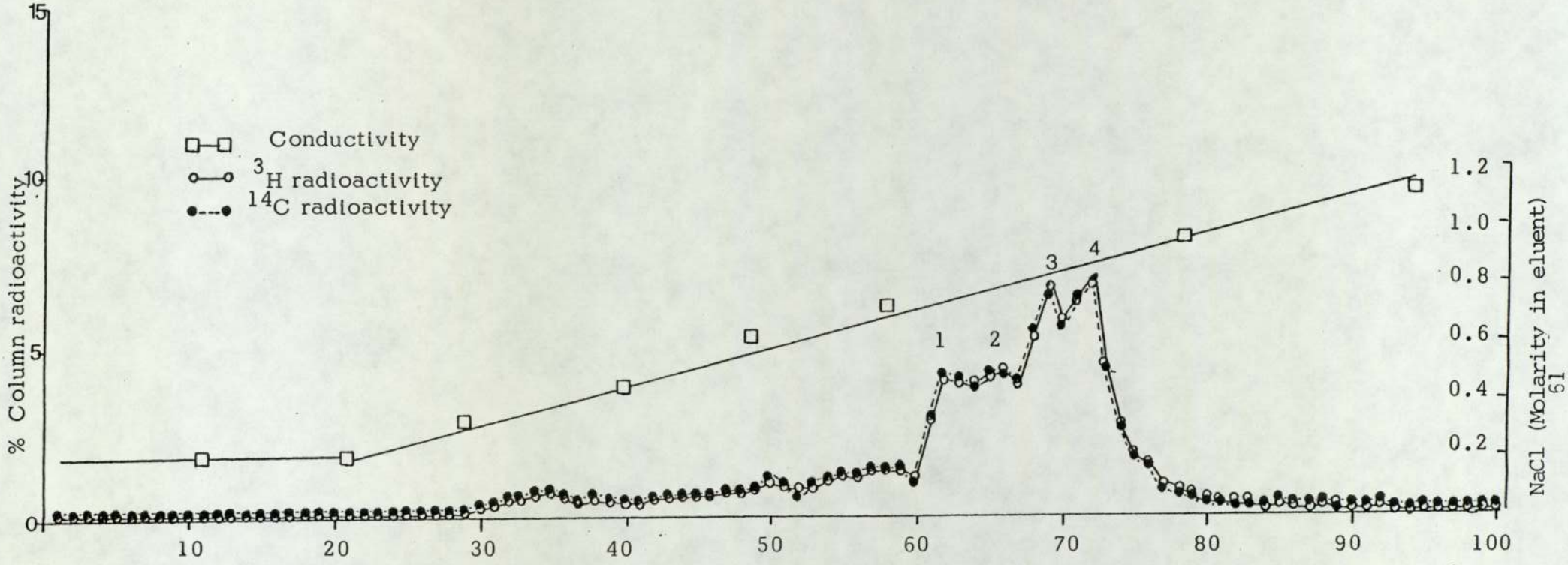


Fig. 3.2. DEAE-cellulose chromatography of major dual labelled peak (fractions 11-19) removed from Sephadex G15 chromatography of 48 hour liver extract (extraction A)
(normal male Wistar rats)

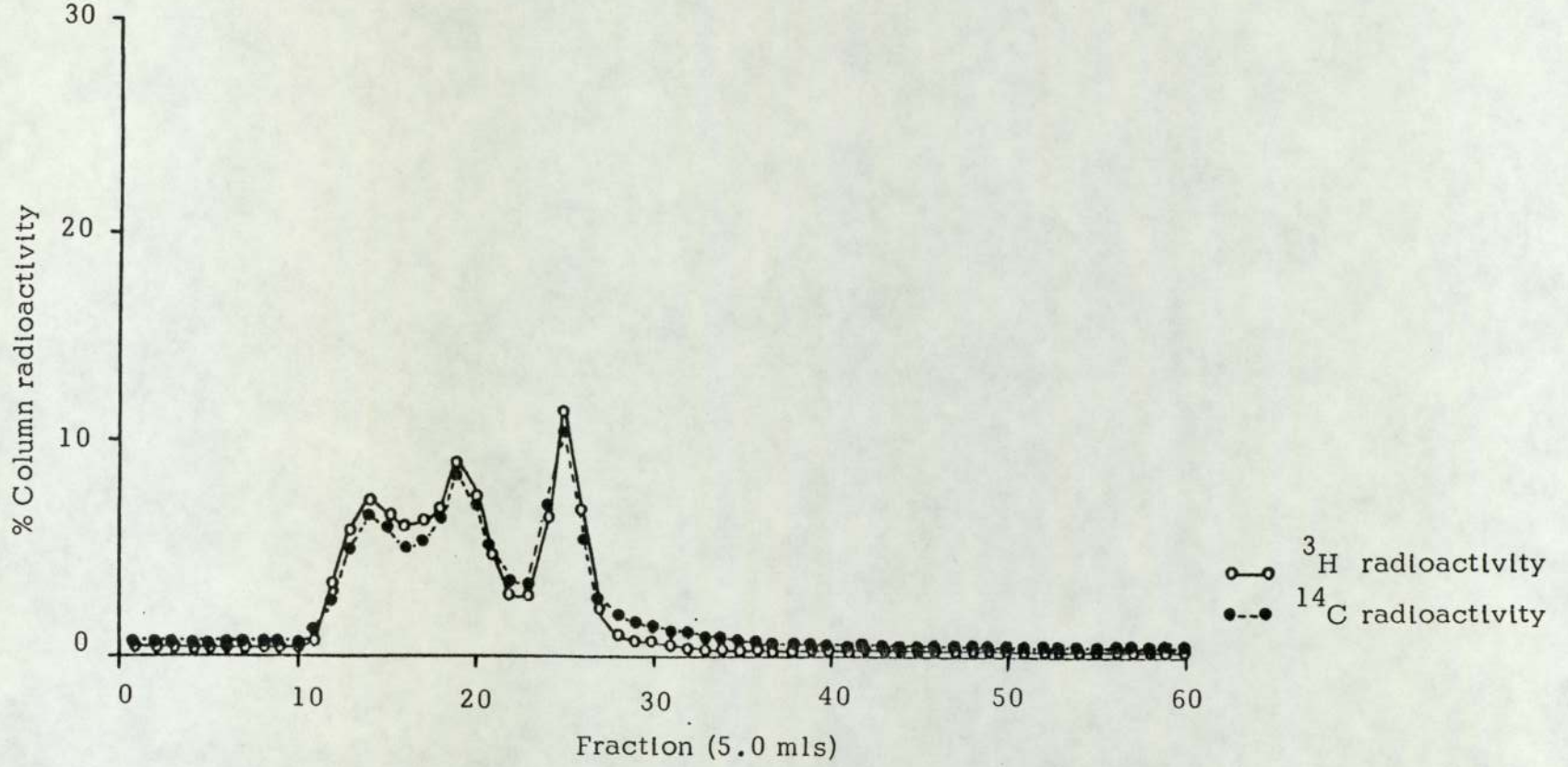


Fig. 3.3. Sephadex G15 chromatography of 24 hour liver extract (extraction B) (normal male Wistar rats).

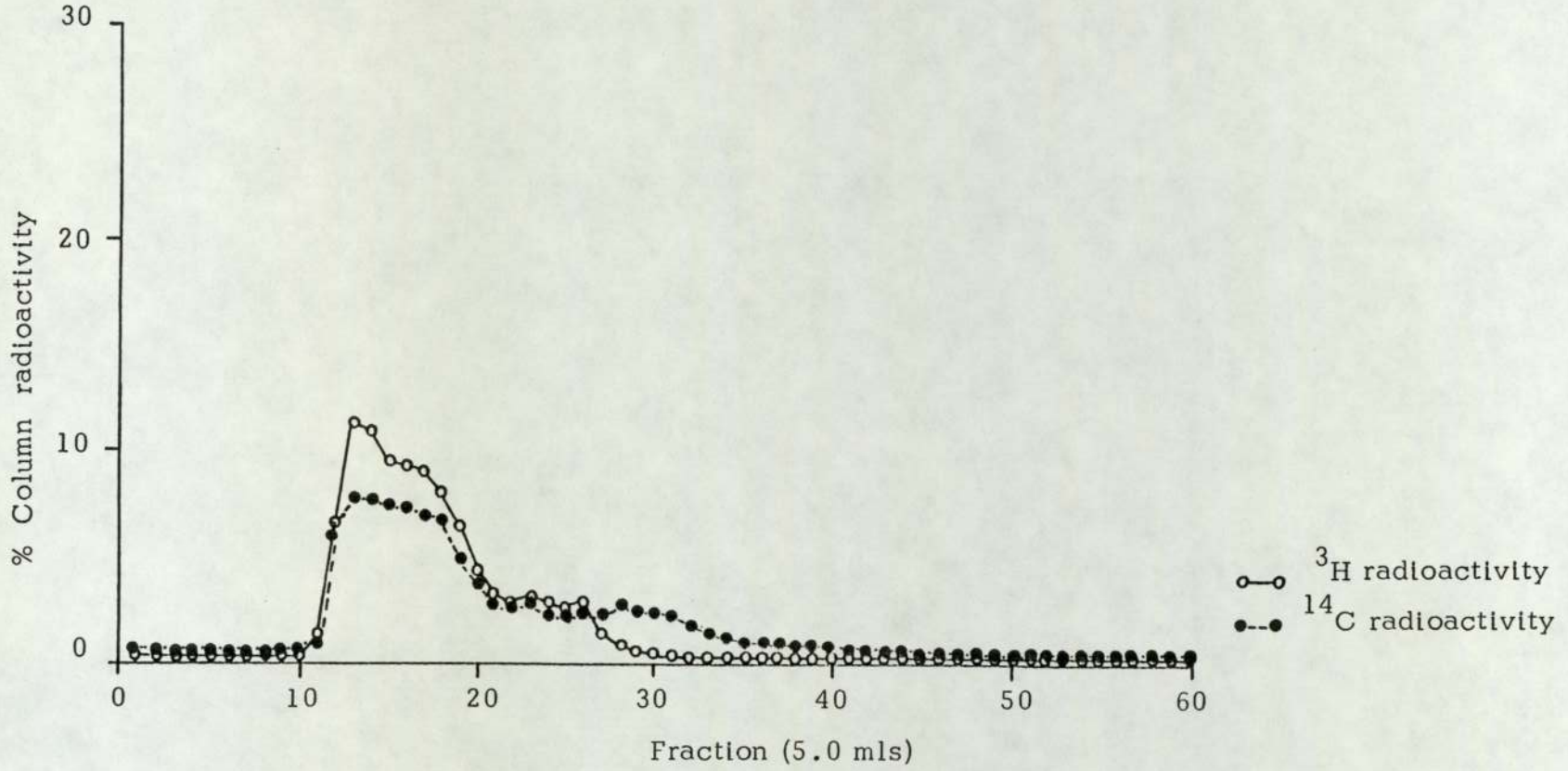


Figure 3.4. Sephadex G15 chromatography of 24 hour liver extract (extraction C) (normal male Wistar rats)

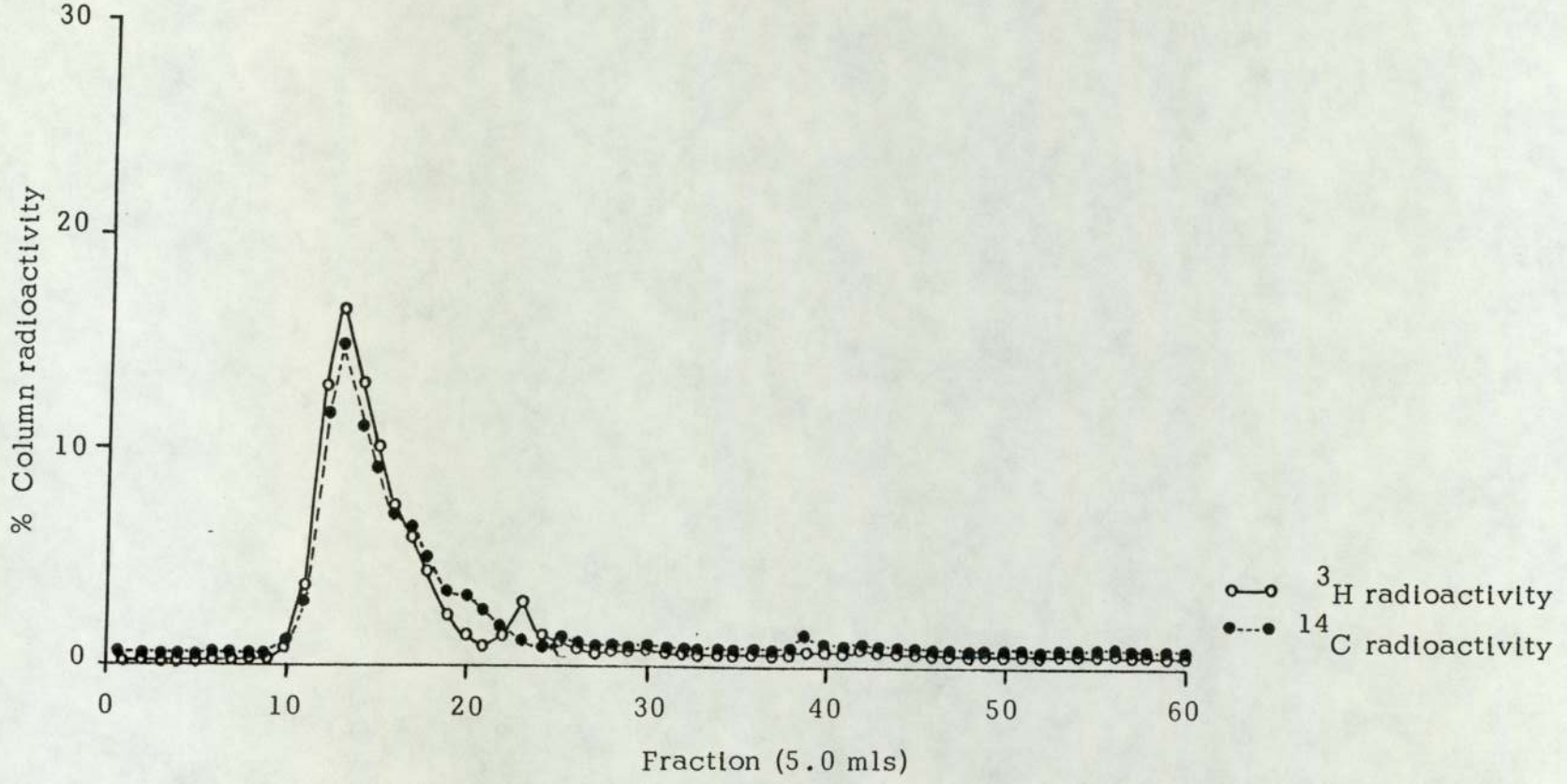


Fig. 3.5. Sephadex G15 chromatography of intestinal extract (extraction A) (normal male Wistar rats).

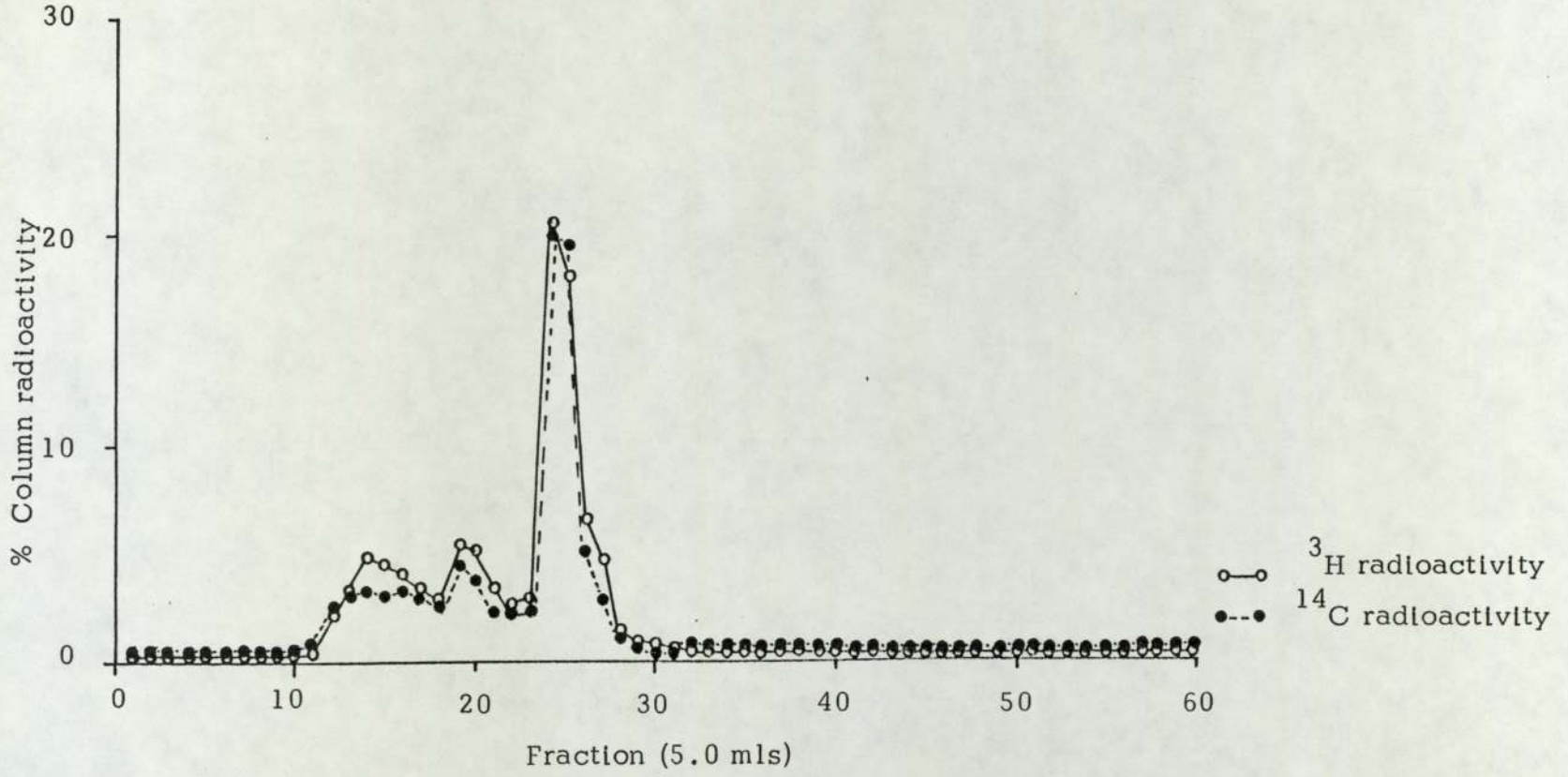


Fig. 3.6. Sephadex G15 chromatography of intestinal extract (extraction C) (normal male Wistar rats).

CHAPTER 4

4.1. The biosynthesis of folate polyglutamates in the tissues of normal and tumour-bearing rats.

4.2. A study of folate binding proteins in the tissues of tumour-bearing rats.

INTRODUCTION

Earlier studies (Experiment 3.1.) demonstrated that the hepatic biosynthesis of folate polyglutamates was complete 24-30 hours after the oral administration of radiolabelled FA as the hepatic levels of these derivatives and the recovery of administered folate were maximal at this time. Experiment 3.2. showed the importance of extraction conditions for the study of folate polyglutamate synthesis. A progressive synthesis of these derivatives between 2-24 hours after FA administration was indicated, however, breakdown of these species was also apparent.

This chapter is concerned with a more detailed investigation of folate polyglutamate synthesis in the tissues of normal and tumour-bearing rats. Further efforts were made to prevent the breakdown of folate polyglutamates by the more rapid heat denaturation of conjugase during extraction; extraction A being employed for these purposes.

The disposition of the folate monoglutamate pool in these tissues and the possibility of tumour induced effects on the folate pool in host tissues, have also been investigated.

A preliminary investigation of folate binding proteins in the tissues of tumour-bearing rats has also been undertaken. These studies employed only two rats for each time period and statistical analysis was not carried out.

METHODS

EXPERIMENT 4.1. The biosynthesis of folate polyglutamates in the tissues of normal and tumour-bearing rats

Normal and tumour-bearing rats groups B (normal male WAB/NOT and male WAB/NOT rats bearing an implanted Sarcoma Mc103B) and C (normal male Wistar rats or male Wistar rats bearing and implanted Walker 256 carcosarcoma) were dosed with a mixture of [2-^{14}C] and [3',5'-7,9-^{3}H] FA. At 2,4,6,10,24 or 48 hours after administration, rats were killed in pairs and the liver and intestine of the normal animals, and the liver, tumour and intestine of the tumour-bearing animals, were extracted (extraction A). Extracts were stored frozen at -20^{o}C.

Analysis of folate extracts (performed between 0-4 months after extract preparation) was carried out using sequentional Sephadex G15 and DEAE-cellulose chromatography. Chromatography columns were calibrated with authentic folate monoglutamates and in some cases, authentic folate polyglutamates.

Sephadex G15 chromatography

Crude tissue extracts were initially chromatographed on Sephadex G15. Fractions 1-60 in the case of normal rats (groups B and C) and fractions 25-50 in the case of tumour-bearing animals (groups B and C) were collected into 1.0 mls sodium ascorbate (2% w/v) to prevent or reduce the oxidation of labile folates.

DEAE-cellulose (DE52) chromatography

Three variations of DE52 chromatography were employed:

DE52 chromatography A: The re-chromatography of folate poly-glutamates eluting as a single peak near the void volume on Sephadex G15.

DE52 chromatography B: The re-chromatography of Sephadex G15 fractions 25-50 (the elution region of FA and $5CH_3THF$).

DE52 chromatography C: In certain cases, whole tissue extracts were chromatographed directly on DEAE-cellulose. Fractions were collected into 1.0 mls sodium ascorbate (2% w/v) in cases where further chromatography of radiolabelled peaks was to be carried out.

EXPERIMENT 4.2. A study of folate binding proteins in the tissues of tumour-bearing rats.

Tumour-bearing rats (group C) (male Wistar) were used; a single rat being employed for each time period. At 2,4,6, 24 and 48 hours after the administration of radiolabelled FA (as described in Section 4.1.), livers, tumours and intestines were extracted (extraction D).

Chromatography

Tissue extracts were chromatographed directly on Sephadex G75 columns. Protein (eluting at the void volume) was detected using the Biuret method (Cornall et al., 1949).

RESULTS

EXPERIMENT 4.1.

Recovery of radioactivity in the tissues

Following oral dosing of radiolabelled FA to normal and tumour-bearing rats, radioactivity was found in the liver, tumour and intestinal tissues. The pattern of recovery of radioactivity in the whole tissues was similar to the pattern of recovery expressed on the basis of unit weight (per gram of tissue, wet weight). These results are presented in Tables 4.1. and 4.2. The tissue weights are given in Table 4.3.

The quantitative recovery of radioactivity between 24-48 hours was highest in the liver and lowest in the intestine of all animals.

LIVER

Hepatic recovery of radioactivity increased progressively, becoming maximal at 24 or 48 hours after administration in all animals. In group B rats, hepatic radioactivity at 2 and 4 hours was lower in normal rats than tumour-bearing rats, however in group C animals, this situation was reversed. At 24 hours, hepatic radioactivity in the tumour-bearing rats was higher than the corresponding normal rats in both groups B and C.

In both groups B and C, hepatic levels of ^{3}H radioactivity were higher than ^{14}C radioactivity at all times. Notably, the hepatic recovery of radioactivity in the normal and tumour-bearing group B rats was normally higher than found in the liver of group C animals.

TUMOUR

In the sarcoma Mc103B, the recovery of radioactivity on the basis of the whole tissue was similar at all times between 2-10 hours and then declined. On the basis of unit weight however, recovery of radioactivity increased progressively becoming maximal at 6 hours and then declined.

In the Walker 256 carcosarcoma, recovery of radioactivity increased progressively and was maximal at 24 hours on the basis of both the whole tissue and unit weight. At 6 and 24 hours, recovery of radioactivity in this tumour was higher than found at the corresponding times in the Sarcoma Mc103B.

INTESTINE

Intestinal radioactivity in the whole tissue and on the basis of unit weight was in most cases maximal at 2-4 hours and then declined. In addition, at 2 and 4 hours in the intestine of groups B and C, recovery of radioactivity was higher in tumour-bearing rats.

Sephadex G15 chromatography

Sephadex G15 chromatography of the crude tissue extracts demonstrated a similar pattern of metabolism in all tissues. This was seen as a progressive increase in the proportion of radioactivity eluting close to V_o (folate polyglutamate) and a progressive decrease in the proportion of radioactivity eluting later (mainly between fractions 25-50) and representing

folate monoglutamates. The levels of radiolabelled folate polyglutamates are presented in Tables 4.4 and 4.5 . A notable exception was that no folate polyglutamates were detected at 6 hours in the Walker 256 carcosarcoma. The chromatograms obtained following Sephadex G15 chromatography of the 2,4,6,10,24 and 48 hour liver extracts (group B rats) are presented in Figs. 4.1. - 4.6.

The proportion of extract radioactivity as folate polyglutamates was maximal at 24 hours in most cases at which time these derivatives represented 58-96% of the tissue radiolabelled folate.

Between 2-6 hours, a minor dual labelled derivative eluting from Sephadex G15 in the position of 10CHOTHF (fractions 18-20) (K_{av} = 0.28) was present in the tumour and intestine (groups B and C) but was not detected in the liver extracts.

DEAE-cellulose (DE52) chromatography

DE52 chromatography A: DE52 chromatography A resolved the dual labelled peak eluting close to V_o on Sephadex G15 into several radiolabelled species in all tissues. The similarity of the chromatographic properties of these derivatives allowed classification as follows:

Folate polyglutamates A and B: These derivatives eluted from DEAE-cellulose between 0.40 and 0.60M NaCl. In most cases, folate polyglutamate A (the earliest eluting species, chromatographing close the the authentic marker 10CHOFA) was the principal derivative.

Folate polyglutamate C: This species eluted from DEAE-cellulose between 0.80 and 1.00M NaCl (just before the authentic marker FA). In certain cases, two derivatives eluted in the region and are referred to as folate polyglutamates C_1 and C_2 in order of elution.

Species T : In most cases a ^{14}C-only labelled species (i.e. a pterin derivative) eluted from DEAE-cellulose between 0.25 and 0.40M NaCl. In some cases, two such derivatives eluted from DEAE-cellulose in this region. In cases where a single such species was present, this is referred to as species T, however when two catabolites were detected; as species T_1 and T_2 in order of elution.

The distribution of folate polyglutamates A, B and C (or C_1 and C_2) and species T (or species T_1 and T_2), as apparent from DE52 chromatography A is as follows :

LIVER

In the liver of normal rats (groups B and C) folate polyglutamates A, B and C (or C_1 and C_2) and species T (or species T_1 and T_2) were detected at all times. Between 2-10 hours in group B normal rats, folate polyglutamates C_1 and C_2 were detected (Fig. 4.7), however, at all other times in the liver of this group and at all times in the liver of group C normal rats, only folate polyglutamate C eluted between 0.80 and 1.00 M NaCl (Fig. 4.8).

In the liver of tumour-bearing rats (groups B and C) the same pattern as that found in the normal liver was apparent, however folate polyglutamate

C (or C_1 and C_2) were not detected.

INTESTINE

In the intestine of normal rats, the pattern of labelled derivatives was similar to that found in the normal liver however, in certain cases folate polyglutamate B was absent and only folate polyglutamate C was detected in the 0.80 - 1.00 M NaCl elution region (see Fig. 4.9).

A similar pattern was also found in the intestine of tumour-bearing rats however, as was the case for the liver of tumour-bearing rats, folate polyglutamate C (or C_1 and C_2) was not detected.

In both normal and tumour-bearing rats, insufficient intestinal radiolabelled folate polyglutamate precluded DE52 chromatography A for this tissue at 2,4 and 6 hours.

TUMOUR

In the tumour of both groups B and C rats, folate polyglutamate A and species T (or T_1 and T_2) were present at all times, however, folate polyglutamate B and C (or C_1 and C_2) were not detected. At two hours, insufficient radioactivity in these tissues precluded DE52 chromatography A. The chromatogram obtained at 24 hours after the administration of radiolabelled FA (Sarcoma Mc103B) is presented in Fig. 4.10.

DE52 chromatography B: DE52 chromatography B demonstrated the presence of $5CH_3THF$ and unmetabolised FA in the three tissues by co-

chromatography with the authentic folates. The chromatogram obtained at 6 hours in the case of the liver (group B tumour-bearing rats) is presented in Fig. 4.11.

Both $5CH_3THF$ and FA demonstrated maximal levels between 2-6 hours and were not detected at 24 or 48 hours. In most cases, a ^{14}C only labelled species eluted between 0.25 - 0.40 M NaCl.

Tissue distribution of $5CH_3THF$

In the normal rats, the proportion of intestinal radiolabelled folate as $5CH_3THF$ between 2-6 hours was at most times higher than found in the liver (Table 4.6). The total amount of radiolabelled $5CH_3THF$ (per gram of tissue, wet weight) between 2-6 hours followed a similar pattern (Table 4.7).

In the tumour-bearing rats, the proportion of tissue radiolabelled folate as $5CH_3THF$ between 2-6 hours was at most times highest in the tumour tissue and lowest in the liver (Table 4.8). The total amount of radiolabelled $5CH_3THF$ (per gram of tissue, wet weight) between 2-6 hours, was highest in the liver and lowest in the tumour of group B animals, but in group C animals, was highest in the intestine and lowest in the liver (Table 4.7).

In the liver of tumour-bearing rats the proportion of hepatic radioactivity as $5CH_3THF$ between 2-6 hours, was at most times higher than found in the corresponding normal rats (Table 4.6 and 4.8). Moreover,

the total amount of radiolabelled $5CH_3THF$ (per gram of tissue, wet weight) between 2-6 hours, was also higher in tumour-bearing than the corresponding normal rats (Table 4.7).

In the intestine of group B animals the proportion of radiolabelled folate as $5CH_3THF$ at 4 hours was higher in normal rats than tumour-bearing rats, however this situation was reversed in group C rats (Table 4.6 and 4.8). The total amount of intestinal $5CH_3THF$ (per gram of tissue; wet weight) between 2-6 hours was similar in normal and tumour-bearing rats of group B animals, however was lower in normal than tumour-bearing group C rats.

Tissue distribution of FA

In both normal and tumour-bearing rats the proportion of tissue radiolabelled folate as unmetabolised FA between 2-6 hours was generally highest in the intestine (Table 4.9 and 4.11).

In tumour-bearing rats the proportion of tissue radiolabelled folate as FA between 2-6 hours was lowest in the liver for group B rats but lowest in the tumour for group C animals (Table 4.11). The total amount of radiolabelled FA (per gram of tissue wet weight) between 2-6 hours followed a similar pattern (Table 4.10).

In the liver of tumour-bearing rats the proportion of radiolabelled folate as FA between 2-6 hours was lower than in the normal liver of group B rats, however, this situation was reversed in group C rats (Table 4.9 and

and 4.11). The total amount of radiolabelled FA (per gram of tissuet; wet weight) in the liver between 2-6 hours, was similar in both normal and tumour bearing rats (table 4.10).

In the intestine between 2-4 hours the proportion of radiolabelled folate as FA was higher in tumour-bearing animals than normal animals (Tables 4.9 and 4.11). The total amount of intestinal FA was similar in both normal and tumour-bearing group B rats, however, in group C animals, was higher in the tumour-bearing rats (Table 4.10).

DE52 chromatography C

DEAE-cellulose chromatography of the 24 hour crude tissue extracts (previously shown to contain only folate polyglutamates) revealed a similar pattern of radiolabelled derivatives as was observed following DE52 chromatography A. However, certain differences were apparent

1) Folate polyglutamate C was detected in all tissues (liver, tumour and intestine) of both groups of tumour-bearing rats

2) A species eluting in the position of folate polyglutamate B was detected in both tumour tissues.

Rate of synthesis of folate polyglutamates

The rate of synthesis of folate polyglutamates was essentially linear in all tissues between 0-6 hours, thus allowing a direct comparison of initial rates. These results in the case of group B tumour-bearing rats are presented graphically in Fig. 4.15.

In most cases, the linear correlation coefficients for a plot of the tissue levels of folate polyglutamates (% of the dose radioactivity as folate polyglutamate, per gram of tissue wet weight) versus time (at 0,2, 4, and 6 hours) were> 0.95. As no folate polyglutamates were detected in the Walker 256 carcasarcoma at 6 hours, this necessitated the calculation of the initial rate of folate polyglutamate synthesis between 0-4 hours. The levels of folate polyglutamate in the tissues expressed as a proportion of the dose radiolabelled folate per gram of tissue (wet weight) and the initial rate of folate polyglutamate synthesis, are presented in Tables 4.12 and 4.13 respectively.

The initial rate of synthesis of folate polyglutamates was highest in the liver of all animals. Furthermore, this was 2-4 times greater in the liver of group B rats (normal and tumour-bearing) than group C rats (normal and tumour-bearing).

In group B animals the initial rate of folate polyglutamate synthesis was similar in both normal and tumour-bearing rats, however, in group C animals this was lower in the tumour-bearing than the normal rats.

In both groups of normal and tumour-bearing rats the initial rate of folate polyglutamate synthesis was similar in the tumour and intestine within each group. Notably, a comparison of the tumour tissues revealed a 2-3 fold greater initial rate of synthesis in the Sarcoma Mcl03B compared to the Walker 256 carcosarcoma.

EXPERIMENT 4.2.

Sephadex G75 chromatography of the crude tissue extracts revealed a different pattern of protein-bound and non-protein-bound folate or folate catabolites in the three tissues investigated. In most cases however, tissue radiolabelled derivatives were predominantly unbound. These results are presented in Table 4.14.

In the liver at all times, approximately 20-30% of the tissue radioactivity co-chromatographed with protein at the void volume (i.e. protein-bound) as a dual labelled peak; the remaining radioactivity eluting later (not preotein-bound) between fractions 15-35 (see Fig. 4.12).

In the tumour tissue protein bound radiolabelled derivatives were detected at all times, however in each case this was predominantly ^{3}H labelled. Protein-bound radioactivity was maximal at 2 hours at which time this represented 33% of the ^{14}C and 85% of the ^{3}H radioactivity in the tissue. It 4,6, 24 and 48 hours, the proportion of bound radioactivity was considerably less than found at 2 hours; representing from 0-11% of the ^{14}C and 9-16% of the ^{3}H radioactivity in the tissue (see Fig. 4.13).

In the intestine, protein-bound radioactivity was virtually undetectable (see Fig. 4.14).

Other studies

1) Folate polyglutamate A present in the liver of group B normal rats at 48 hours, co-chromatographed on DEAE-cellulose with authentic $10CHOFA(glu)_4$. Differential microbiological assay of folate polyglutamate A, present at 24 hours in the liver of normal group B rats, showed it to be active for both L. casei and S. faecalis (but inactive for P. cerevisiae), producing a several fold increase in activity after conjugase treatment. Folate polyglutamate B, also present in this tissue at 24 hours, produced a similar microbiological response.

2) Further study of folate polyglutamate C present in the normal liver (group B) at 24 hours, revealed that it re-chromatographed at the void volume on Sephadex G15; confirming it to be a folate polyglutamate. This species gave the microbiological response expected for a $5CH_3THF$ polyglutamate (i.e. active for L. casei but inactive for S. faecalis and P. cerevisiae and producing a several fold increase in activity after conjugase treatment) but did not co-chromatograph on DEAE-cellulose with authentic $5CH_3THF(glu)_4$ or $FA(glu)_4$.

3) Species T, present at 48 hours in the liver of group B tumour-bearing rats, co-chromatographed on DEAE-cellulose with authentic pterin (2-amino-4-hydroxy pteridine).

DISCUSSION

These studies show that following the oral administration of radiolabelled FA to normal and tumour-bearing rats the distribution of radiolabelled derivatives between 2-48 hours was similar in all tissues. An overview of this process shows a progressive metabolism of FA to one or two reduced folate monoglutamates and finally to the synthesis of folate polyglutamates.

The levels of all radiolabelled folate monoglutamates were maximal between 2-6 hours and were not detected at 24 or 48 hours. The dual labelled derivative present at 2,4 and 6 hours and eluting in fraction 18-20 following Sephadex G15 chromatography of tumour and intestinal extracts is probably 10CHOTHF as no other folate monoglutamates elute in this region. However, the possiblity that this derivative was a short chain folate polyglutamate cannot be excluded.

The ^{14}C only labelled derivative (species T) was shown to be pterin (2-amino-4-hydroxy pteridine) since in the case of the 48 hour liver extract (tumour-bearing rats, group B) this species co-chromatographed with the authentic compound. On account of the similar elution properties, species T_1 or T_2 may also be pterin.

The presence of a tumour mass appears to influence the quantitative recovery of folate in the tissues. Thus at 24 hours, radiolabelled folate recovered in the liver of tumour-bearing rats was higher than found in the liver of the corresponding normal animals. Tumour-induced effects on the

distribution of radiolabelled folate derivatives were also evident. The increase in the proportion of radiolabelled $5CH_3THF$ in the liver of tumour-bearing rats between 2-6 hours, compared to the corresponding normal liver, was at the expense of FA in group B animals (i.e. the levels of folate polyglutamates were similar) but at the expense of folate polyglutamates in group C animals.

Sephadex G15 chromatography demonstrated a progressive synthesis of folate polyglutamates in all tissues; this appearing complete at 24 hours at which time these were the principal tissue folate derivatives and quantitative tissue recovery of administered folate maximal. The absence of folate polyglutamates at 6 hours in the Walker 256 carcosarcoma is probably an isolated case of erroneous breakdown occurring during the extraction procedure, as has previously been observed (Experiment3.2).

Certain qualitative differences in the pattern of folate polyglutamates in the tissues were apparent following DE52 chromatography A. Thus, in the liver folate polyglutamates A and B were detected, in the intestine folate polyglutamate A (or A and B) were detected whereas in the tumour tissue, only folate polyglutamate A was detected. The contradictory observation following DE52 chromatography C (i.e. chromatography of whole extracts) of the 24 hour tumour extracts; namely the detection of a species with similar chromatographic properties as folate polyglutamate B, may reflect either partial breakdown or the presence of a folate species which was not detected by the initial DE52 chromatography A on account of prior resolution from the

folate polyglutamates by the initial Sephadex G15 step.

DE52 chromatography A also demonstrated folate polyglutamates eluting between 0.80 and 1.00M NaCl (i.e. folate polyglutamate C or C_1 and C_2) which were not detected in the tissues of tumour-bearing animals. The non-detection of these derivatives in these tissues probably resulted from oxidation breakdown due to the absence of appropriate precautions to prevent this occurring during or after the initial Sephadex G15 step. In this respect,during the initial Sephadex G15 chromatography step of normal tissue extracts, folate polyglutamates eluting at the void volume were collected into ascorbate, whereas this was not the case for the tissue extracts of tumour-bearing animals. Further indication that the loss of folate polyglutamate C (or C_1 and C_2) resulted from breakdown and loss is apparent following DE52 chromatography C, which revealed folate polyglutamate C in all 24 hour tissue extracts. In this case the single chromatographic procedure was probably more favourable for preventing the breakdown of these derivatives. A corollary of this observation is that folate polyglutamate C (or C_1 and C_2) is very labile and readily breaks down unless appropriate precautions are taken to prevent this occurring. The detection of folate polyglutamate C_1 and C_2 in the liver of group B normal rats between 2-10 hours and similarly absent in the corresponding host liver shows that both are labile and may therefore be of the same class(one or both differing in the length of the γ-glutamyl chain) as folate polyglutamate C.

The detection of pterin following DE52 chromatography A shows that

cleavage of the central C_9-N_{10} bond occurred during chromatography. Although a ^3H labelled p-aminobenzoyl derivative, the second fragment of the cleavage process was not clearly resolved following DE52 chromatography A, the excess ^3H radioactivity associated with folate polyglutamate A indicates the presence of such a derivative. Moreover, this would appear to be a p-aminobenzoyl polyglutamate derivative.

EXPERIMENT 4.2

The proportion of tissue radiolabelled derivatives bound to protein-differed considerably between the three tissues. The detection of a dual labelled protein-bound peak in the case of the liver indicates the binding of intact folate whereas in the tumour tissue the bound radioactivity indicated the predominant binding of a p-aminobenzoyl derivative (or derivatives). Intestinal bound folate appeared negligible from this study.

		GROUP B rats				GROUP C rats			
Tissue	Time	^{14}C		^{3}H		^{14}C		^{3}H	
Liver	2	4.8	(0.37)	5.0	(0.39)	5.4	(0.62)	7.2	(0.82)
	4	10.1	(1.00)	10.6	(1.05)	6.2	(0.78)	7.3	(0.92)
	6	16.7	(1.62)	17.1	(1.66)	8.0	(1.02)	9.3	(1.19)
	10	14.9	(2.16)	16.5	(2.39)	9.3	(1.24)	10.2	(1.36)
	24	16.7	(1.46)	18.6	(1.63)	9.9	(1.08)	12.5	(1.35)
	48	-		-		12.4[x]	(1.46)	14.2[x]	(1.68)
Intestine	2	2.7	(0.43)	2.7	(0.43)	4.0	(0.83)	4.2	(0.87)
	4	4.6	(0.82)	4.0	(0.71)	4.2	(0.83)	4.3	(0.85)
	6	3.9	(0.82)	3.9	(0.82)	2.3	(0.40)	2.5	(0.44)
	10	3.6	(0.67)	3.9	(0.72)	4.5	(0.73)	5.2	(0.84)
	24	2.6	(0.47)	2.9	(0.52)	4.1	(0.60)	4.4	(0.64)
	48	-		-		1.6[x]	(0.24)	2.5[x]	(0.37)

Table 4.1. The recovery of radioactivity in the liver and intestine of group B (male WAB/NOT) and C (male Wistar) normal rats between 2-48 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: % dose radioactivity in whole tissue; values in parenthesis indicate the percentage of dose radioactivity per gram (wet weight) of tissue. All values are an average of 2 rats except x (determined from a single rat).

Tissue	Time (h)	GROUP B RATS ^{14}C	GROUP B RATS ^{3}H	GROUP C RATS[x] ^{14}C	GROUP C RATS[x] ^{3}H
Liver	2	9.4 (0.93)	10.9 (1.08)	2.6 (0.16)	3.2 (0.20)
	4	12.5 (1.30)	15.6 (1.62)	4.1 (0.45)	5.2 (0.57)
	6	12.7 (1.78)	14.7 (2.07)	5.2 (0.65)	8.2 (1.02)
	10	14.3 (1.96)	16.2 (2.21)	-	-
	24	18.5 (1.65)	20.5 (1.83)	12.2 (1.15)	16.6 (1.47)
	48	-	-	12.6 (1.39)	15.5 (1.72)
Tumour	2	2.8 (0.47)	3.3 (0.55)	1.7 (0.19)	2.0 (0.22)
	4	2.5 (0.53)	2.8 (0.59)	2.9 (0.44)	3.2 (0.48)
	6	2.2 (0.73)	2.6 (0.87)	5.3 (0.85)	5.6 (0.90)
	10	3.0 (0.68)	3.4 (0.77)	-	-
	24	1.8 (0.45)	2.2 (0.55)	7.6 (0.96)	8.4 (1.06)
	48	-	-	3.4 (0.60)	4.0 (0.70)
Intestine	2	4.9 (0.85)	4.8 (0.84)	10.7 (1.97)	8.9 (1.64)
	4	5.5 (0.96)	5.5 (0.96)	5.4 (1.27)	4.1 (0.97)
	6	3.3 (0.70)	3.9 (0.83)	3.6 (0.87)	3.5 (0.84)
	10	2.8 (0.55)	3.3 (0.65)	-	-
	24	2.9 (0.44)	3.4 (0.51)	2.1 (0.45)	2.4 (0.51)
	48	-	-	1.5 (0.30)	1.7 (0.34)

Table 4.2. The recovery of radioactivity in the liver, tumour and intestine of group B (WAB/NOT) and C (male Wistar) tumour-bearing rats between 2-48 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: % dose radioactivity in whole tissue; values in parenthesis indicate the percentage of dose radioactivity per gram (wet weight) of tissue. All values are an average of 2 rats except x (determined from a singe rat).

TISSUE WEIGHTS (g).

Tissue	Time (h)	GROUP B		GROUP C	
		N	TB	N	TB
Liver	2	12.9	10.1	8.7	16.2
	4	10.1	9.6	7.9	9.1
	6	10.3	7.1	7.8	8.0
	10	6.9	7.3	7.5	-
	24	11.4	11.2	9.2	10.6
	48	-	-	8.5	9.0
Tumour	2	-	6.0	-	8.9
	4	-	4.7	-	6.6
	6	-	3.0	-	6,2
	10	-	4.4	-	-
	24	-	4.0	-	7.9
	48	-	-	-	5.7
Intestine	2	6.3	5.7	4.8	5.4
	4	5.6	5.7	5.1	4.2
	6	4.7	4.7	5.7	4.1
	10	5.4	5.1	6.2	-
	24	5.5	6.6	6.8	4.7
	48	-	-	6.7	5.0

Table 4.3. The whole tissue weights (wet weight) of group B (male WAB/NOT) and C (male Wistar) normal and tumour-bearing rats. Values are an average of 2 rats.

Tissue	Time (h)	GROUP B RATS ^{14}C	GROUP B RATS ^{3}H	GROUP C RATS ^{14}C	GROUP C RATS ^{3}H
Liver	2	53 (2.5)	53 (2.6)	30 (1.6)	40 (2.9)
	4	65 (6.6)	66 (7.0)	37 (2.3)	46 (3.3)
	6	79(13.2)	80(13.7)	78 (6.2)	78 (7.2)
	10	93(13.9)	92(15.2)	91 (8.5)	92 (9.4)
	24	96(16.0)	95(17.7)	58 (5.7)	78 (9.7)
	48	-	-	78[X](9.7)	89[X](12.6)
Intestine	2	11 (0.3)	13 (0.3)	5 (0.2)	9 (0.4)
	4	14 (0.6)	17 (0.7)	8 (0.3)	10 (0.4)
	6	42 (1.6)	37 (1.4)	42 (1.0)	47 (1.2)
	10	50 (1.8)	57 (2.2)	89 (4.0)	85 (4.4)
	24	92 (2.4)	77 (2.2)	93 (3.8)	87 (3.8)
	48	-	-	-	-

Table 4.4. The distribution of radiolabelled folate polyglutamates in group B (male WAB/NOT) and C (male Wistar) normal rats between 2-48 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: Percentage of total radioactivity in the tissues as radiolabelled folate polyglutamate; values in parenthesis indicate the percentage of dose radioactivity in the whole tissue as radiolabelled folate polyglutamate. All values are an average of two rats except X (determined from a single rat).

Tissue	Time (h)	GROUP B RATS ^{14}C	GROUP B RATS ^{3}H	GROUP C RATS [x] ^{14}C	GROUP C RATS [x] ^{3}H
Liver	2	44 (4.2)	52 (5.7)	38 (1.0)	37 (1.2)
	4	57 (7.1)	67(10.4)	34 (1.4)	43 (2.2)
	6	68 (8.7)	78(11.5)	52 (2.7)	51 (4.2)
	10	91(13.0)	92(14.9)	-	-
	24	93(17.2)	97(19.8)	91(11.1)	95(15.8)
	48	-	-	52 (6.6)	71(11.1)
Tumour	2	22 (0.6)	22 (0.7)	12 (0.2)	15 (0.3)
	4	32 (0.8)	32 (0.9)	14 (0.4)	22 (0.7)
	6	36 (0.8)	46 (1.2)	ND	ND
	10	78 (2.3)	77 (2.6)	-	-
	24	84 (1.5)	75 (1.7)	75 (5.7)	86 (7.2)
	48	-		32 (1.1)	32 (1.3)
Intestine	2	8 (0.4)	13 (0.6)	6 (0.6)	18 (1.6)
	4	19 (1.0)	20 (1.1)	5 (0.3)	3 (0.3)
	6	55 (1.8)	51 (2.0)	11 (0.4)	17 (0.6)
	10	74 (2.1)	72 (2.4)	-	-
	24	91 (2.6)	85 (2.9)	71 (1.5)	87 (2.1)
	48	-	-	80 (1.2)	82 (1.4)

Table 4.5. The distribution of radiolabelled folate polyglutamates in group B (male WAB/NOT) and C (male Wistar) tumour-bearing rats between 2-48 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: Percentage of total radioactivity in the tissues as radiolabelled folate polyglutamate. All values are an average of two rats except x (determined from a single rat) ND : not detected.

		GROUP B RATS		GROUP C RATS	
Tissue	Time (hrs)	^{14}C	^{3}H	^{14}C	^{3}H
Liver	2	6.6 (0.32)	5.6 (0.28)	11.5 (0.62)	6.5 (0.47)
	4	5.7 (0.88)	7.7 (0.82)	11.5 (0.71)	12.1 (0.88)
	6	3.2 (0.54)	3.6 (0.61)	ND	ND
	10	1.1 (0.17)	0.7 (0.12)	ND	ND
	24	ND	ND	ND	ND
	48	-	-	ND[x]	ND[x]
Intestine	2	16.6 (0.45)	15.9 (0.43)	24.4 (0.98)	22.4 (0.94)
	4	23.2 (1.07)	15.7 (0.63)	13.4 (0.56)	10.3 (0.44)
	6	11.2 (0.44)	7.6 (0.30)	5.5 (0.13)	2.7 (0.07)
	10	ND	ND	ND	ND
	24	ND	ND	ND	ND
	48	-	-	ND[x]	ND[x]

Table 4.6. The distribution of radiolabelled $5CH_3THF$ in the liver and intestine of group B (male WAB/NOT) and C (male Wistar) normal rats between 2-48 hours after the oral administration of a mixture of [2-^{14}C] an [3',5',7,9-^{3}H] FA. Units: percentage of total radioactivity in whole tissues as radiolabelled $5CH_3THF$; values in parenthesis indicate percentage of dose radioactivity in whole tissues as radiolabelled $5CH_3THF$. All values are an average of two rats except x (determined from a single rat). ND: not detected.

	Total amount of radiolabelled $5CH_3THF$ between 2-6 hrs, per gram of tissue			
	GROUP B RATS		GROUP C RATS	
Noral rats				
Liver	0.15	0.15	0.16	0.19
Intestine	0.35	0.24	0.38	0.32
Tumour-bearing rats				
Liver	0.29	0.38	0.22^x	0.26^x
Tumour	0.19	0.21	0.29^x	0.29^x
Intestine	0.26	0.24	0.72^x	0.68^x

Table 4.7. The total amounts of radiolabelled $5CH_3THF$ in the tissues of group B (male WAB/NOT) and C (male Wistar) normal and tumour-bearing rats between 2- 6 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: Cumulative amount of tissue radioactivity as $5CH_3THF$ (as a percentage of the dose radioactivity) per gram (wet weight)of tissue . All values are an average of two rats except x (determined from a single rat).

Tissue	Time (h)	GROUP B RATS ^{14}C	GROUP B RATS ^{3}H	GROUP C RATS [x] ^{14}C	GROUP C RATS [x] ^{3}H
Liver	2	11.7 (1.10)	14.0 (1.53)	24.2 (0.63)	29.6 (0.95)
	4	6.5 (0.82)	8.1 (1.27)	12.2 (0.50)	10.7 (0.56)
	6	5.9 (0.75)	4.5 (0.67)	12.8 (0.67)	16.7 (1.37)
	10	ND	ND	-	-
	24	ND	ND	ND	ND
	48	-	-	ND	ND
Tumour	2	14.2 (0.40)	13.6 (0.45)	22.9 (0.39)	18.5 (0.37)
	4	11.6 (0.29)	13.6 (0.38)	23.4 (0.68)	22.5 (0.72)
	6	7.7 (0.17)	5.7 (0.15)	20.0 (1.06)	18.7 (1.05)
	10	ND	ND	-	-
	24	ND	ND	ND	ND
	48	-	-	ND	ND
Intestine	2	10.0 (0.49)	8.1 (0.39)	18.6 (2.00)	22.5 (2.01)
	4	9.6 (0.53)	9.8 (0.54)	14.2 (0.77)	15.8 (0.65)
	6	11.5 (0.38)	9.4 (0.37)	14.4 (0.52)	13.1 (0.46)
	10	ND	ND	-	-
	24	ND	ND	ND	ND
	48	-	-	ND	ND

Table 4.8. The distribution of radiolabelled $5CH_3THF$ in the liver, tumour and intestine of group B (male WAB/NOT) and C (male Wistar) tumour-bearing rats between 2-48 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]FA. Units: percentage of total radioactivity in whole tissues as radiolabelled $5CH_3THF$; values in parenthesis indicate the percentage of dose radioactivity in the whole tissue as radiolabelled $5CH_3THF$. All values are an average of two rats except x (determined from a single rat). ND = Not detected.

Tissue	Time (h)	GROUP B RATS ^{14}C	GROUP B RATS ^{3}H	GROUP C RATS ^{14}C	GROUP C RATS ^{3}H
Liver	2	6.4 (0.31)	4.4 (0.22)	7.6 (0.41)	3.5 (0.25)
	4	4.0 (0.41)	3.4 (0.37)	6.9 (0.42)	5.1 (0.37)
	6	1.9 (0.32)	2.2 (0.38)	ND	ND
	10	0.6 (0.10)	0.4 (0.07)	ND	ND
	24		ND	ND	ND
	48	-	-	ND^{x}	ND^{x}
Intestine	2	26.6 (0.72)	17.7 (0.48)	14.2 (0.57)	6.1 (0.26)
	4	17.6 (0.81)	8.7 (0.35)	19.9 (0.85)	13.5 (0.58)
	6	14.6 (0.57)	8.7 (0.34)	6.4 (0.15)	4.9 (0.12)
	10	ND	ND	ND	ND
	24	ND	ND	ND	ND
	48	-	-	ND^{x}	ND^{x}

Table 4.9. The distribution of radiolabelled FA in the liver and intestine of group B (male WAB/NOT) and C (male Wistar) normal rats between 2-48 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: Percentage of total radioactivity in whole tissues as radiolabelled FA; values in parenthesis indicate percentage of dose radioactivity in whole tissues as radiolabelled FA. All values are an average of two rats except x (determined from a single rat). ND; not detected.

	Total amount of radiolabelled FA between 2-6 hours, per gram of tissue			
	GROUP B RATS		GROUP C RATS	
	^{14}C	^{3}H	^{14}C	^{3}H
Normal rats				
Liver	0.09	0.09	0.11	0.08
Intestine	0.37	0.21	0.46	0.28
Tumour-bearing rats				
Liver	0.08	0.09	0.11^{x}	0.19^{x}
Tumour	0.21	0.30	0.04^{x}	0.06^{x}
	0.57	0.20	1.97^{x}	0.80^{x}

Table 4.10 The total amounts of radiolabelled FA in the tissues of group B (male WAB/NOT) and C (male Wistar) normal and tumour-bearing rats between 2-6 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: Cumulative amount of tissue radioactivity as FA (as a percentage of the dose radioactivity) per gram (wet weight) of tissue . All values are an average of two rats except x (determined from a single rat).

		GROUP B RATS		GROUP C RATS[x]	
Tissue	Time (hrs)	^{14}C	^{3}H	^{14}C	^{3}H
Liver	2	3.5 (0.32)	2.7 (0.30)	23.8 (0.62)	30.3 (0.97)
	4	1.9 (0.24)	2.5 (0.39)	7.0 (0.29)	7.3 (0.38)
	6	1.5 (0.19)	1.4 (0.20)	6.7 (0.35)	9.8 (0.81)
	10	ND	ND	-	-
	24	ND	ND	ND	ND
	48	-	-	ND	ND
Tumour	2	15.0 (0.42)	18.4 (0.61)	7.6 (0.13)	8.5 (0.17)
	4	16.0 (0.40)	20.0 (0.56)	5.8 (0.17)	8.4 (0.27)
	6	8.1 (0.18)	6.1 (0.16)	ND	ND
	10	ND	ND	-	-
	24	ND	ND	ND	ND
	48	-	-	ND	ND
Intestine	2	35.1 (1.72)	7.5 (0.36)	55.8 (5.97)	30.3 (2.47)
	4	20.9 (1.15)	10.2 (0.56)	44.4 (2.40)	21.4 (0.88)
	6	6.1 (0.20)	4.8 (0.19)	17.7 (0.64)	9.1 (0.32)
	10	ND	ND	-	-
	24	ND	ND	ND	ND
	48	-	-	ND	ND

Table 4.11 The distribution of radiolabelled FA in the liver, tumour and intestine of group B (male WAB/NOT) and C (male Wistar) tumour-bearing rats between 2-48 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: Percentage of total radioactivity in whole tissues as radiolabelled FA; values in parenthesis indicate percentage of dose radioactivity in whole tissue as radiolabelled FA. All values are an average of two rats except x (determined from a single rat).

	Time (hrs)	GROUP B RATS ^{14}C	GROUP B RATS ^{3}H	GROUP C RATS ^{14}C	GROUP C RATS ^{3}H
Normal rats					
Liver	2	0.20	0.21	0.19	0.32
	4	0.65	0.69	0.29	0.42
	6	1.28	1.33	0.79	0.93
Intestine	2	0.05	0.05	0.04	0.08
	4	0.11	0.12	0.07	0.08
	6	0.34	0.30	0.17	0.21
Tumour-bearing rats					
Liver	2	0.41	0.56	0.06	0.07
	4	0.74	1.08	0.15	0.24
	6	1.21	1.61	0.34	0.54
Tumour	2	0.10	0.12	0.02	0.03
	4	0.17	0.19	0.06	0.10
	6	0.26	0.40	ND	ND
Intestine	2	0.07	0.11	0.11	0.29
	4	0.18	0.19	0.06	0.03
	6	0.38	0.42	0.09	0.14

Table 4.12 The levels of radiolabelled folate polyglutamate in the tissues of group B (male WAB/NOT) and C (male Wistar) normal and tumour-bearing rats at 2,4 and 6 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: Percentage of dose radioactivity as radiolabelled folate polyglutamate per gram (wet weight) of tissue: ND : not detected.

	GROUP B RATS		GROUP C RATS	
	^{14}C	^{3}H	^{14}C	^{3}H
Normal rats				
Liver	0.21 (0.97)	0.22 (0.98)	0.12 (0.94)	0.14 (0.97)
Intestine	0.05 (0.93)	0.05 (0.95)	0.03 (0.96)	0.03 (0.93)
Tumour-bearing rats				
Liver	0.20 (0.99)	0.27 (0.99)	0.05 (0.96)	0.09 ((0.96)
Tumour	0.04 (0.99)	0.06 (0.98)	0.02 (0.98)*	0.02 (0.97)*
Intestine	0.06 (0.97)	0.07 (0.97)	0.01 (0.59)	0.01 (0.15)

Table 4.13 The initial rates of synthesis of radiolabelled folate polyglutamate in group B (male WAB/NOT) and C (male Wistar) normal and tumour-bearing rats between 0-6 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. Units: Percentage of dose radioactivity converted to radiolabelled folate polyglutamate, per gram of tissue, per hour: values in parenthesis give the corresponding linear correlation coefficients calculated from a linear plot of the levels of folate polyglutamate in the tissues (as % of dose radioactivity per gram) versus time (hours): * calculated from the levels of radiolabelled folate polyglutamate in the tissues at 2 and 4 hours only.

Tissue	Time (hrs)	% tissue radiolabelled folate bound to protein (eluting at V_o)		% tissue radiolabelled folate not bound to protein (eluting in fractions 15-35)	
		^{14}C	^{3}H	^{14}C	^{3}H
Liver	2	19	30	74	54
	4	21	24	63	62
	6	26	25	66	66
	24	31	27	65	71
	48	34	23	55	66
Tumour	2	33	84	55	14
	4	11	15	87	80
	6	3	10	93	83
	24	3	9	91	87
	48	ND	16	97	81
Intestine	2	1	3	96	91
	4	3	5	91	91
	6	2	2	92	96
	24	1	2	98	98
	48	3	3	91	90

Table 4.14 The distribution of protein-bound and non-protein-bound radioactivity in the liver, tumour and intestine of group C (male Wistar) tumour-bearing rats between 2-48 hours after the oral administration of a mixture of [2-^{14}C] and [3',5',7,-9,-^{3}H] FA. Units: Percentage of total tissue radioactivity eluting at the void volume (protein-bound) and in fractions 15-35 (non-protein-bound). All values determined from a single rat.

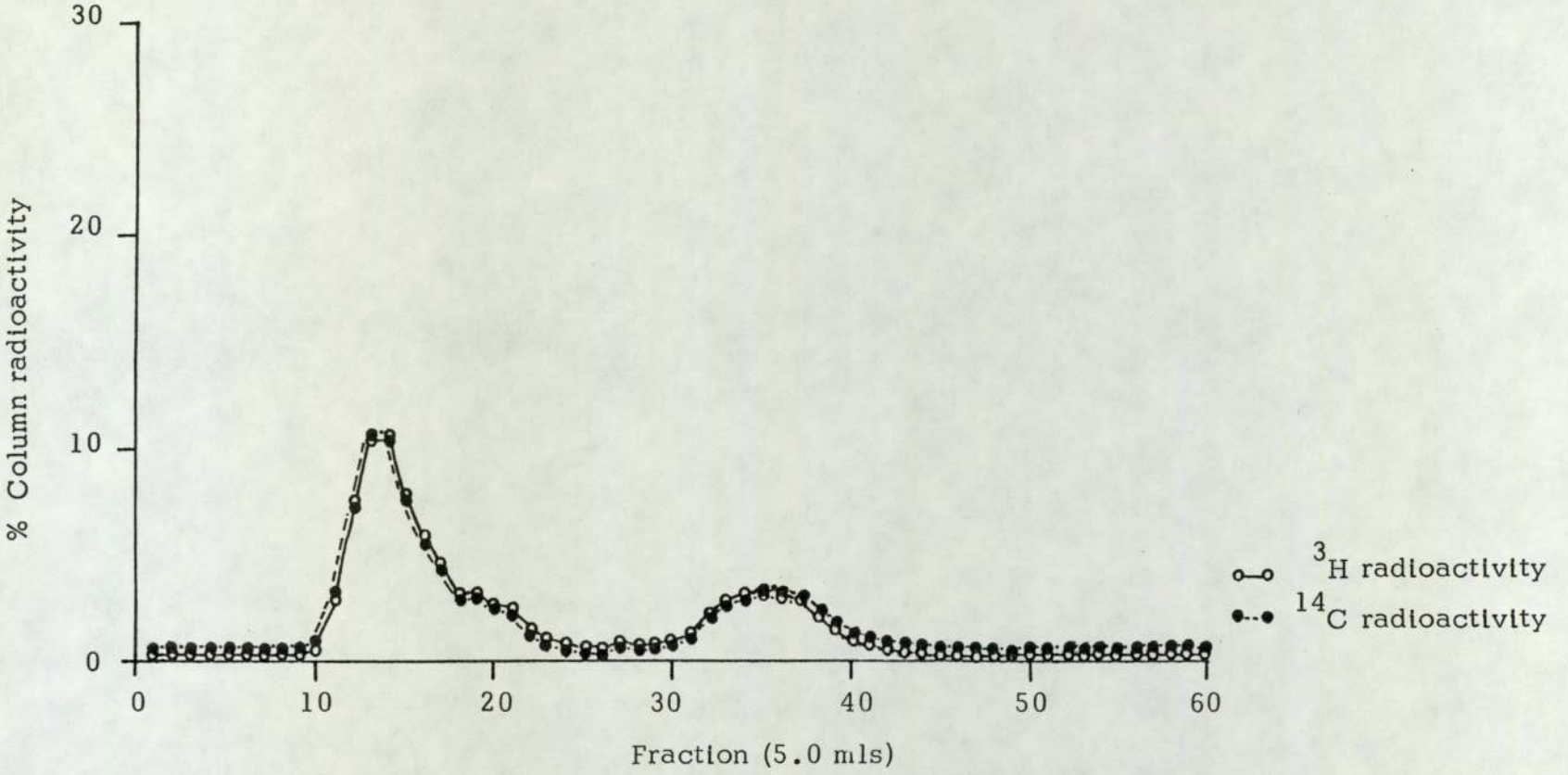


Fig. 4.1. Sephadex G15 chromatography of 2 hour liver extract (extraction A) (normal Male WAB/NOT rats).

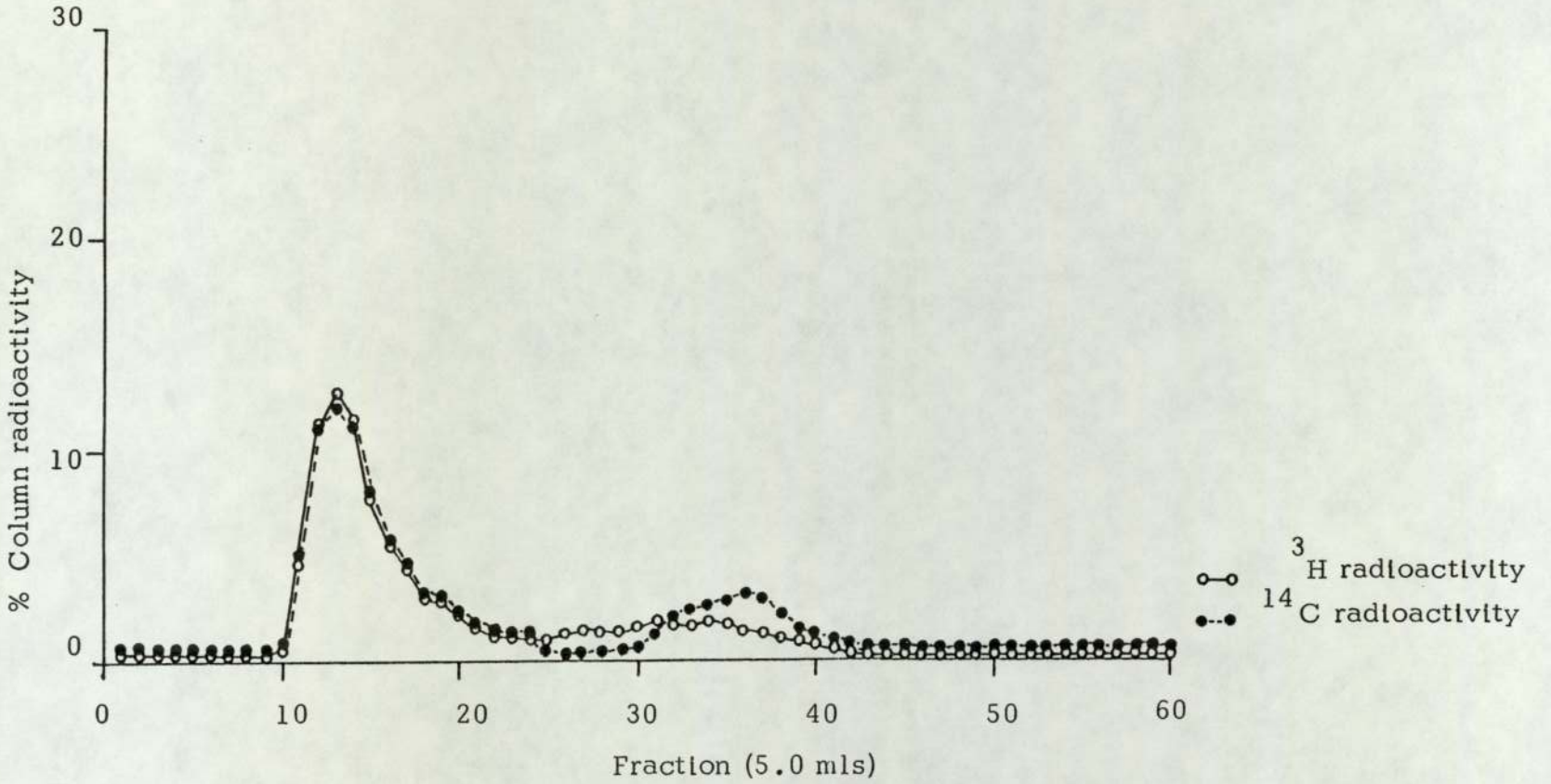


Fig. 4.2. Sephadex G15 chromatography of 4 hour liver extract (extraction A) (normal male WAB/NOT rats)

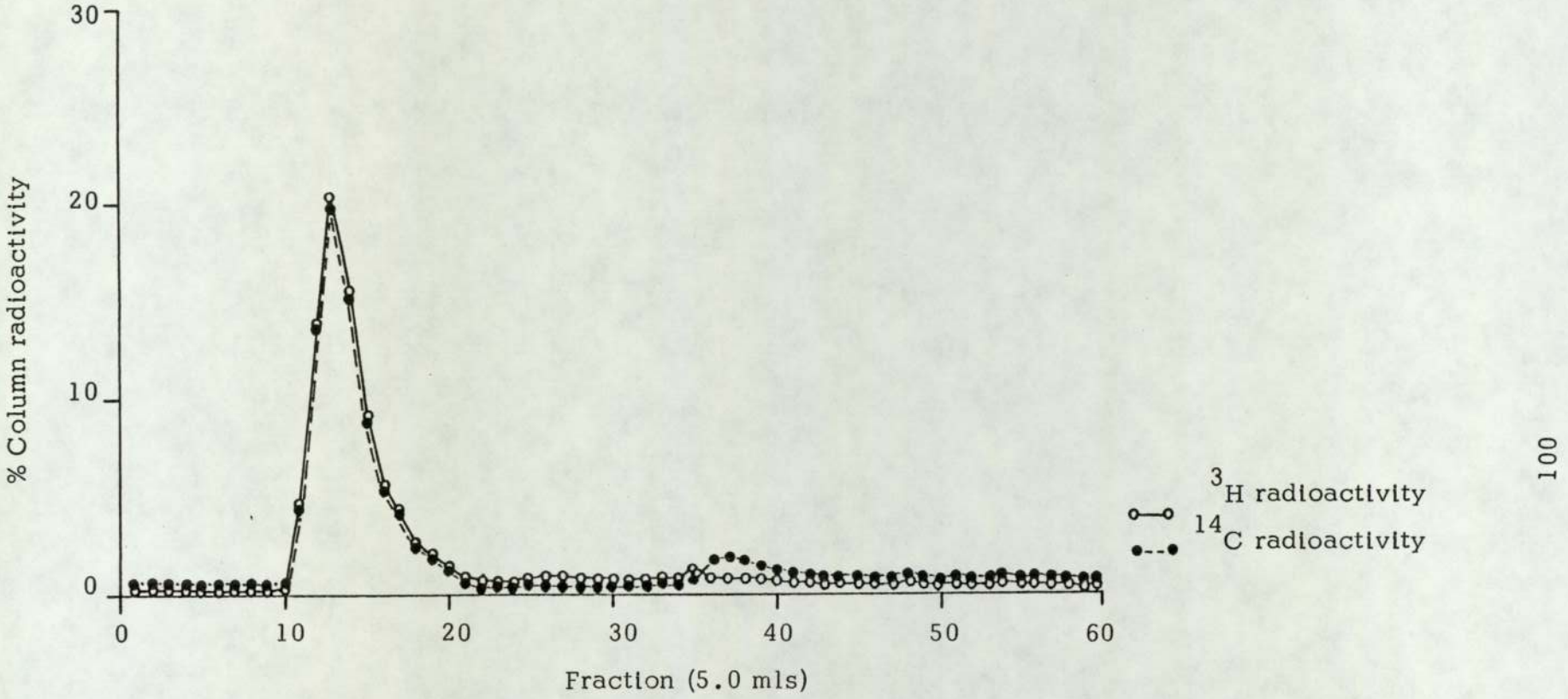


Fig. 4.3. Sephadex G15 chromatography of 6 hour liver extract (extraction A) (normal male WAB/NOT rats).

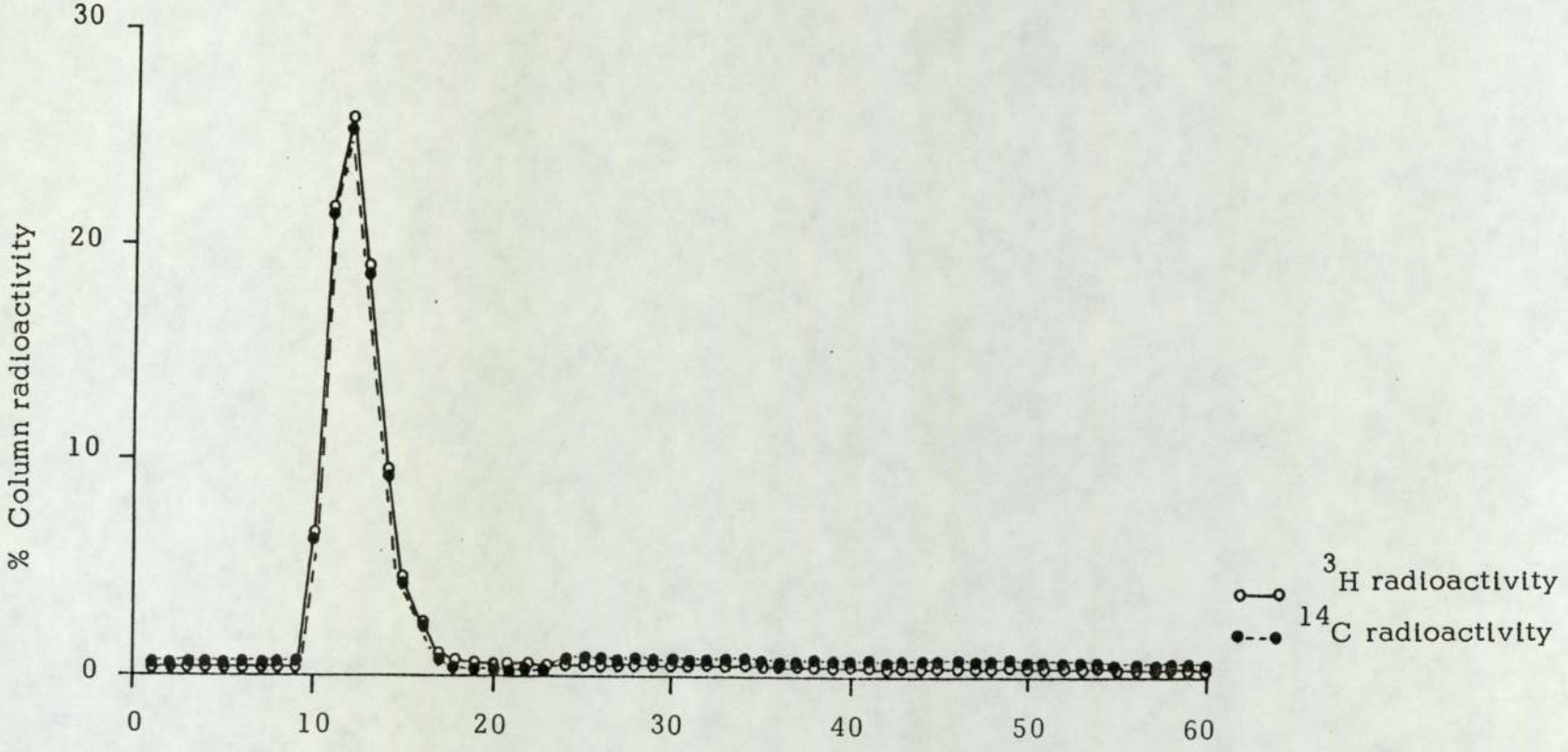


Fig. 4.4. Sephadex G15 chromatography of 10 hour liver extract (extraction A) (normal male WAB/NOT rats)

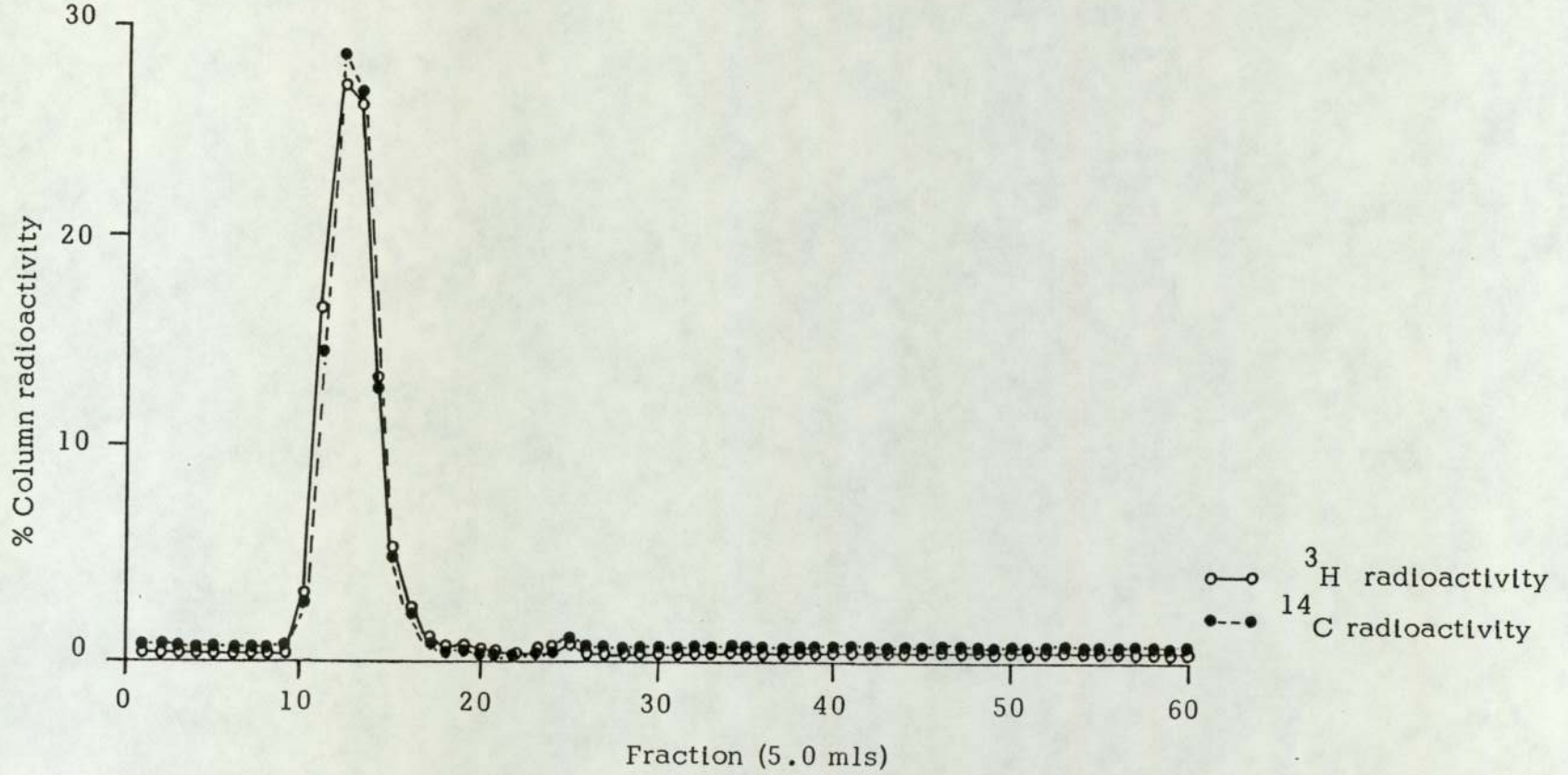


Fig. 4.5 Sephadex G15 chromatography of 24 hour liver extract (extraction A) (normal male WAB/NOT rats)

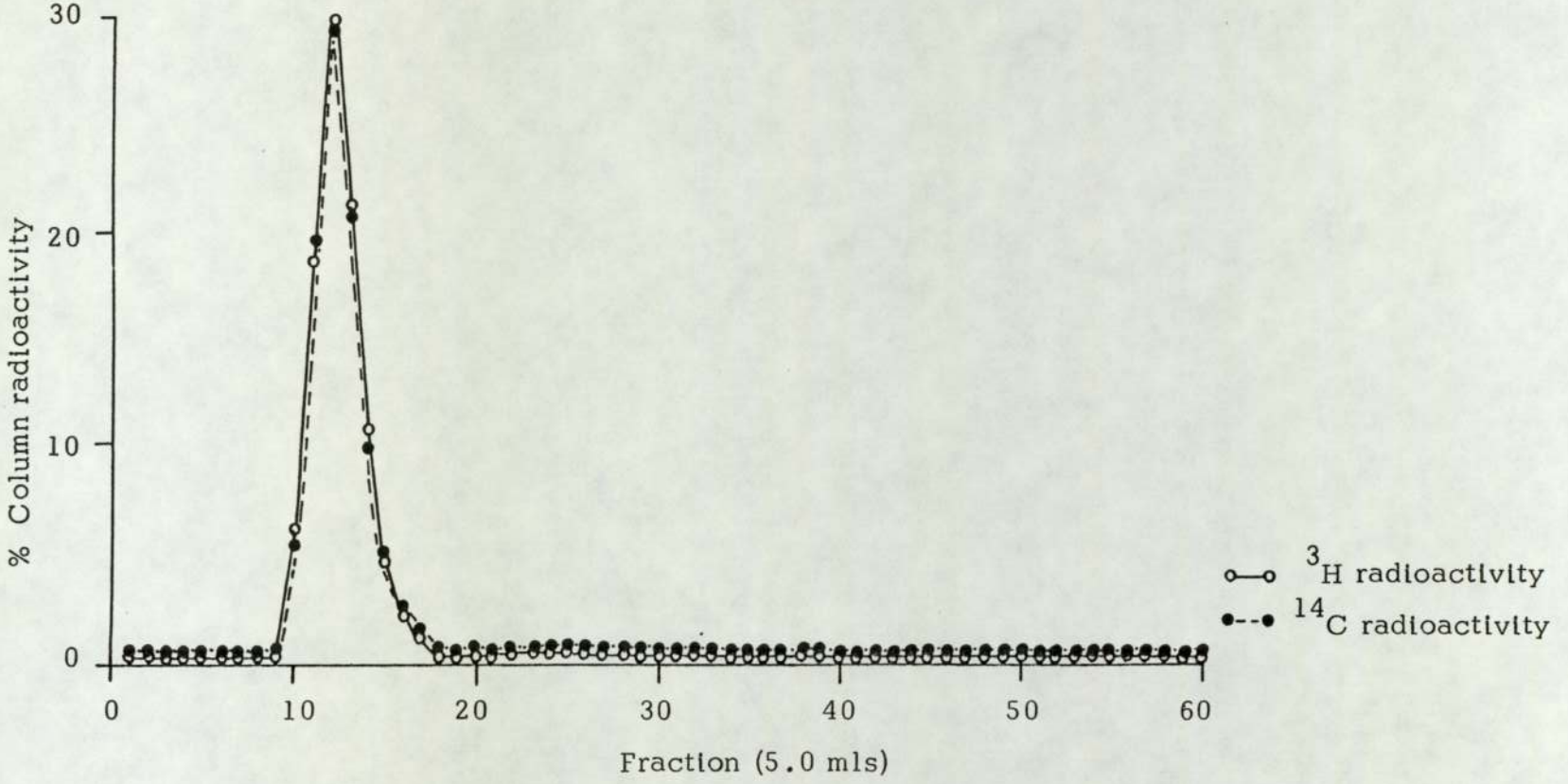


Fig. 4.6 Sephadex G15 chromatography of 48 hour liver extract (extraction A)(normal male WAB/NOT rats)

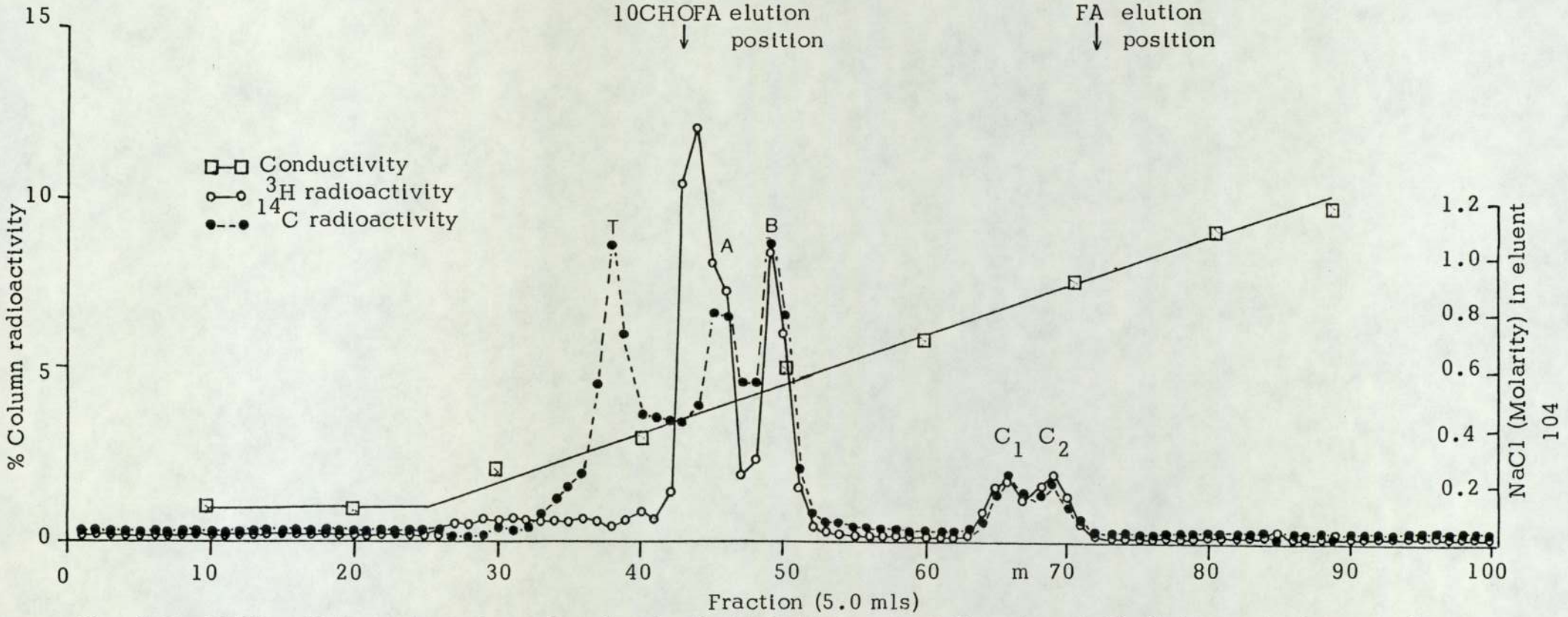


Fig. 4.7. DEAE-cellulose chromatography of major dual labelled peak eluting close to V_o (folate polyglutamates) (fractions 11-18) following Sephadex G15 chromatography of 6 hour liver extract (extraction A) (normal WAB/NOT rats)

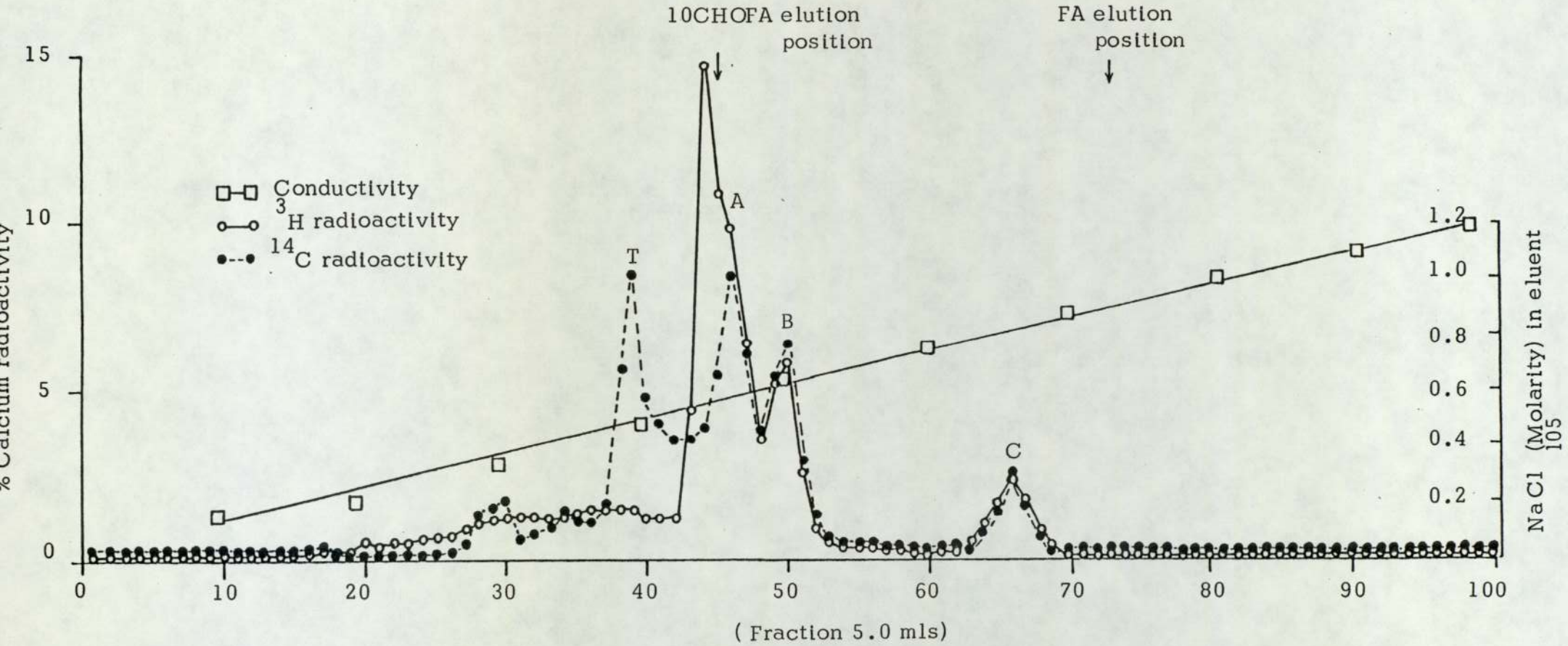


Fig. 4.8. DEAE-cellulose chromatography of major dual labelled peak eluting close to V_o (folate polyglutamate) (fractions 11-18) following Sephadex G15 chromatography of 48 hour liver extract (extraction A) (normal WAB/NOT rats).

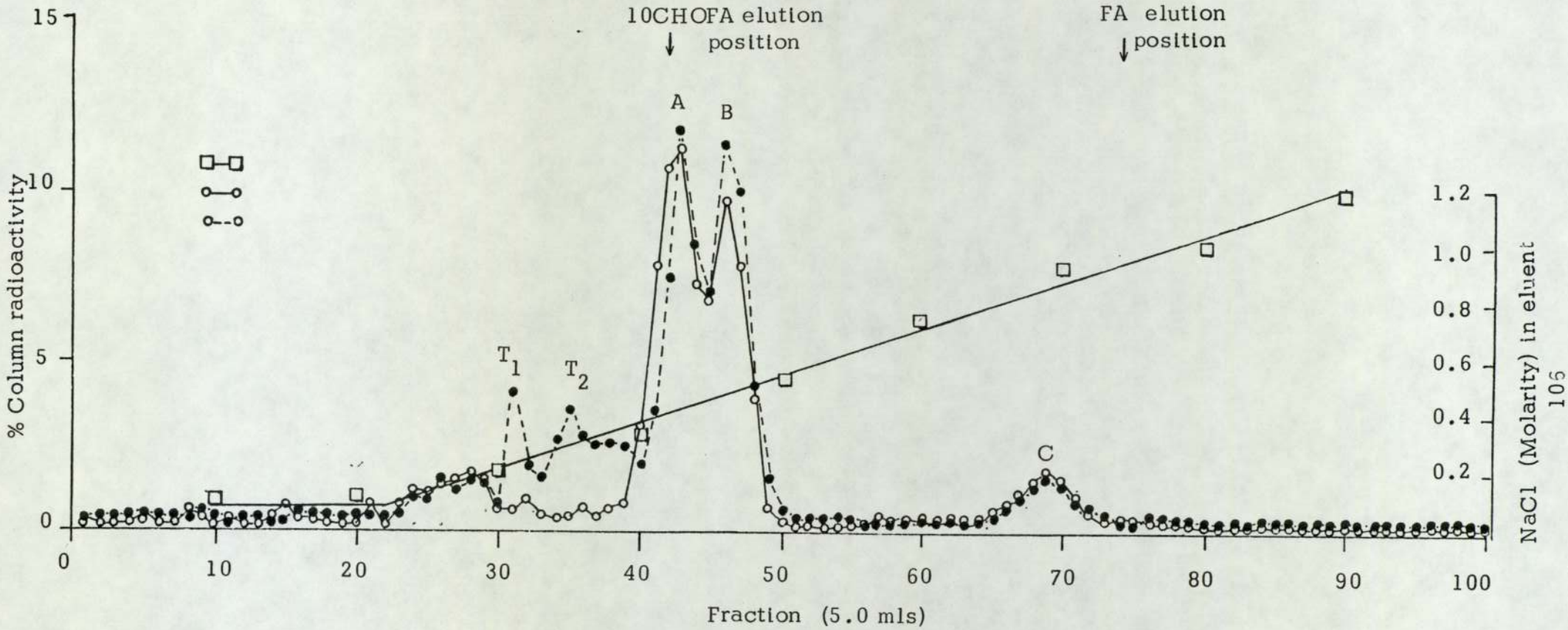


Fig. 4.9 DEAE-cellulose chromatography of major dual labelled peak eluting close to V_o (folate polyglutamate) (fractions 11-18) following Sephadex G15 chromatography of 48 hour intestinal extract (extraction A) (normal male WAB/NOT rats) (group B)

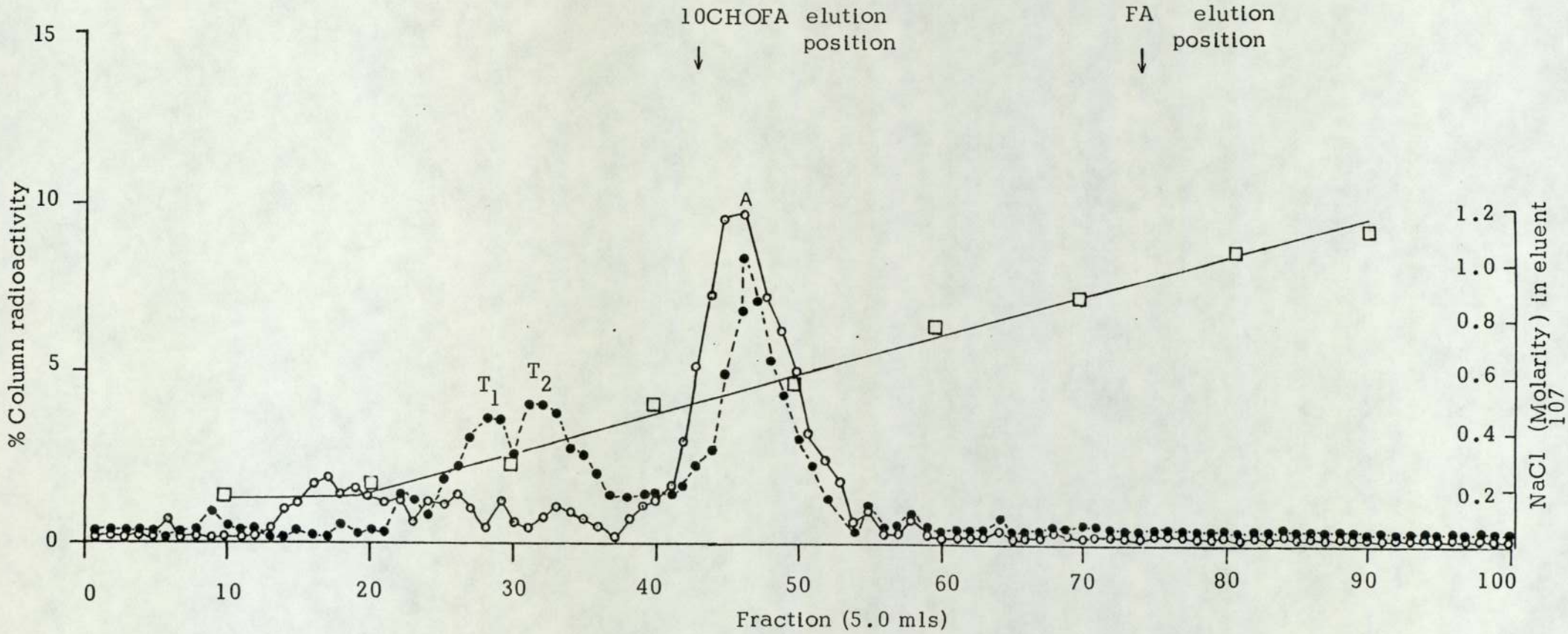


Fig. 4.10 DEAE-cellulose chromatography of major dual labelled peak eluting close to V_o (folate polyglutamates) (fraction 11-18) following Sephadex G15 chromatography of 24 hour tumour (Sarcoma Mc103B) extract (extraction A) (Sarcoma Mc103B bearing male WAB/NOT rats) (group B)

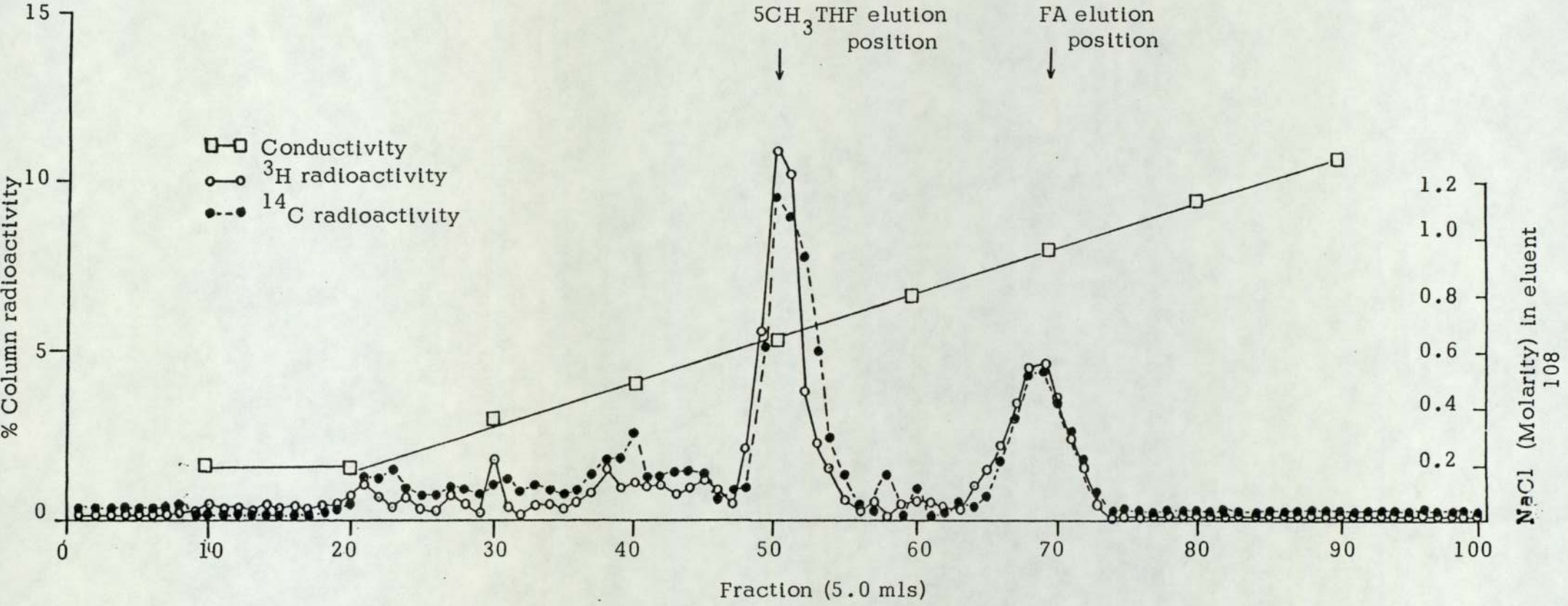


Fig. 4.11 DEAE-cellulose chromatography of fractions 25-50 obtained following Sephadex G15 chromatography of 6 hour liver extract (extraction A) (Sarcoma Mc103B bearing male WAB/NOT rats) (group B).

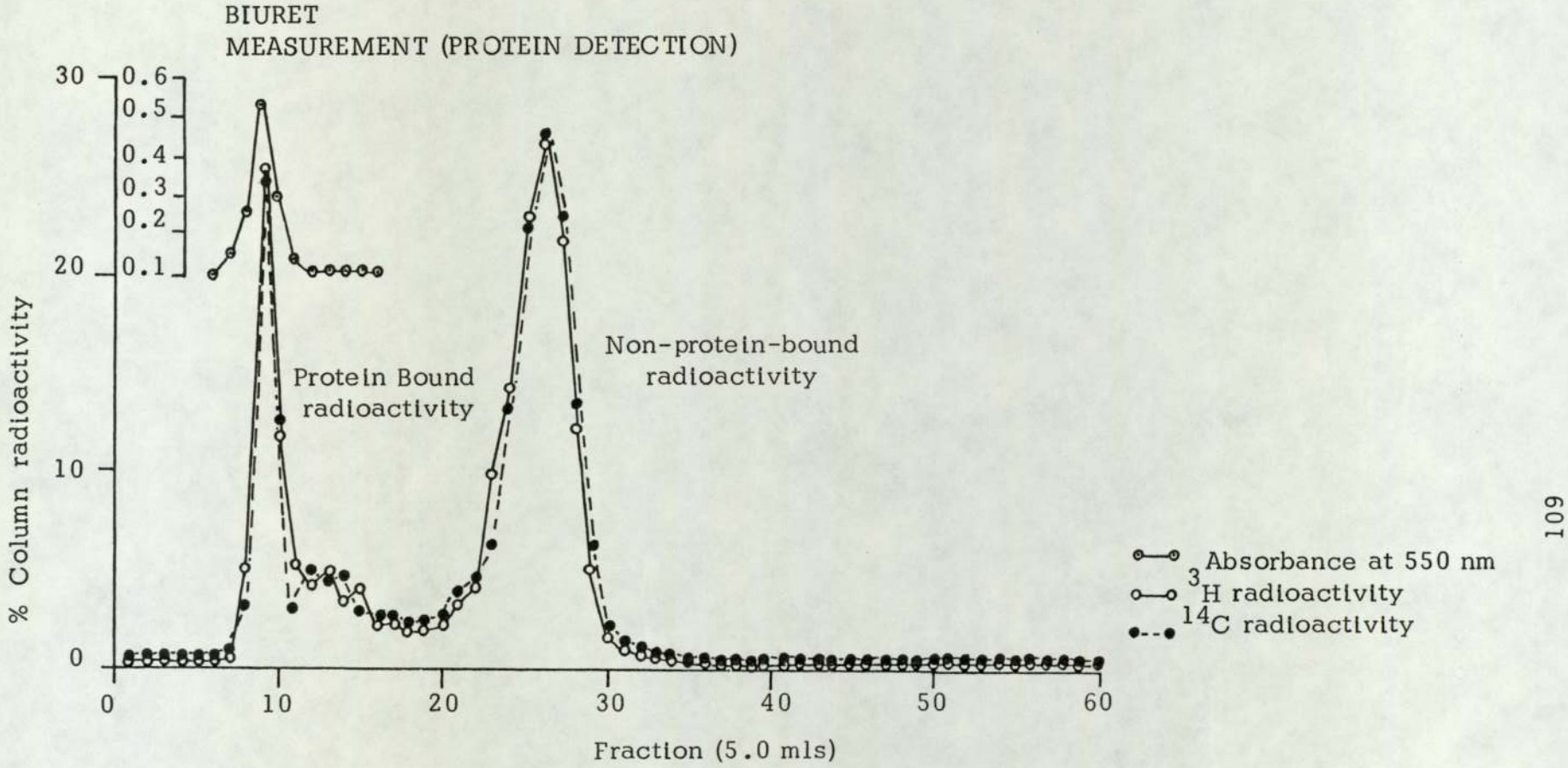


Fig. 4.12 Sephadex G75 chromatography of 4 hour liver extract (extraction D) (Walker 256 carcosarcoma bearing male Wistar rats) (group C)

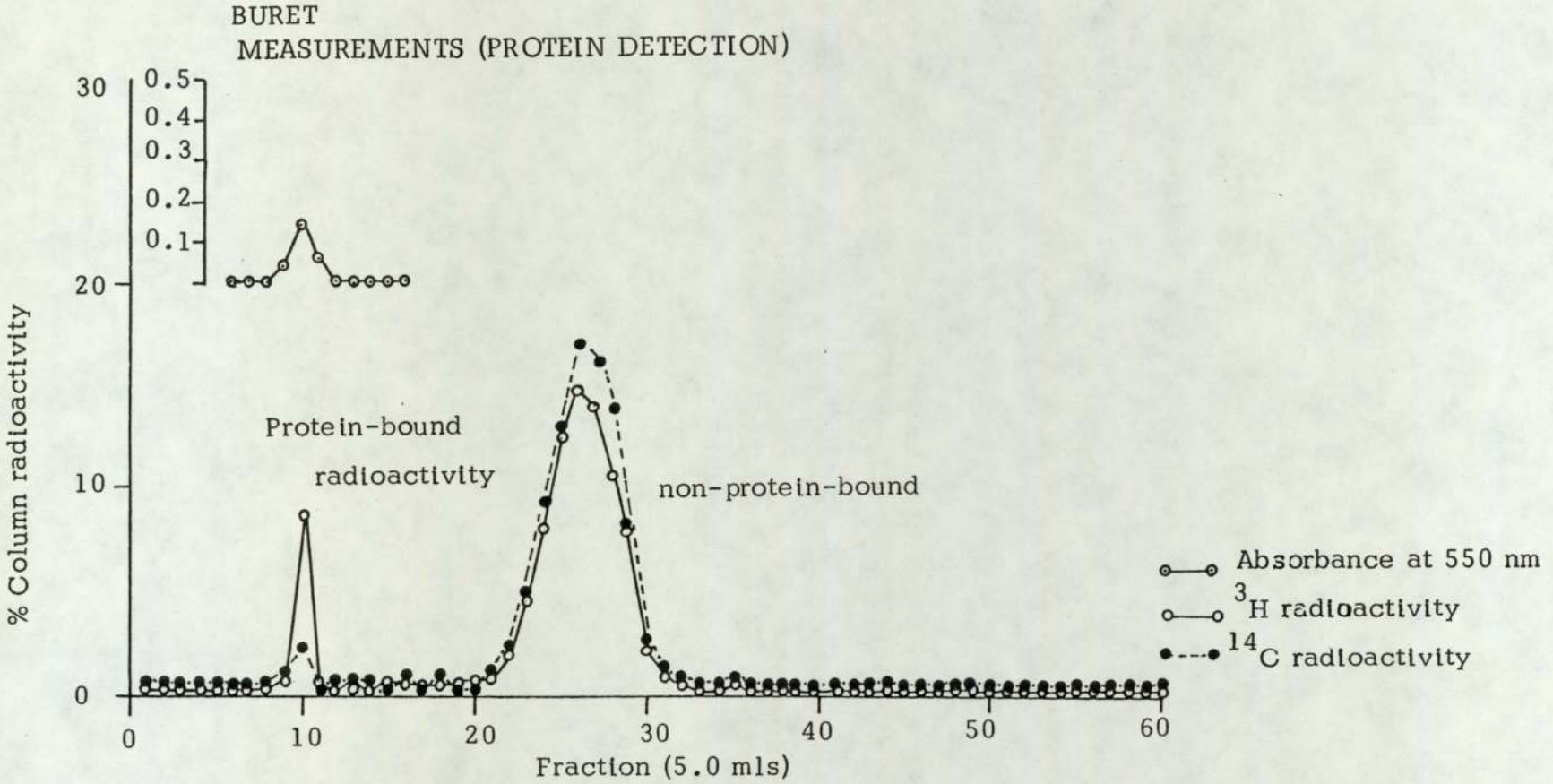


Fig. 4.13 Sephadex G75 chromatography of 6 hour tumour (Walker 256 carcosarcoma) extract (extraction D) (tumour-bearing male Wistar rats) (group C)

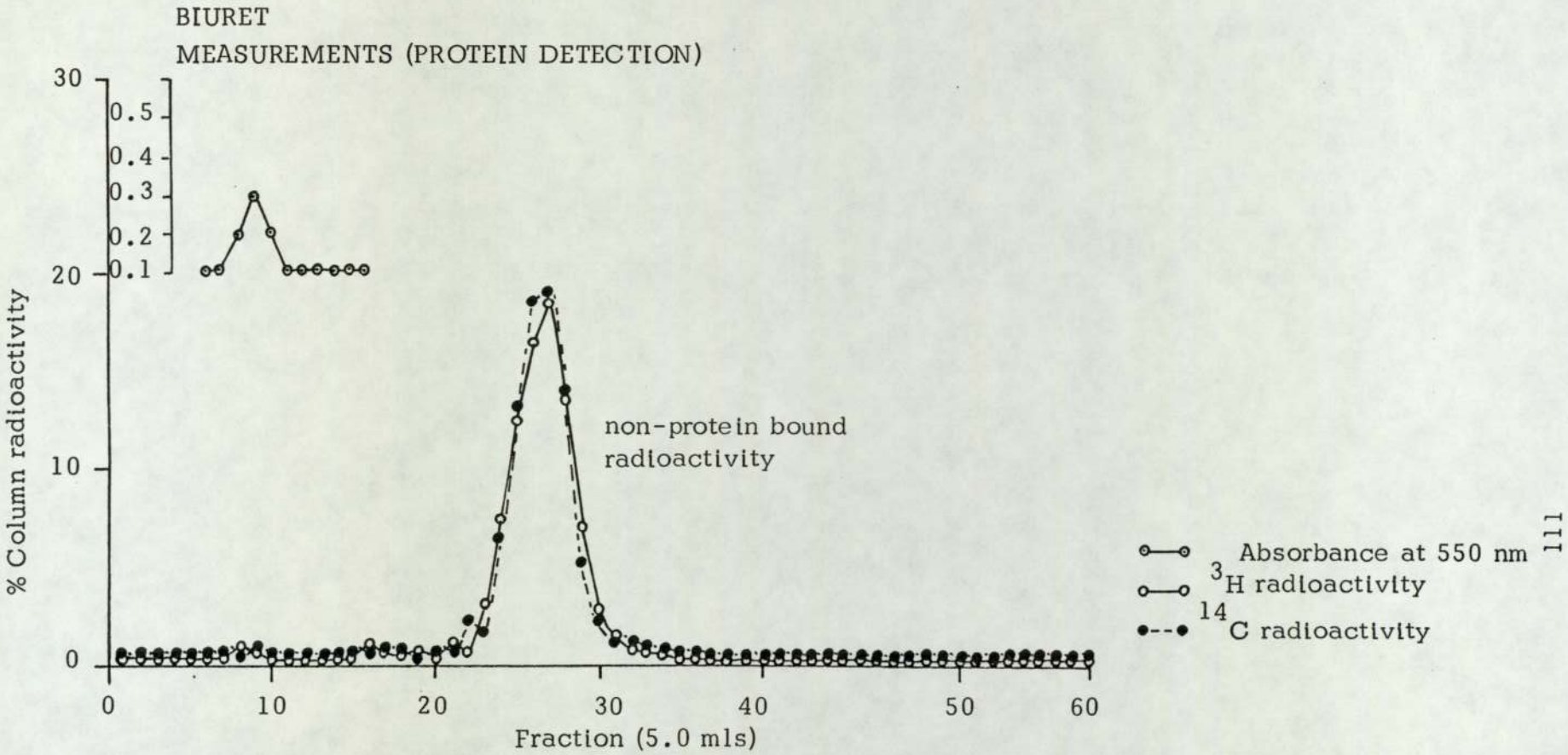


Fig. 4.14 Sephadex G75 Chromatography of 48 hour intestinal extract (extraction D) (Walker 256 carcosarcoma bearing male Wistar rats) (group C).

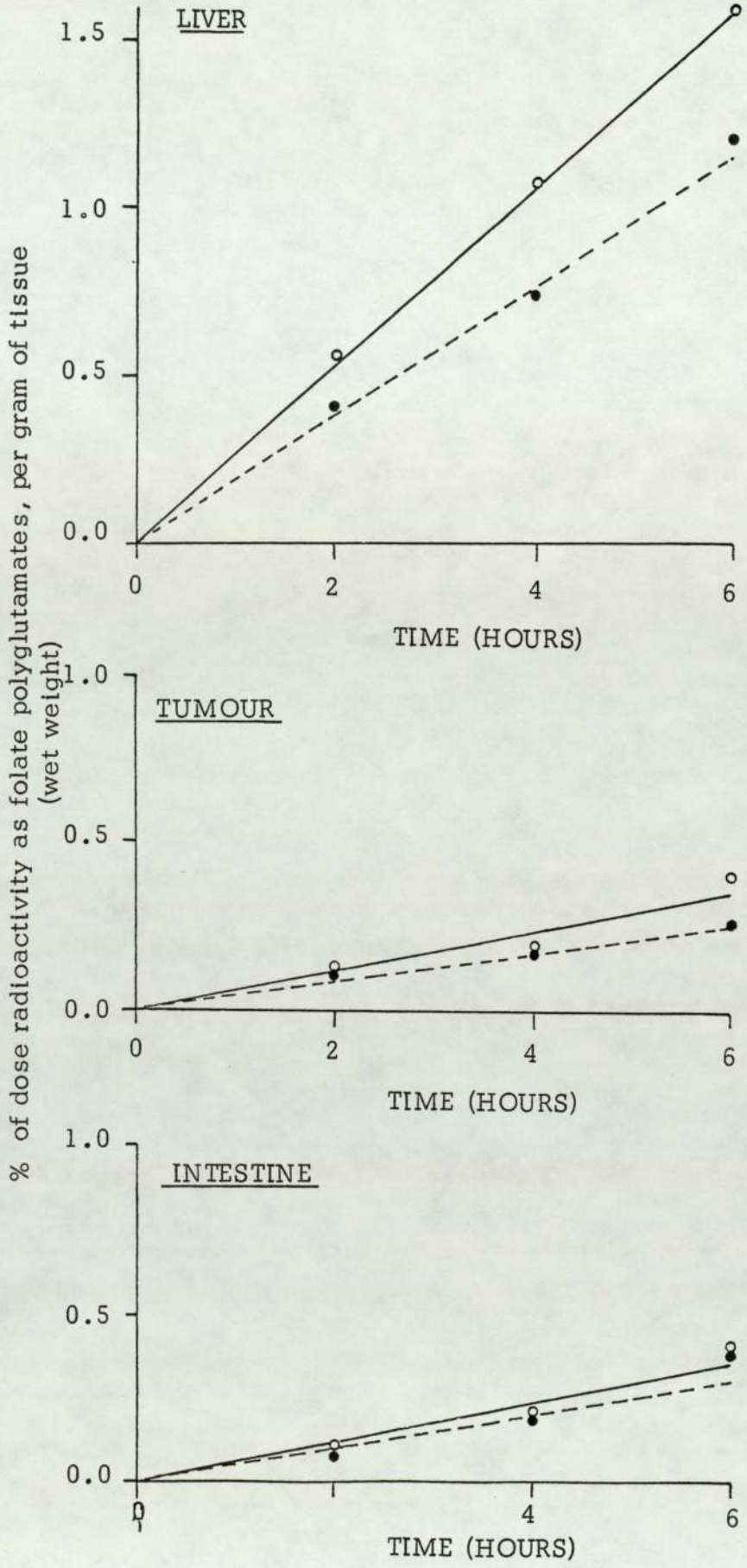


Fig. 4.15. The initial rates of folate polyglutamate synthesis in the liver, tumour (sarcoma Mc 103B) and intestine of group B (male WAB/NOT) tumour-bearing rats, following the oral administration of a mixture of [2^{14}C] and [3',5',7,9-^{3}H] FA. o—o = ^{3}H ; •···• = ^{14}C

CHAPTER 5

5.1. The long-term disposition of the hepatic folate polyglutamate pool in the normal rat.

INTRODUCTION

Earlier studies showed that orally administered FA is metabolised to folate polyglutamates; these derivatives representing the major, proportion of the radiolabelled folate in the liver, tumour and intestine at 24 hours after administration.

The present study is concerned with the long-term disposition of the hepatic folate polyglutamate pool up to 22 days after the oral administration of radiolabelled FA.

In addition, the radiolabelled metabolites excreted in the urine over this time period were investigated by Adley Saleh, Chemistry Department, University of Aston in Birmingham.

METHODS

EXPERIMENT 5.1. The long term disposition of the hepatic folate polyglutamate pool in the normal rat

18 normal group B (WAB/NOT) rats were dosed with a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. At 1, 3, 5, 7, 9, 11, 13, 15 and 22 days after administration, rats were killed and the tissue extracts (stored frozen at -20^{o}C) were analysed between 0-3 months after extract preparation.

Chromatography

Crude liver extracts were initially chromatographed on Sephadex G15 and the major dual labelled peak eluting close to V_o (folate polyglutamate) was re-chromatographed on DEAE-cellulose (days 1-11 only) as previously described.

Analysis of urinary excreted metabolism (analysis carried out by Adley Saleh

5 rats were kept in cages designed for the separate collection of faeces and urine (Jencons Metabowls; Jencons (Scientific) Limited, Herts, U.K). Urine samples were collected into flasks containing sodium ascorbate (2% w/v) and dithiothreitol (5 mg % w/v). To prevent light degradation of folates in the urine, flasks were surrounded by aluminium foil.

Urine flasks changed daily and samples from days 1, 2 + 3, 4 + 5, 6 + 7, 8 + 9 and 10 + 11 were pooled and stored frozen (-20°C) until required. Faeces were collected at the end of days 1, 3, 5, 7, 9 and 11. Urine samples were analysed using DEAE-cellulose, Sephadex G15 and paper chromatography (Whatman No. 1) (descending method, soluent. proparol: acetic acid in the ratio 200 : 1 : 99).

RESULTS

Hepatic recovery of radioactivity

The hepatic recovery of radioactivity was maximal at 3 days on the basis of the whole tissue and at 5 days on the basis of unit weight (per gram). After 5 days, hepatic radioactivity declined exponentially (Fig. 5.1). These results are presented in Table 5.1. A semi-logarithmic plot of hepatic radioactivity (per gram of tissue, wet weight) versus time (days 5-22) revealed a linear relationship (linear correlation coefficient = 0.96) giving a half-life ($t_{\frac{1}{2}}$) of 7.1 days.

Sephadex G15 chromatography

Sephadex G15 chromatography of the crude tissue extracts demonstrated a similar pattern of radiolabelled derivatives at all times. Folate polyglutamates were the predominant radiolabelled derivatives eluting as a single peak close to V_o. Folate monoglutamates were not detected. The proportion of hepatic radioactivity as folate polyglutamate was lowest at 13, 15 and 22 days; the remaining radioactivity eluting as "non-defined" radioactivity (i.e. did not elute a distinguishable peak). The proportion of hepatic radioactivity as folate polyglutamate between 1-22 days after administration is presented in Table 5.2.

DEAE-cellulose chromatography

DEAE-cellulose chromatography of the folate polyglutamate peak resolved on Sephadex G15, revealed a similar pattern of radiolabelled derivatives at all times investigated (1-11 days). The pattern and elution positions of these species were very similar to that previously obtained in the case of the liver at 24 hours (Chapter 4, Fig. 4.8); showing the presence of folate polyglutamates A, B and C.

Urinary excreted metabolites (analysed by Adley Saleh)

Chromatographic analysis of urinary samples revealed intact folate monoglutamates ($5CH_3THF$, FA $10CHOFA$ and $5,10CH_2THF$) and folate catabolites (two pterin derivatives, p-AcB, p-AcBG, ^3H_2O and ^{14}C

labelled urea). Folate monoglutamates demonstrated maximal urinary levels between 0-1 days after administration; $5CH_3THF$ and 10CHOFA being the major species at this time. These results are presented in Table 5.3. The levels of radioactivity present in the urine and faeces are presented in Table 5.4.

DISCUSSION

These studies confirm previous findings that folate polyglutamates are the principal folate derivatives in the cell. The qualitative distribution of folate polyglutamates A, B and C remained very constant from 1-11 days after administration indicating that the dose folate had established equilibrium with the endogenous folate and that this represents the steady state endogenous hepatic distribution of folate coenzymes. These results are further discussed in Chapter 7.

Time (days)	PERCENTAGE OF DOSE RADIOACTIVITY IN THE LIVER	
	^{14}C	^{3}H
1	24.6 (2.1)	25.8 (2.2)
3	27.8 (2.7)	28.4 (2.7)
5	24.6 (3.2)	23.0 (3.0)
7	17.7 (1.8)	15.0 (1.6)
9	12.2 (1.4)	11.8 (1.4)
11	15.9 (1.3)	17.2 (1.4)
13	9.4 (1.0)	8.2 (0.9)
15	9.8 (1.0)	9.8 (1.0)
22	4.0 (0.5)	4.3 (0.5)

Table 5.1. Recovery of radioactivity in the liver at 1-22 days after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA to normal group B (WAB/NOT) rats. Units: % dose radioactivity in the whole tissue; values in parenthesis indicating the % of dose radioactivity per gram (wet weight) of tissue. All values are an average of two rats.

Time (days)	% tissue radioactivity as folate polyglutamate: ^{14}C	^{3}H
1	91	92
3	64	74
5	72	77
7	81	86
9	89	81
11	88	76
13	58	64
15	56	59
22	30	39

Table 5.2. Summary of the percentage of hepatic radiolabelled folate as folate polyglutamate at 1-22 days after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA to normal group B (WAB/NOT) rats.

Folate derivatives	Day 1		Day 2 + 3		Day 4 + 5		Day 6 + 7		Day 8 + 9		Day 10+11	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
FA	2.9	3.2	0.08	0.05	ND	ND	ND	ND	ND	ND	ND	ND
$5CH_3THF$	11.9	12.0	0.48	0.43	0.35	0.25	0.65	0.66	0.66	0.47	0.53	0.47
10CHOFA	8.1	8.7	0.96	0.96	0.89	0.53						
$5,10CH_2THF$	2.6	2.8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
pAcB	ND	4.4	ND	1.46	ND	0.34	ND	0.31	ND	0.38	ND	0.15
pAcBG	ND	5.9	ND	1.94	ND	1.21	ND	0.96	ND	0.97	ND	0.47
Pterin A	3.1	ND	1.60	ND	0.88	ND	0.68	ND	0.33	ND	0.22	ND
Pterin B	2.8	ND	0.90	ND	0.27	ND	-	-	-	-	-	-
Urea	0.66	ND	0.44	ND	0.34	ND	-	-	-	-	-	-
$^{3}H_2O$	ND	1.50	ND	1.20	ND	0.75	ND	0.61	ND	0.41	ND	0.49

Table 5.3. The distribution of radiolabelled metabolites in the urine 0-11 days after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA to group B (WAB/NOT) normal rats. Each value was determined from the pooled urine of 5 rats. ND = not detected.

Time (days)	Urinary recovery		Faeces recovery	
	^{14}C	^{3}H	^{14}C	^{3}H
1	35.1	42.8	8.5	8.8
2	2.7	3.9	16.4	8.5
3	2.1	2.7		
4	1.6	1.8	3.5	2.3
5	1.5	1.9		
6	1.3	1.6	2.5	1.4
7	0.9	1.1		
8	0.8	1.1	1.4	1.0
9	1.0	1.1		
10	0.8	0.9	1.0	0.8
11	0.8	0.8		
12	0.7	0.7		
13	0.7	0.8		
14	0.6	0.6		
15	0.7	0.7		

Table 5.4. The recovery of radioactivity in the urine and faeces 1-15 days after the oral administration of a mixture of 2-^{14}C and 3',5',7,9-^{3}H FA to normal group B (male WAB/NOT) rats. Each value is an average of 5 rats.

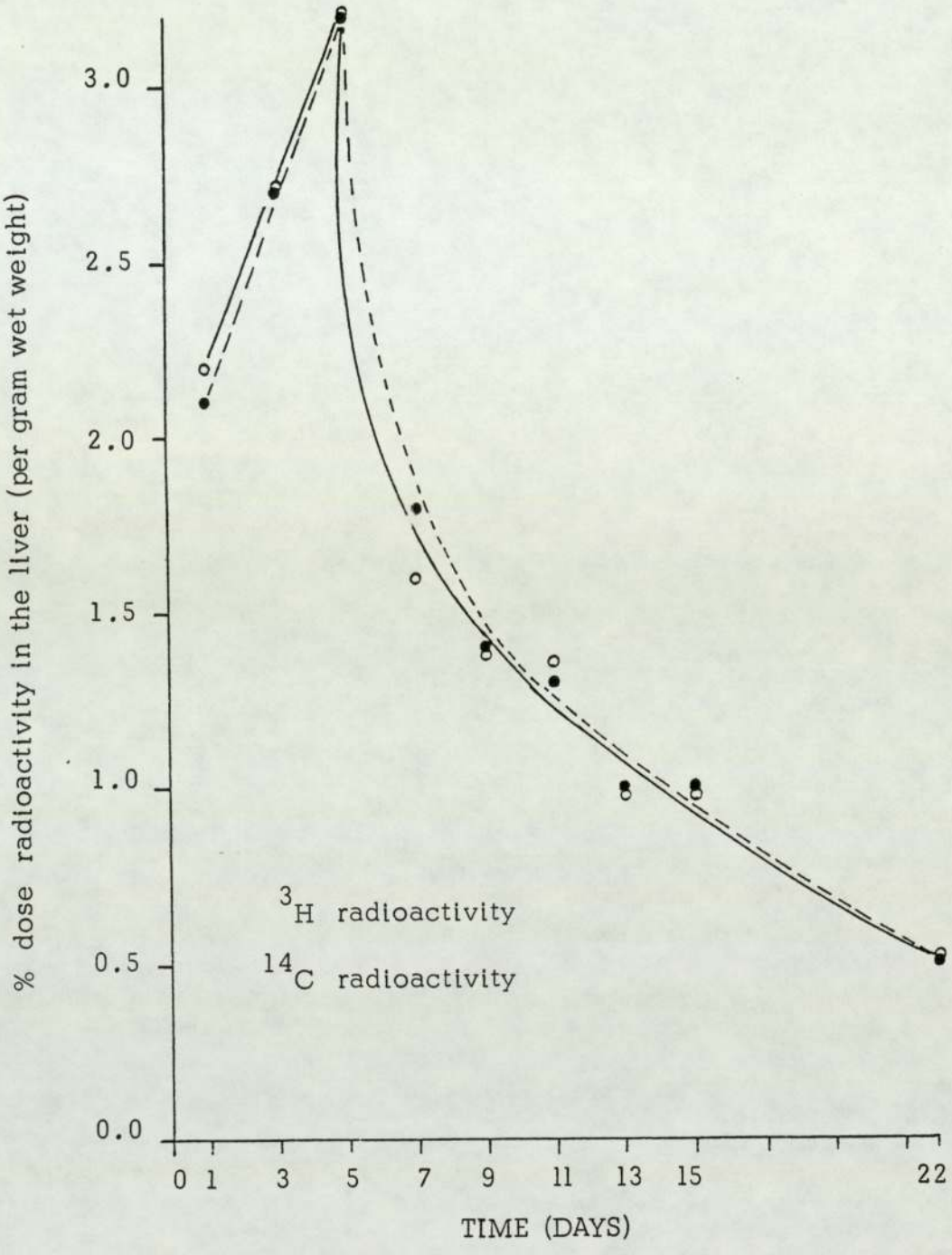


Fig. 5.1. The levels of radioactivity in the liver of group B (male WAB/NOT) normal rats at 1-22 days after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA.

CHAPTER 6

6.1. The analysis of folate polyglutamates using high-performance liquid chromatography.

6.2. Further studies of hepatic folate polyglutamates in the normal rat.

INTRODUCTION

The recent introduction of high efficiency microparticulate columns has allowed the development of high-performance liquid chromatography (HPLC) for the analysis of folates. HPLC offers high selectivity and resolution, and speed of analysis; thus giving important advantages over more conventional methods. Under suitable conditions, HPLC allows the separation of folate monoglutamates (Reed and Archer, 1976; Archer and Reed, 1979). Moreover, HPLC has been demonstrated to resolve folate polyglutamates differing in the number of glutamate residues (Stout et al., 1976; Bush et al., 1979; Cashmore et al., 1979).

The present study is concerned principally with the development of HPLC for the identification of folate polyglutamates isolated from mammalian tissues. In this instance, the hepatic folate polyglutamates present 48 hours after the oral administration of radiolabelled FA have been investigated.

EXPERIMENT 6.1. The analysis of folates using high performance liquid chromatography

Initial studies were concerned with the development of suitable chromatographic conditions allowing the separation of folate polyglutamates from folate monoglutamates and also the individual resolution of folate polyglutamates.

Isocratic elution

Isocratic elution (single solvent system) was investigated using phosphate buffer (0.2, 0.4 and 0.6 M) (pH 5.0) previously found to resolve certain folate monoglutamates under conditions of anion-exchange HPLC (Dr. M. J. Connor, personal communication).

Gradient elution

Linear gradient elution using Na_2SO_4 in phosphate buffer was also investigated as these conditions have been found to resolve folate polyglutamates differing in the number of glutamate residues (Mr D Phillips, ARC Laboratories, Norwich, personal communication). On completion of the gradient time (1 hour), elution was continued at maximum salt concentration (0.50 M) for 1 hour.

EXPERIMENT 6.2. The analysis of hepatic folate polyglutamates present in group B (WAB/NOT) normal rats 48 hours after the oral administration of radiolabelled FA

6 Normal male WAB/NOT rats were dosed orally with a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] FA. At 48 hours after administration, livers were extracted (extraction A) and the crude extract (stored frozen at -20^{o}C) retained for analysis. Analysis was complete between 0-3 months after extract preparation.

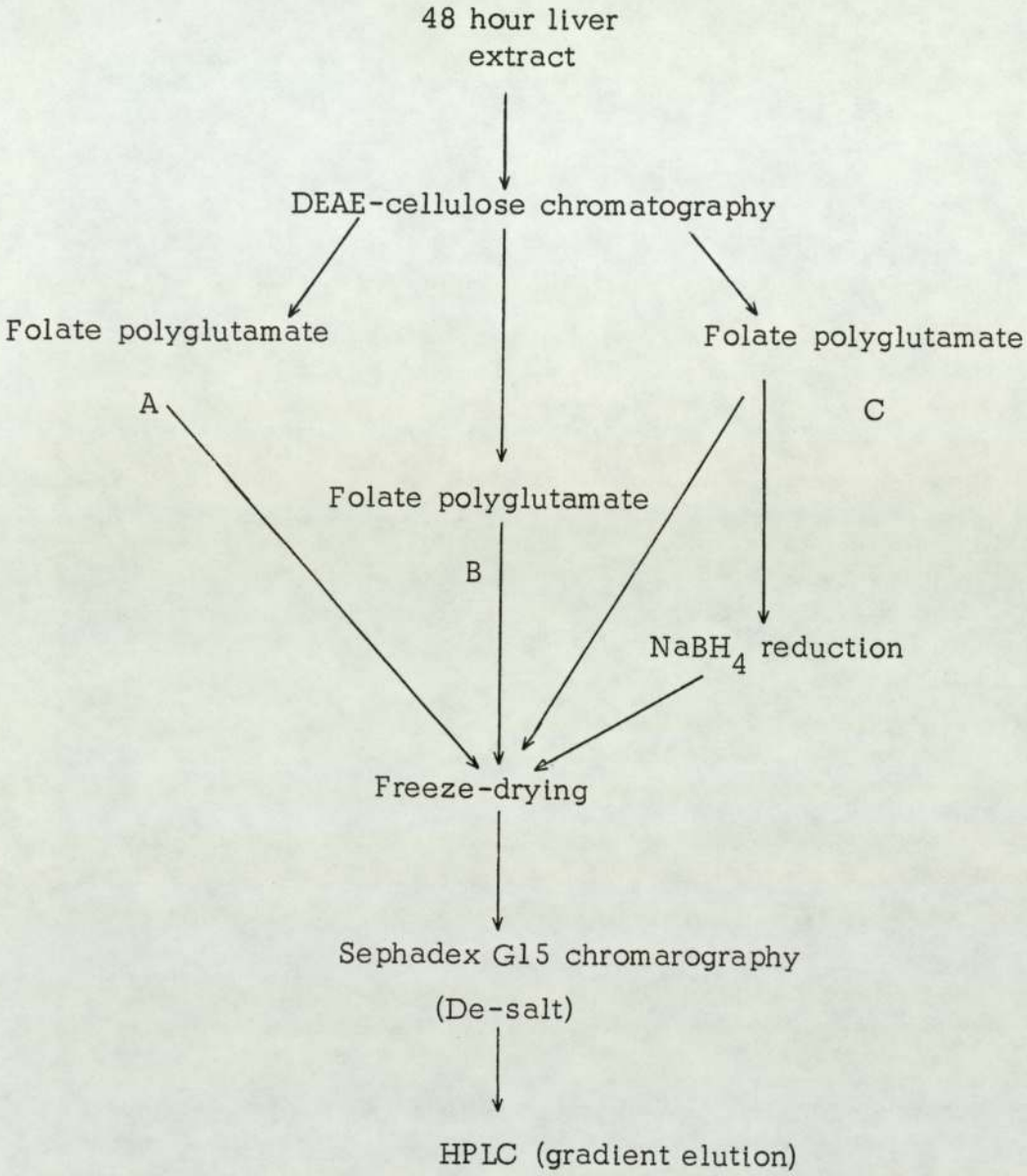
ANALYSIS

The crude tissue extract was chromatographed initially on Sephadex G15 and by HPLC (gradient elution). The remainder of the extract was chromatographed directly on DEAE-cellulose. Due to the lability of folate polyglutamate C, the appropriate fractions were collected into 1.0 mls sodium ascorbate (10% w/v) in 0.05M phosphate buffer (pH 7.0). The subsequent experimental steps are presented overleaf.

The possibility that folate polyglutamate C is a 5, $10CH_2THF$ derivative was investigated by the attempted $NaBH_4$ reduction of the species. Under these conditions, a $5,10CH_2THF$ polyglutamate should be converted to the corresponding $5CH_3THF$ polyglutamate; the latter species being available as authentic chromatographic markers. Following DEAE-cellulose chromatography of the crude liver extract, fractions containing folate polyglutamate C were pool and an aliquot was incubated at 45°C for 1 hour in the presence of $NaBH_4$ (1.2 g per 25 mls of solution). Oxygen free nitrogen was passed through the solution during incubation (Blair and Saunders, 1970).

Other methods

The crude liver extracts retained from the long-term study (Chapter 5) were chromatographed by HPLC (gradient elution). These extracts (stored frozen at -20°C) were analysed 6-8 months after extract preparation.



Experimental steps undertaken to identify folate polyglutamates A, B and C using the combined procedure of Sephadex G15 DEAE-cellulose chromatography and HPLC.

RESULTS

EXPERIMENT 6.1

Isocratic elution HPLC allowed the separation of the folate monoglutamates 10CHOFA, FA and $5CH_3THF$ at phosphate buffer (the eluant) concentrations of 0.2, 0.4 and 0.6M (pH 5.0). At 0.6M phosphate buffer, $FA(glu)_4$ was resolved from 10CHOFA, FA and $5CH_3THF$, however 10CHOFA-$(glu)_4$ eluted very close to 10CHOFA. These results are presented in Table 6.1.

The retention times of the folate monoglutamates increased with decreasing phosphate buffer concentration. Thus, the retention times of $5CH_3THF$ at 0.2, 0.4 and 0.6M phosphate buffer were 65, 51 and 44 minutes respectively.

Gradient elution

Gradient elution HPLC proved to be a very effective procedure for the separation of folates (Tables 6.2 and 6.3). The folate monoglutamates 10CHOTHF, 10CHOFA, $5CH_3THF$ and FA were resolved from each other. These conditions also allowed good separation of folate polyglutamates from folate monoglutamates. Thus, 10CHOFA and $10CHOFA(glu)_4$ had retention times of 42 and 67 minutes respectively. More importantly, folate polyglutamates differing in the number of glutamate residues were also resolved. In this respect, $FA(glu)_3$, $FA(glu)_4$ and $FA(glu)_5$ had retention times of 66, 77 and 82 minutes respectively. A similar pattern was observed for

$10CHOFA(glu)_n$ (n = 3, 4 and 5), and $5CH_3THF(glu)_n$ (n = 3 and 4).

Certain folate polyglutamates were not resolved however. Thus, $10CHOFA(glu)_5$ and $5CH_3THF(glu)_4$ both had the same retention time (75 minutes) and similarly, $FA(glu)_3$ (R_t = 66 minutes) was poorly resolved from $10CHOFA(glu)_4$ (R_t = 67 min.). The u.v. detector trace showing the separation of 10CHOTHF, 10CHOFA, $5CH_3THF$, FA and $FA(glu)_4$ is presented in Fig. 6.1.

This chromatographic procedure also allowed the separation of several folate catabolites. These results are presented in Table 6.2. Pterin (2-amino-4-hydroxypteridine) (R_t = 35 min.) was resolved from xanthopterin (R_t = 31 min.), p-aminobenzoic acid (R_t = 35 min.) was resolved from p-aminobenzoyl-L-glutamate (R_t = 42 min.) and p-acetamidobenzoic acid (R_t = 35 min.) was resolved from p-acetamidobenzoyl-L-glutamate (R_t = 44 min.). However, the N-acetylated derivatives of p-aminobenzoic acid and p-aminobenzoyl-L-glutamate were not resolved from the corresponding free amines.

The retention times of several folates chromatographed in three separate consecutive runs demonstrated a standard deviation, expressed as a % of the mean retention time, of $\pm$ 1.3-3.2% (Table 6.4). In certain cases, a greater variance in the retention time of the same folate chromatographed under these conditions was observed from day-to-day or week-to-week. However, the likelihood of this arising was reduced by the use of a blank run (without sample injection) prior to the first analytical run.

A plot of the number of glutamate residues versus the retention time (minutes) for FA, 10CHOFA and $5CH_3THF$ and their corresponding folate polyglutamate derivatives revealed a good linear relationship for each series, with linear correlation coefficients of 0.994, 0.999 and 0.992 respectively (Table 6.3a). These results are presented graphically in Fig. 6.2. A table of predicted retention times for FA, 10CHOFA and $5CH_3THF$ and their respective folate polyglutamates obtained by interpolation and extrapolation of the experimentally determined linear relationships are presented in Table 6.3b.

EXPERIMENT 6.2.

Sephadex G15 chromatography of the 48 hour liver extract revealed a major dual labelled peak eluting close to V_o (folate polyglutamate) representing 90% of the tissue radiolabelled folate. DEAE-cellulose chromatography showed the same pattern of radiolabelled hepatic derivatives (folate polyglutamates A, B and C) as previously found at 24 hours in the liver (Fig. 4.8). Hepatic recovery of orally administered radioactivity was 17.8 and 19.1% of the ^{14}C and ^{3}H dose radioactivity respectively, in the whole tissue.

Sephadex G15 chromatography (de-salt) of the freeze-dried fractions containing folate polyglutamates A, B and C (before and after $NaBH_4$ reduction) demonstrated a dual labelled derivative eluting close to V_o, in each case associated with excess of ^{3}H radioactivity. In addition, 2-^{14}C only labelled species (i.e. pterins) eluting between fractions 16-22

were present in the case of folate polyglutamates A and B whereas for folate polyglutamate C before $NaBH_4$ reduction, a broad ^{14}C only labelled species eluted between fractions 30-50 however, after the $NaBH_4$ step, a similar species eluted between fractions 20-40. The levels of radioactivity eluting close to V_o as a single peak following the Sephadex G15 de-salt step, are given in Table 6.6.

Further chromatography of folate polyglutamates A (after de-salt step) under conditions of gradient elution HPLC revealed a ^{3}H labelled species (R_t = 30-32) and a dual labelled species (R_t = 38-40); both therefore eluting considerably earlier than the authentic folate polyglutamate investigated. Chromatography of folate polyglutamate B (after the de-salt step) under the same conditions showed that this species co-chromatographed with authentic $5CH_3THF(glu)_3$. In both these chromatographic runs, $FA(glu)_4$ eluted reproducibly at the normal retention time, indicating constancy of chromatographic separation. Folate polyglutamate C was not investigated under these conditions due to the marked breakdown of this species both before and after $NaBH_4$ reduction.

Chromatography of the 48 hour liver extract under conditions of gradient elution HPLC resolved three dual labelled derivatives eluting in the

folate polyglutamate region (Fig. 6.3). These had retention times of 64-66 (close to the elution position of 10CHOFA$(glu)_4$) 78-80 (the principal species) and 84-86 min. An additional derivative was also indicated in this region, appearing as a "shoulder" (indicating poor resolution) eluting before the principal peak, approximately in the region 72-74 minutes (close to the elution position of $5CH_3THF(glu)_3$).

Other studies

Analysis of the liver extracts retained from the long-term study of hepatic folate polyglutamates (Chapter 5) revealed a similar pattern of radiolabelled derivatives at all times. In each case, the major proportion of the tissue radioactivity eluted in the folate polyglutamate region supporting the earlier observations.

At 1, 3, 5 and 7 days, thee principal dual labelled derivatives were present with retention times of 76-78, 84-86 and 90-92 min. At 9, 11, 13 and 15 days, the principal dual labelled derivatives had retention times of 86-88, 92-94 and 96-98 min.

At all times one or two ^3H labelled species (p-aminobenzoyl derivativative) eluted between fractions 38-43 (R_t = 74-86 min.) and a broad ^{14}C only labelled derivative (a pterin) eluted between fractions 21-30 (R_t = 46-48 min.). In each chromatographic run the folate marker 10CHOFA eluted reproducibly at the normal retention time indicating constancy of chromatographic conditions. The chromatogram obtained following HPLC

of day 5 liver extract is presented in Fig. 6.4.

DISCUSSION

EXPERIMENT 6.1.

These studies demonstrate the capability of HPLC to rapidly resolve folate coenzymes. Isocratic elution using phosphate buffer, though able to resolve certain folate monoglutamates was less effective for the separation of folate polyglutamates from folate monoglutamates.

Gradient elution HPLC offered considerably improved resolution of folate polyglutamates from folate monoglutamates. Moreover, these conditions allowed the separation of folate polyglutamates differing in the number of glutamate residues. This procedure may therefore be of considerable value for the identification of folate polyglutamates. The failure of these conditions to resolve all folate polyglutamates illustrates certain limitations of this procedure. In addition, the observed day-to-day or week-to-week variability in the retention time of folate markers raises difficulties with regard to prior column calibration for the identification of tissue folates. This chromatographic system therefore necessitates the use of the appropriate folate markers during each analytical run.

EXPERIMENT 6.2

Sephadex G15 and DEAE-cellulose chromatography of the 48 hour liver extract confirmed previous observations; that folate polyglutamates

A, B and C are the principal hepatic folate derivatives at the time. The Sephadex G15 de-salt step showed that partial breakdown of folate polyglutamates A, B and C had occured , this being apparent from the excess ^{3}H radioactivity associated with the dual labelled peak eluting close to V_o. The excess ^{3}H radioactivity shows the presence of a p-aminobenzoyl derivative, presumably a p-aminobenzoylpolyglutamate. In the case of folate polyglutamate C (both before and after the $NaBH_4$ step), breakdown occurred to a greater extent than in the case of folate polyglutamates A and B. This indicates that folate polyglutamate C is more labile than folate polyglutamates A and B, as previously observed.

The breakdown of folate polyglutamates A, B and C during the de-salt step is also evident from the detection of ^{14}C only labelled species, presumably pterin derivatives formed by scission of the C_9-N_{10} bond. The two ^{14}C only species eluting between fractions 16-22 following the Sephadex G15 de-salt step of folate polyglutamates A and B exhibit properties markedly different from pterin (2-amino-4-hydroxy-pteridine) (which elutes in fraction 35) and xanthopterin (which elutes in fraction 57). Connor (1979) however, reported that xanthopterin elutes from Sephadex G15, under the same conditions employed in the present study, in fraction 21. This conflict may have arisen from concentration dependent effects which can alter the elution position of xanthopterin (Blakley, 1969). Thus, according to the findings of Connor (1979), one of these ^{14}C only labelled species may be xanthopterin.

The ^{14}C only labelled species derived from the breakdown of folate polyglutamate C during the de-salt step, is indicated to be pterin from the elution position of this species. The change in the chromatographic properties of this derivative after the $NaBH_4$ step may have resulted from chemical modification during the reaction.

The resolution of folate polyglutamates A (after the de-salt step) by gradient elution HPLC into a ^{3}H labelled species (R_t = 30-32 minutes) and a dual labelled species (R_t = 38-40 minutes) was unexpected as both these derivatives eluted considerably earlier than any of the folate polyglutamate markers. This result is also difficult to interpret in relation to the results obtained following gradient elution HPLC of the 48 hour liver extract, which did not reveal any radiolabelled derivatives eluting in this region. This shows that breakdown of folate polyglutamate A occurred during analysis giving rise to species with markedly changed chromatographic properties.

HPLC of folate polyglutamate B suggested this species to be $5CH_3THF(glu)_3$, however this can only be confirmed by more rigorous chromatographic analysis. As $5CH_3THF(glu)_3$ is well resolved from other folate polyglutamate markers, this reduces the ambiguity of this identification under these conditions.

Gradient elution HPLC of the 48 hour liver extract showed the presence of two radiolabelled derivatives with similar elution positions as $10CHOFA(glu)_4$ and $5CH_3THF(glu)_3$. This therefore lends support to

previous conclusions regarding the identity of folate polyglutamates A and B. The identity of the major dual labelled peak detected in this extract under these conditions is unclear, however, this may comprise two or more unresolved folate polyglutamates; one possibly being folate polyglutamate C. Alternatively, this more rapid analytical procedure may have favoured minimal breakdown of folate polyglutamate C, or some other labile folate polyglutamate, which may therefore appear as a major peak.

Other studies

Gradient elution HPLC of the crude liver extracts (days 1-15) retained from the long-term study confirmed that folate polyglutamates are the principle derivatives in the liver at these times.

Little information of the identity of these derivatives is apparent from these studies as authentic folate polyglutamates were not chromatographed with the extracts. Surprisingly, these dual labelled derivatives eluted considerably later than any of the folate polyglutamate markers investigated under these conditions. This may indicate that long-term metabolism of folate produces folate polyglutamates with a higher number of glutamate residues (and therefore higher retention times); changes not apparent from the initial DEAE-cellulose chromatography, possibly due to the poorer resolving power of DEAE-cellulose. Alternatively, changes in the chromatographic properties of the long-term derivatives may have resulted from degradative effects due to the longer storage of these extracts. That breakdown had occurred was apparent from the detection of both ^{3}H and ^{14}C only labelled species in these extracts.

Folate	Phosphate buffer concentration (pH 5.0)		
	0.2M	0.4M	0.6M
FA	59	43	36
10CHOFA	49	37	33
$5CH_3THF$	65	51	44
$FA(glu)_4$	-	-	50
$10CHOFA(glu)_4$	-	-	32

Table 6.1. Summary of the retention times (min) of several folates following anion-exchange HPLC under conditions of isocratic elution using phosphate buffer (0.2M, 0.4M and 0.6M) at pH 5.0.

Compound	R_t(min)	R_s(T)	N
FA	48	3.53	1412
10CHOTHF	30	0	769
10CHOFA	42	2.82	2399
$5CH_3THF$	44	3.00	1710
5CHOTHF	46	3.98	3425
Pterin	35	0.94	642
Xanthopterin	31	0.22	786
pABA	35	1.15	1379
pAc.BA	35	1.07	1085
pABG	42	3.28	3118
pAcBG	44	2.75	2798

Table 6.2. Chromatographic properties of several folate monoglutamates and folate catabolites chromatographed under conditions of gradient elution (0-0.5M Na_2SO_4 in 0.05M phosphate buffer, pH 4.5) anion-exchange HPLC. R_t = component retention time (min); R_s(T) = component resolution from 10CHOTHF; N = column efficiency with respect to the component.

$$R_s(T) = R_t(T) - R_t' / W(T) + W' \quad \text{and} \quad N = 5.54\left(\frac{Rt}{WHH}\right)^2$$

Where R_t(T) and R_t' are the retention times of 10CHOTHF and the component resolved from 10CHOTHF respectively; W(T) and W' represent the baseline width (measured by chart recorder) of 10CHOTHF and the component resolved from 10CHOTHF respectively; WHH = peak width of component (measured by chart recorder) at half height.

Folate	R_t(min)	R_s(T)	N
$FA(glu)_3$	66	9.03	10613
$FA(glu)_4$	77	10.78	8211
$FA(glu)_5$	82	12.58	14367
$10CHO(glu)_3$	61	8.11	16299
$10CHOFA(glu)_4$	67	9.44	13250
$10CHOFA(glu)_5$	75	12.25	16603
$5CH_3THF(glu)_3$	71	10.98	14878
$5CH_3THF(glu)_4$	75	12.46	13850

Table 6.3. Chromatographic properties of several folate polyglutamates chromatographed under conditions of gradient elution (0-0.5M Na_2SO_4 in 0.05M phosphate buffer; pH 4.5) anion-exchange HPLC. R_t = component retention time (min); R_s(T) = component resolution from 10CHOTHF; N = column efficiency with respect to the component.

$$R_s(T) = R_t(T) - R_t' / W(T) + W' \text{ and } W = 5.54\left(\frac{R_t}{WHH}\right)^2$$

Where R_t(T) and R_t' are the retention times of 10CHOTHF and the component resolved from 10CHOTHF respectively; W(T) and W' represent the baseline width (measured by chart recorder) of 10CHOTHF and the component resolved from 10CHOTHF respectively; WHH = peak width of component (measured by chart recorder) at half heitht.

a) Folate	Retention times (min) of folates chromatographed three times (1-3) under conditions of gradient elution HPLC		
	1	2	3
10CHOTHF	30.25	29.50	29.75
10CHOFA	40.25	40.75	43.50
$5CH_3THF$	44.75	43.25	45.50
FA	47.75	47.00	49.00
$FA(glu)_4$	76.50	76.50	78.50

b)	R_t (mean $\pm$ S.D)(min.)	S.D. as % of mean
10CHOTHF	29.83 $\pm$ 0.38	$\pm$ 1.3
10CHOFA	42.16 $\pm$ 1.37	$\pm$ 3.2
$5CH_3THF$	44.50 $\pm$ 1.14	$\pm$ 2.5
FA	47.91 $\pm$ 1.01	$\pm$ 2.1
$FA(glu)_4$	77.17 $\pm$ 1.15	$\pm$ 1.5

Table 6.4. a) Retention characteristics of several folates chromatographed three times (1-3) under constant conditions of gradient elution (0-0.5M Na_2SO_4 in 0.05M phosphate buffer, pH 4.5) anion exchange HPLC b) Retention times (R_t) (represented in 6.2a) calculated as the mean $\pm$ S.D. and the S.D. expressed as a % of the mean.

a) Folate

Folate	Number of glutamate residues				Correlation Coefficient
	1	4	5	6	
10CHOFA	42	61	67	75	0.999
FA	48	66	77	82	0.994
$5CH_3THF$	44	71	75	-	0.992

b)

Number of glutamate residues	10CHOFA R_t (min)	FA R_t (min)	$5CH_3THF$ R_t (min)
1	41.7	47.2	44.2
2	48.2	54.2	52.4
3	54.7	61.2	60.6
4	61.2	68.2	68.8
5	67.7	75.3	76.9
6	74.3	82.2	85.1
7	80.8	89.3	93.2
8	87.3	96.4	101.4
9	93.8	103.2	109.6
10	100.3	110.3	117.7

Table 6.5 a) The retention times (R_t) (min) of 10CHOFA, FA, $5CH_3THF$ and their respective folate polyglutamate derivatives, with respect to the number of glutamate residues. For each series, the correlation coefficient for the linear plot is presented. Retention times were determined under conditions of gradient elution (0-0.5M Na_2SO_4 in 0.05M phosphate buffer, pH 4.5) anion-exchange HPLC, b) predicted retention times for 10CHOFA, FA, $5CH_3$ THF and their respective folate polyglutamate derivatives, by interpolation and extrapolation of the experimentally determined linear relationship (presented in Table 6.5a)

Folate polyglutamate	% total radioactivity eluting close to V_o	
	^{14}C	^{3}H
A	66	84
B	39	72
C[a]	18	52
C[b]	24	64

Table 6.6. Summary of the percentage of total column radioactivity eluting close to V_o following Sephadex G15 chromatography (de-salt) (column dimensions 60 x 1.5 cm) of folate polyglutamates A, B and C (before (a) and after (b) $NaBH_4$ reduction).

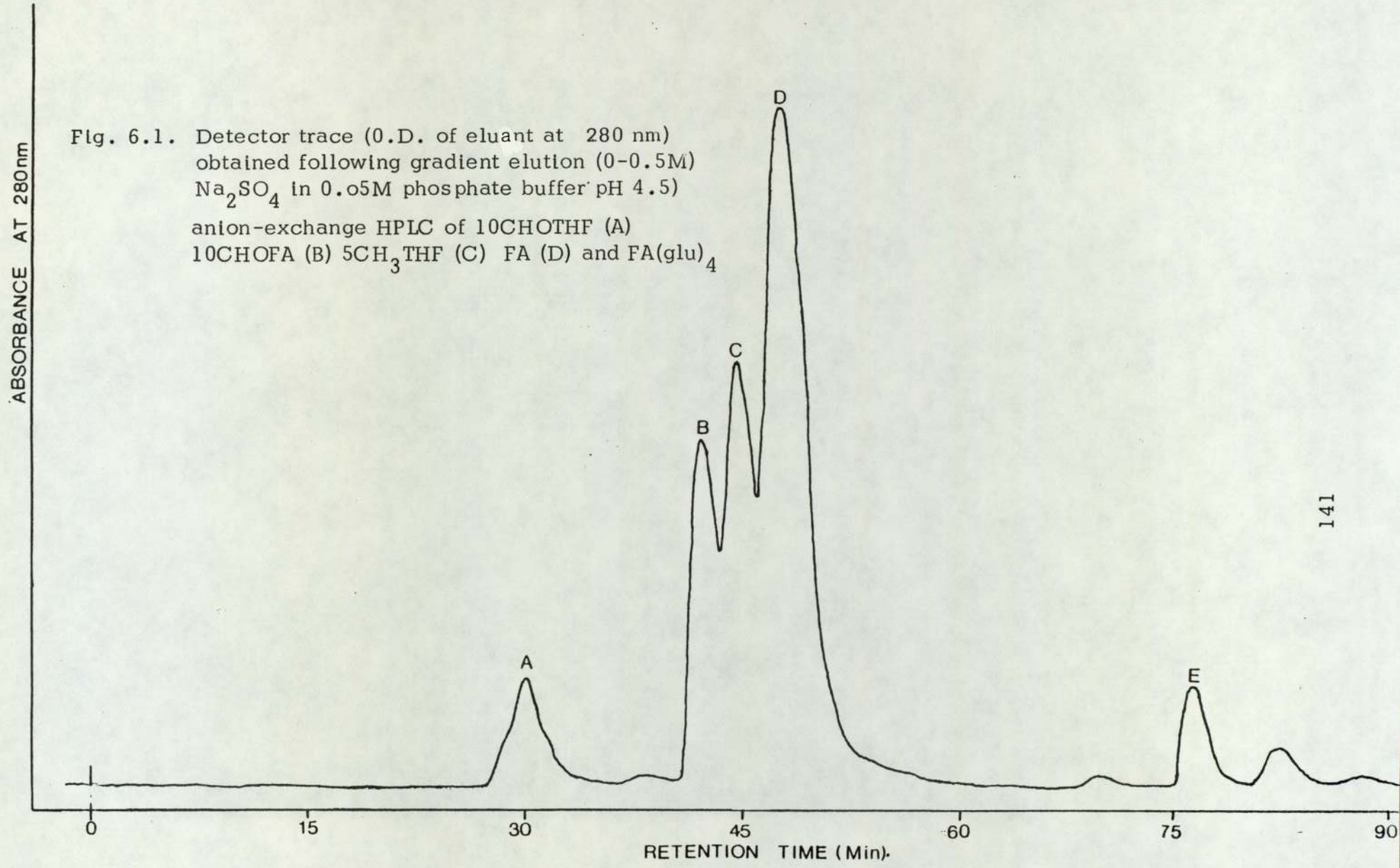


Fig. 6.1. Detector trace (0.D. of eluant at 280 nm) obtained following gradient elution (0-0.5M) Na_2SO_4 in 0.o5M phosphate buffer pH 4.5) anion-exchange HPLC of 10CHOTHF (A) 10CHOFA (B) $5CH_3THF$ (C) FA (D) and $FA(glu)_4$

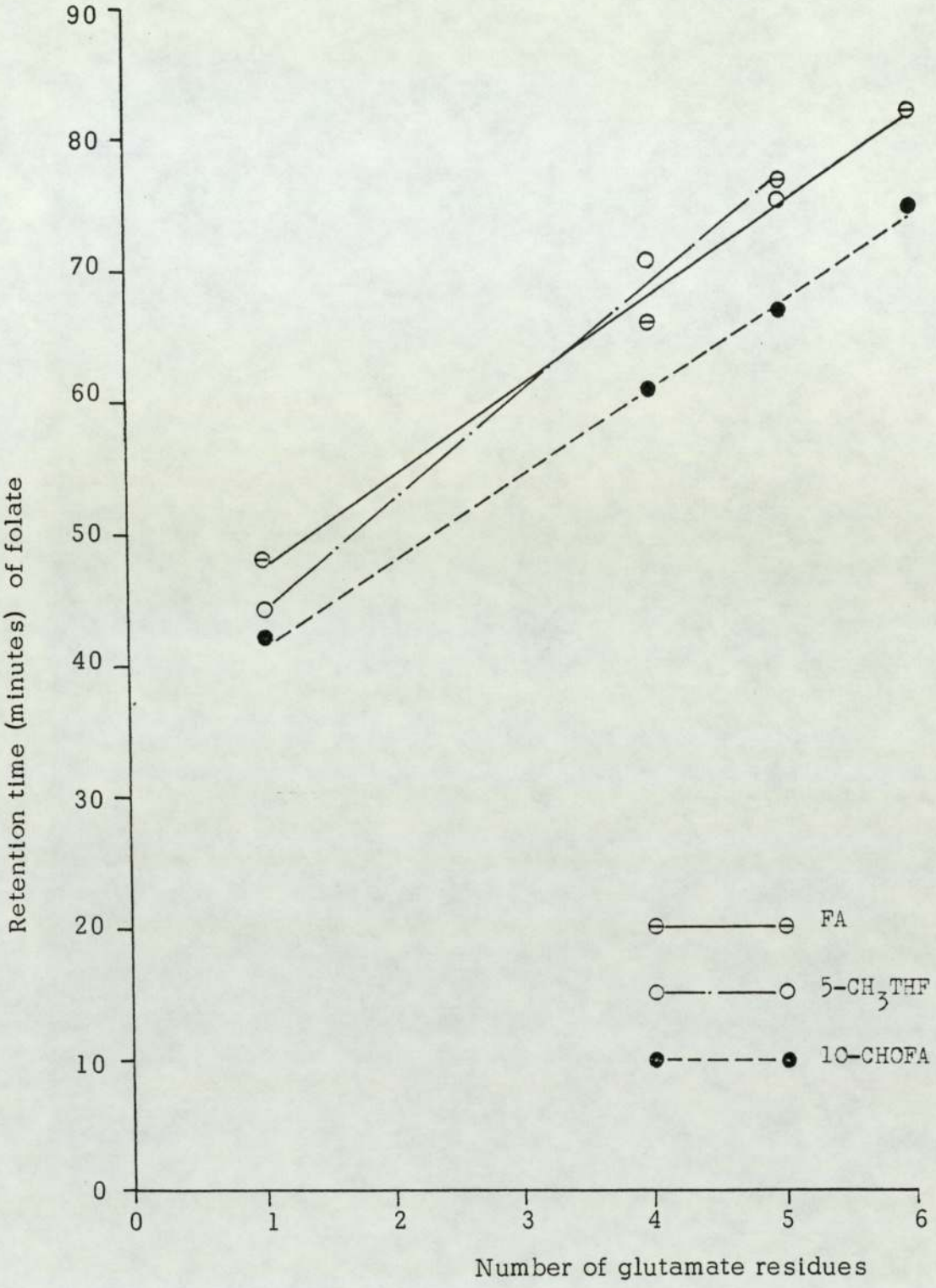


Fig. 6.2. Graphical plot showing the linear relationship between the retention time and the number of glutamate residues of $10CHOFA(glu)_n$ (n = 0, 3, 4 and 5), $FA(glu)_n$ (n = 0, 3, 4 and 5) and $5CH_3THF(glu)_n$ (n = 0, 3 and 4) when chromatographed by gradient elution HPLC.

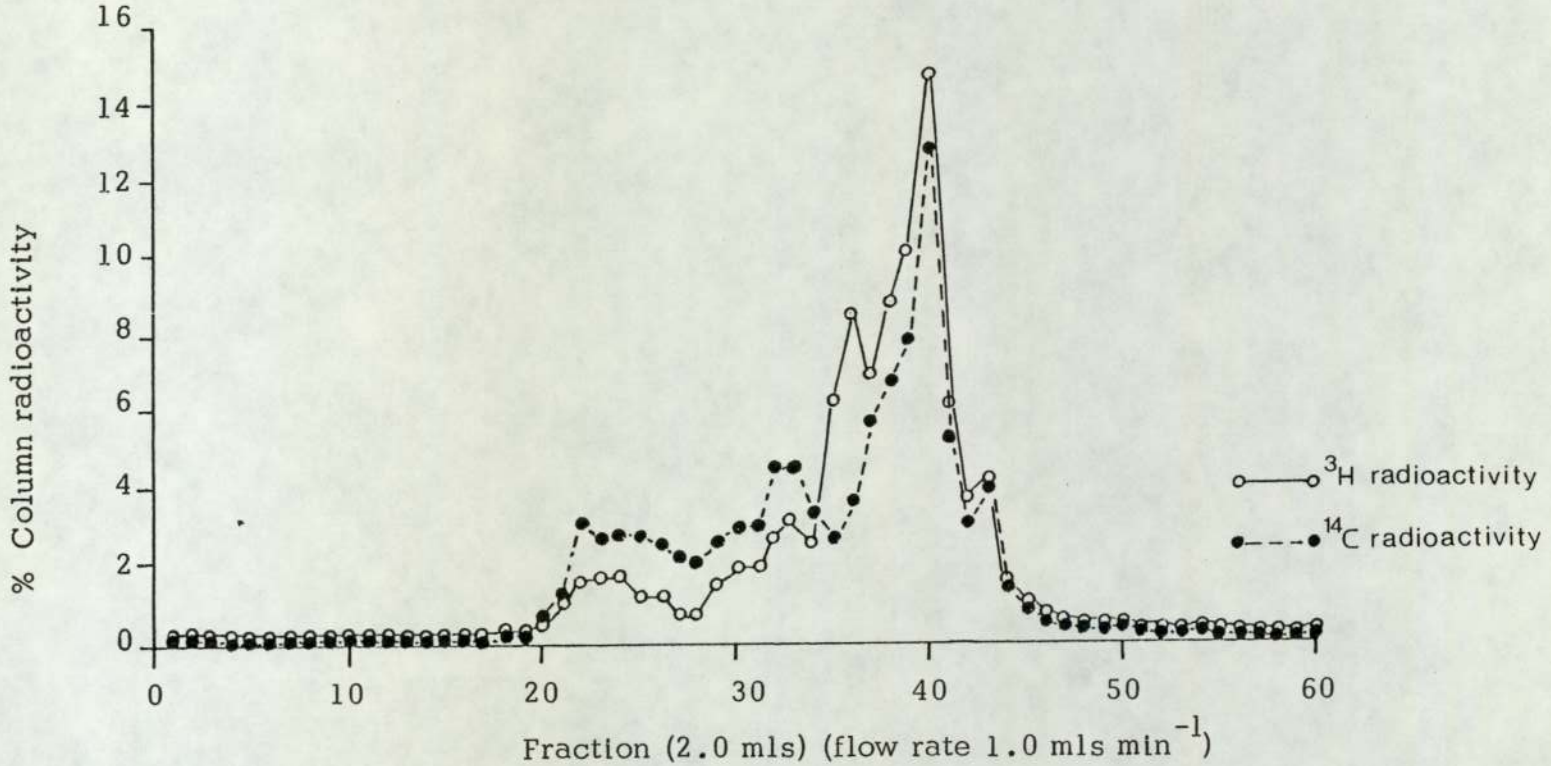


Fig. 6.3. HPLC (anion-exchange) of 48 hour liver extract under conditions of gradient elution extraction A (normal male WAB/NOT rats)

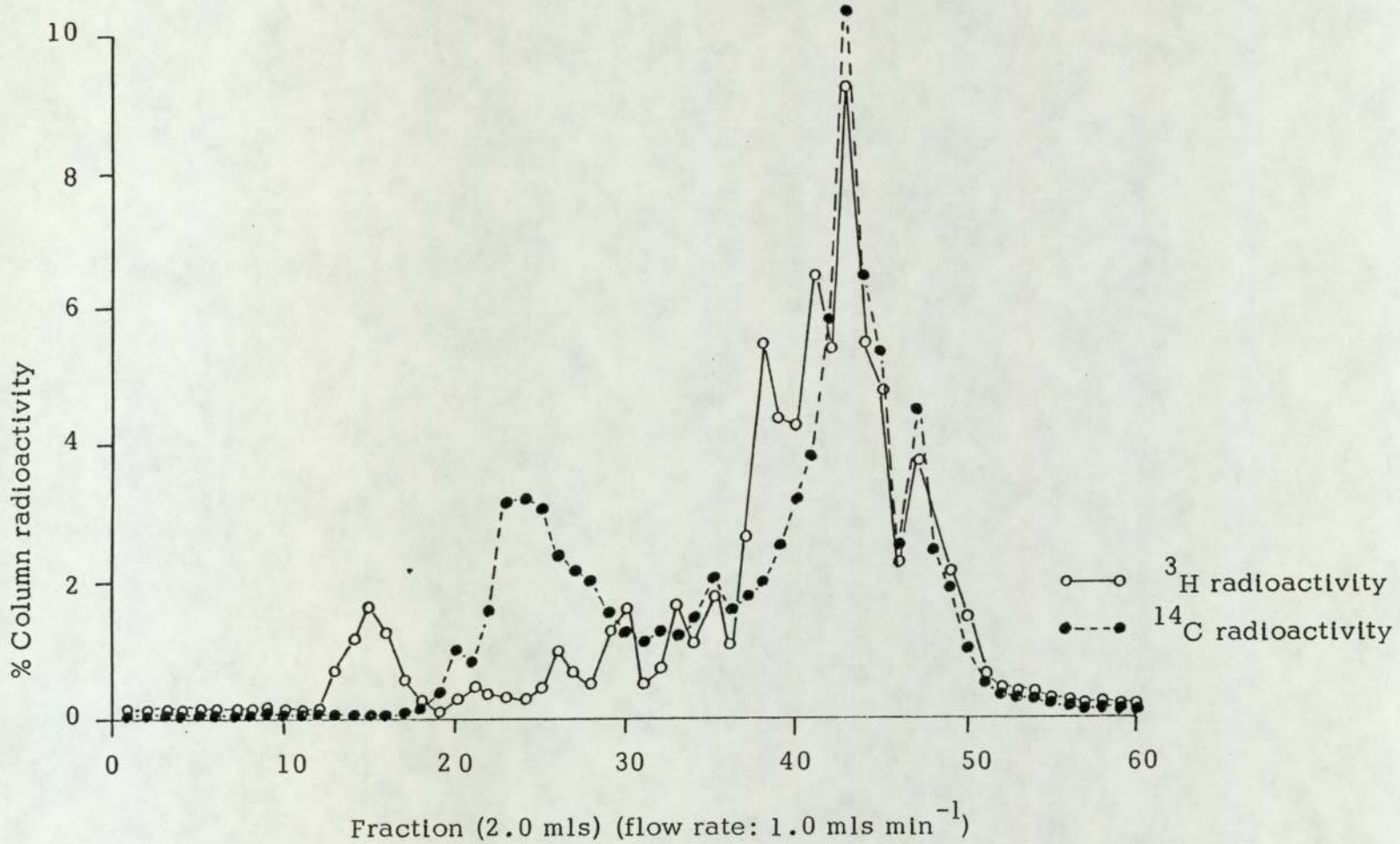


Fig. 6.4. HPLC (anion-exchange) of day 5 liver extract under conditions of gradient elution (extraction A) (normal male WAB/NOT rats).

CHAPTER 7

DISCUSSION

The present study illustrates several areas of folate metabolism in the tissues of normal and tumour-bearing rats; in particular the biosynthesis of folate polyglutamates. In addition, this work indicates possible tumour effects on the disposition of the folate pool in tumour-bearing rats.

7.1. The folate monoglutamate pool

The detection of unmetabolised FA in the liver and tumour tissues between 2-6 hours after administration demonstrates that prior metabolism of FA to naturally occurring folates is not essential for adsorption. This is consistent with previous studies (Blair and Matty, 1974; Blair, 1975) which showed that FA is absorbed partially unchanged through the intestine; and also with the detection of unmetabolised FA in the urine of the rat (Connor, 1979; Saleh *et al.*, 1981) and human (Saleh *et al.*, 1980) following the oral administration of FA. The underlying basis for the specific distribution of unmetabolised FA in the tissues is difficult to interpret as it may reflect many parameters (e.g. tissue uptake, loss and capacity to metabolise FA) not investigated in this work. This is particularly true in the case of the intestine on account of the absorptive function of this tissue, independent of metabolism.

The principal initial route of FA metabolism involves reduction and methylation to give $5CH_3THF$. Similar observations have been made in other studies of the rat (Hillman *et al.*, 1977, Steinberg *et al.*, 1978).

In addition, $5CH_3THF$ has been reported as the principal urinary reduced folate monoglutamate of the rat (Blair and Dransfield 1971; Retief et al., 1976; Barford et al., 1978) and human (Saleh et al., 1980) following the oral administration of FA.

The first step probably proceeds by the reduction of FA by DHF reductase to give DHF and then THF; both of which would thereby gain entrance to the endogenous folate pool. In support of this; oral administration of the DHF reductase inhibitor methotrexate prior to the oral administration of radiolabelled FA to rats, caused increased urinary excretion of unmetabolised FA (Barford et al., 1980). Wright and Anderson (1957) reported an alternative route of FA reduction to DHF (but not THF) in Clostridium which utilizes pyruvate and coenzyme A; however, this has not been reported in the mammal.

In addition, FA also appears to be metabolised to 10CHOTHF following the detection of a dual labelled derivative with the same chromatographic properties as this species in the tumour and intestine 2-6 hours after administration. This derivative has also been detected in rat liver and bile (Hillman et al., 1977) and rat urine (Connor, 1979) following FA administration. Saleh et al., (1981) reported the detection of 10CHOFA and not 10CHOTHF in rat urine following oral administration of FA. In this case, oxidative conversion of 10CHOTHF to 10CHOFA may have taken place.

The intracellular levels of 10CHOTHF may be tissue specific as this derivative was not detected in the liver. However, as the presence of 10CHOTHF in the tumour and intestine was only apparent as a small peak or "shoulder" eluting just after the folate polyglutamate peak on Sephadex G15, the possibility that the higher levels of radiolabelled folate polyglutamates in the liver may have masked the detection of this species in this tissue cannot be excluded. The apparent shift towards 10CHOTHF in the tumour and intestinal tissue may reflect a greater requirement for purine synthesis; both tissues being more rapidly dividing than the liver. Ratanasthien (1975) also reported increased levels of 10CHOTHF in the plasma of humans suffering from malignant disease. The report (Barford and Blair, 1978) of increased urinary excretion of 10CHOTHF by rats bearing the Walker 256 carcosarcoma is further support for a tumour induced shift in favour of 10CHOTHF; however, Saleh et al., (1980) were unable to confirm these observations in humans suffering from malignancy.

The physiological basis for the shift towards 10CHOTHF in the tumour and intestine is unclear; however this may reflect different activities of specific folate dependent enzymes in these tissues compared to the liver. This was indicated following an investigation of the products of autolysis of rat liver and intestinal folate extracts (Dr. A. E. Pheasant, personal communication). In the case of the liver extract, autolysis for two hours at 37°C revealed predominantly $5CH_3THF$ whereas in the intestine, the principal folate was 10CHOTHF. Thus the activities of specific

folate dependent enzymes in the intestine may favour a channelling of folate towards 10CHOTHF in this tissue, compared to the liver.

Jackson and Neithammer (1979) investigated folate-dependent enzymes in several rat hepatoma lines and the normal liver. In all hepatomas, the enzyme activities associated with histidine catabolism (i.e. formiminotransferase, cyclodeaminase), 10CHOTHF synthetase, 10CHOTHF dehydrogenase and $5,10CH_2THF$ dehydrogenase were markedly reduced compared to the normal liver whereas serine hydroxymethyltransferase was in most cases very similar to the liver. This may explain an observed shift in favour of a urinary folate considered to be $5,10CH_2THF$ in rats bearing the Novikoff hepatoma (Dr. A. E. Pheasant, personal communication).

The mechanism of $5CH_3THF$ synthesis from FA and the extent of this reaction in the different tissues is not revealed by these studies and is also not reported in the literature; however, the final step involves the reduction of $5,10CH_2THF$ by $5,10CH_2THF$ reductase. The non-detection of $5,10CH_2THF$ in the tissues, despite the intermediate role of this species in the synthesis of $5CH_3THF$, indicates very low intracellular levels of this derivative. Therefore, following the reduction of FA, the synthesis of $5CH_3THF$ appears to be extremely rapid.

Saleh et al., (1981) has confirmed that $5,10CH_2THF$ is synthesized from FA following the identification of this metabolite in rat urine following

FA administration. Therefore, these and other studies show that $5CH_3THF$, 10CHOTHF and $5,10CH_2THF$ are the principal reduced folate monoglutamates derived in vivo from orally administered FA in the rat. The possible routes of $5CH_3THF$ synthesis are presented in Fig. 7.1.

7.2. The folate polyglutamate pool

This work confirms many previous reports (Corrocher et al., 1972; Richardson et al., 1979; Connor and Blair, 1980) that folate polyglutamates are the principal folate derivatives in mammalian tissues. This also indirectly indicates that folate polyglutamates are the principal active folate coenzymes.

Rate of synthesis

In both normal and tumour-bearing rats, the rate of synthesis of folate polyglutamates in the liver was considerably higher than found in the tumour and intestinal tissue. Hepatic synthesis was generally 3-4 times higher than found in the tumour (when present) and the intestine of the same group. In tumour and intestinal tissues the rates of synthesis were very similar.

Between 0-6 hours the rate of synthesis of folate polyglutamates was essentially linear in all tissues investigated; however in the intestine of group C animals, this was not the case. In this group of rats the intestinal levels of radiolabelled folate at 2 hours was markedly greater than found in the intestine of other animals at this time. This may

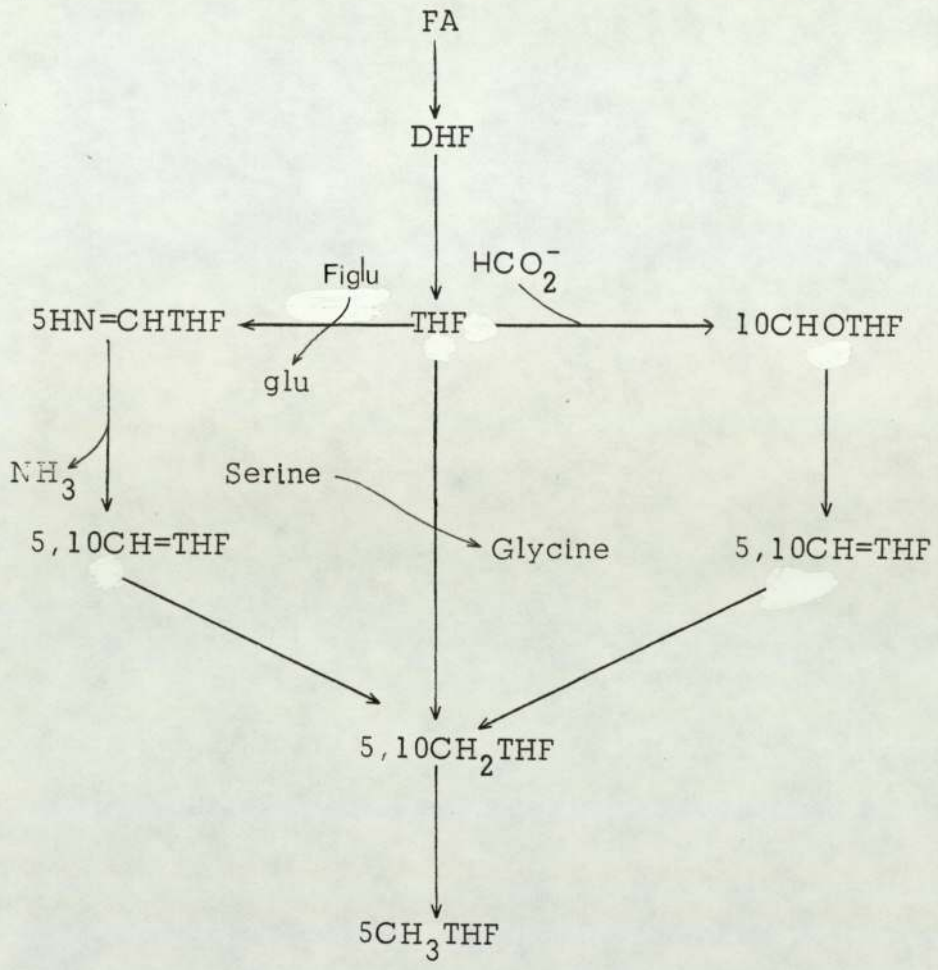


Fig. 7.1. Possible routes of $5CH_3THF$ synthesis from FA in the tissues of normal and tumour-bearing rats.
Figlu = formiminoglutamate; glu = glutamate.

explain the higher level of folate polyglutamates in this tissue at 2 hours and hence, the non-linearity of the synthesis of these derivatives. Shin et al., (1976) also observed a linear synthesis of folate polyglutamates in the rat between 0-5 hours after the oral administration of radiolabelled FA.

The rate of synthesis of ^{3}H-labelled folate polyglutamates was seen to be greater than the rate of synthesis of ^{14}C labelled folate polyglutamates. This reflects the higher levels of ^{3}H radioactivity retained in the tissues and also the slight excess of ^{3}H radioactivity (over ^{14}C radioactivity) eluting close to V_o (i.e. associated with the folate polyglutamate peak) which presumably resulted from the oxidative scission of folate polyglutamates during extraction or analysis. The latter process effectively magnified the levels of ^{3}H labelled folate polyglutamates. A greater tissue incorporation of ^{3}H radioactivity than ^{14}C radioactivity following oral administration of a mixture of [2-^{14}C] and [3' 5',7,9-^{3}H] FA has been observed in other studies (Connor, 1979). These findings may reflect secondary isotope effects as it has been found (Connor et al., 1980) that [3',5',7,9-^{3}H] FA is transported into the intestine more rapidly than [2-^{14}C] FA.

The rate of synthesis of folate polyglutamates is expected to be dependent on several factors. In this respect the intracellular levels of folate substrate (or substrates) and folylpolyglutamate synthetase (or synthetases) would most likely be of principal importance. Whilst the present studies have shown the levels of radiolabelled $5CH_3THF$ and FA in

the tissues, these derivatives are poor substrates for polyglutamate synthesis (Masureker and Brown, 1975; McGuire et al., 1980).

A clearer understanding of the rate of synthesis would require information on the tissue levels of the active folate substrates. Circulating folate in rat and human plasma has been shown to be principally $5CH_3THF$ and 10CHOTHF (Ratanasthien, 1975). This is also supported by the detection of these derivatives in the bile (Hillman et al., 1977) and urine (Connor, 1979) of the rat. Ratanasthien et al., (1974) reported constant levels of 10CHOTHF in human plasma, even when considerable variance of the total plasma levels was seen; the variable folate was found to be $5CH_3THF$. The plasma levels of 10CHOTHF also remained essentially constant even during short or long term folate administration.

These results suggest a careful homeostatic control of circulating 10CHOTHF in the mammal. As this derivative has been demonstrated to have good substrate properties for the synthesis of folate polyglutamates (McGuire et al., 1980) the rate of synthesis of these derivatives in the tissues may depend on the individual capacity of each tissue to sequestrate and utilize circulating 10CHOTHF.

In addition, the capacity of the individual tissues to utilize circulating $5CH_3THF$ may also have an important bearing on the rate of folate polyglutamate synthesis. However, the poorer substrate properties of $5CH_3THF$ would suggest that prior conversion of this derivative to a more

active substrate (or substrates) would be an important factor.

In mammalian tissues the only interconversion step of $5CH_3THF$ is in the synthesis of methionine, during which $5CH_3THF$ is converted to THF; a considerably more active substrate than $5CH_3THF$. Thus the individual tissue levels of methionine synthetase may affect the rate of synthesis of folate polyglutamates.

Grossman et al., (1974) investigated the levels of methionine synthetase in normal and neoplastic mammalian tissues. The levels of this enzyme were generally higher in the normal liver compared to several hepatoma tumour lines, however, in certain cases, this situation was reversed. Notably, methionine synthetase is absent in intestinal tissue (Finkelstein et al., 1978). Therefore, it appears that the liver may have a generally greater capacity to utilize $5CH_3THF$ than tumour tissue and that this step is absent in the intestine.

The greater rate of folate polyglutamate synthesis in the liver compared to the tumour and intestine may therefore reflect a more efficient utilization of circulating 10CHOTHF and $5CH_3THF$, the latter folate possibly undergoing prior interconversion to a more suitable substrate. This may reflect higher levels of folylpolyglutamate synthetase and methionine synthetase inthis tissue compared to the tumour and intestine.

The capacity of the tissues to sequestrate circulating folate monoglutamates prior to conversion to folate polyglutamates may also have

been involved in this process. In this respect, folate binding proteins may have an important role and therefore, possibly explain the higher rate of folate polyglutamate synthesis in the liver; in which the level of folate binding is highest.

Folate polyglutamate identity

These investigations have revealed a similar qualitative distribution of folate polyglutamates in the tissues investigated. Collectively in the three tissues, three principal folate polyglutamates were detected; namely folate polyglutamates A, B and C.

Folate polyglutamates A and B

Folate polyglutamates A and B exhibited very similar chromatographic properties on DEAE-cellulose as two folate polyglutamates previously identified in rat liver 48 hours after the oral administration of radiolabelled FA as 10CHOFA$(glu)_4$ (corresponding to folate polyglutamate A) and 10CHOFA$(glu)_3$ (corresponding to folate polyglutamate B) (Connor and Blair, 1980).

In respect of folate polyglutamate A, the findings of Connor and Blair (1980) were confirmed as this derivative co-chromatographed on DEAE-cellulose with authentic 10CHOFA$(glu)_4$. Further evidence from these studies supporting the identity of folate polyglutamate A is somewhat contradictory. Thus, HPLC of liver extracts retained from the long-term study (Chapter 5), which were previously demonstrated to

contain folate polyglutamate A, did not reveal a dual labelled species eluting in the position of 10CHOFA$(glu)_4$. The reason for this is unclear, however this may have resulted from folate degradation giving rise to changes in chromatographic properties, since these extracts were stored for considerably longer (6-8 months) prior to HPLC analysis, than before the initial analysis (0-3 months).

This would not explain the similarly conflicting results following efforts to identify folate polyglutamate A from a freshly prepared 48 hour liver extract, using HPLC (Chapter 6). In this instance, breakdown or degradation was evident on account of the markedly changed chromatographic properties of this species on HPLC following initial chromatography of the extract on DEAE-cellulose. Whilst a species with similar chromatographic properties as 10CHOFA$(glu)_4$ was detected following HPLC of the freshly prepared 48 hour liver extract, this was not a major peak. Differential microbiological assay of folate polyglutamate A revealed the expected properties of a 10CHOFA polyglutamate, however this is not exhaustive proof of identity.

Therefore, these results render difficult any firm conclusions regarding the identity of folate polyglutamate A; although preliminary results support the results of Connor and Blair (1980), that it is 10CHOFA$(glu)_4$.

Folate polyglutamate B was indicated to be 5CH_3THF$(glu)_3$ by the co-chromatography of this species on HPLC with the authentic compound.

$5CH_3$THF polyglutamates have been previously reported in rat liver (Shin et al., 1972; Hillman et al., 1977) although Connor and Blair (1980) were unable to confirm these findings. The existence of a folate triglutamate co-existing in the cell with a folate tetraglutamate (i.e. folate polyglutamate A) is difficult to explain as this would imply the loss or gain of a single glutamate residue simultaneously with the interconversion of these two derivatives. A possible explanation for this is that folate polyglutamate B lost a single glutamate residue during the extraction process (i.e. due to the partial action of endogenous conjugase). Alternatively, folate polyglutamates A and B may exist in metabolically distinct folate pools.

Microbiological assay showed folate polyglutamate B, in the 24 hour liver extract of group B normal rats to have the same properties as folate polyglutamate A. This may represent an individual case of the existence of a species with similar chromatographic properties as folate polyglutamate B, but of different identity.

Folate polyglutamate C

Folate polyglutamate C demonstrated marked lability. This was apparent from the observation that this species was not detected unless precautions (i.e. the collection of folate polyglutamates, eluting from Sephadex G15, into ascorbate) were taken to prevent breakdown during chromatography. A folate polyglutamate with similar chromatographic properties as this species was not detected in similar studies by Connor

and Blair (1980) however in their studies, similar precautions to prevent the breakdown of labile folate polyglutamates were not taken.

The marked lability of folate polyglutamate C was also apparent from later studies (Chapter 6) in which re-chromatography of this species (initially particularly purified on DEAE-cellulose) on Sephadex G15 demonstrated a predominantly ^{3}H labelled peak eluting close to V_o and a broad ^{14}C only labelled peak eluting later; showing that considerable breakdown had taken place. This presumably resulted from the oxidative scission of the C_9-N_{10} bond, yielding a ^{3}H labelled p-aminobenzoyl polyglutamate and a pterin derivative.

Following the use of authentic markers on DEAE-cellulose, folate polyglutamate C was shown not to be either $5CH_3THF(glu)_4$ or $FA(glu)_4$. In addition, no change was observed following $NaBH_4$ treatment suggesting that folate polyglutamate C is not a $5,10CH_2THF$ derivative.

The considerable lability of folate polyglutamate C is indicative of a THF polyglutamate; THF being very labile and readily undergoing oxidative breakdown to yield a pterin and a p-aminobenzoyl derivative (Blakley, 1969). Wittwer and Wagner (1980) recently reported the identification of $THF(glu)_4$ in rat liver, however, other studies of hepatic folates in the rat (Connor and Blair, 1980) and the guinea pig (Corrocher et al., 1972) were unable to confirm this; possibly on account of the lability of this species. Notably, Shin et al., (1972) identified a THF polyglutamate in

rat liver; a species exhibiting very similar chromatographic properties on DEAE-cellulose as folate polyglutamate C.

Further indication of a THF polyglutamate stems from the identification of pterin (2-amino-4-hydroxy pteridine) following DE52 chromatography A (and with the detection of a species with similar chromatographic properties to pterin following the breakdown of folate polyglutamate C (chapter 6)) as this species has been identified as a specific scission product of THF (Blair and Pearson, 1974). Although microbiological assay of folate polyglutamate C suggested it might be a $5CH_3$THF polyglutamate, the chromatographic behaviour and extreme lability of this species excludes this possibility.

The detection of two labile folate polyglutamates (C_1 and C_2) eluting from DEAE-cellulose between 0.80 and 1.00M NaCl in the liver of group B normal rats between 2-10 hours suggests the existence of two folate polyglutamate (differing in the number of glutamate residues) of the same class as folate polyglutamate C; namely, THF polyglutamates. As only folate polyglutamate C eluted in this region at 24 hours, this indicates an intermediate role of one or both folate polyglutamates C_1 and C_2 in the synthesis of folate polyglutamate C.

Tissue distribution of folate polyglutamates

Comparison of the distribution of folate polyglutamates A, B and C in the three tissues revealed certain differences. In the liver, folate polyglutamates A, B and C were present whereas in the tumour tissue,

folate polyglutamate B was absent; the intestine representing an intermediate situation.

Thus, in tumour tissue and to a lesser extent in intestinal tissue, a shift in favour of folate polyglutamate A is apparent. If the proposed identity of folate polyglutamate A as $10CHOFA(glu)_4$ is correct, this may effectively represent a shift in favour of $10CHOTHF(glu)_4$ as the identification of $10CHOFA(glu)_4$ has been attributed to the oxidation of $10CHOTHF(glu)_4$ during analysis (Connor and Blair 1980).

A shift in favour of 10CHOTHF polyglutamates may reflect the apparent shift in favour of 10CHOTHF in the tumour and intestine as was evident at the earlier time periods, Similarly, this may reflect the greater requirement for purine synthesis in these tissues compared to the liver.

The studies described in this section reveal many of the inherent difficulties associated with the identification of folate polyglutamates extracted from mammalian tissues. Preliminary evidence suggests that folate polyglutamates A, B and C are $10CHOFA(glu)_4$, $5CH_3THF(glu)_3$ and $THF(glu)_n$ respectively, however, more definitive identifications are required.

7.3. Tumour effects on folate metabolism

The present studies have shown several differences in the incorporation and metabolism of administered folate by the liver and intestine of tumour-bearing rats compared to normal rats. These observations raise the possibility of specific tumour effects on folate metabolism.

The presence of a tumour mass might be expected (and has been shown) to affect whole body folate metabolism in a number of ways (1-3).

1. Competition for circulating folate : A tumour mass will act as an additional tissue and therefore compete with the host tissues for circulating folate. This would be expected to diminish the amount of administered folate available to the host tissues and thereby ultimately decrease the degree of folate incorporation into these tissues.

2. Increased FA reduction : The presence of a tumour mass (i.e. an additional tissue) would also be expected to increase the degree of reduction of administered FA; thereby expanding the circulating pool of reduced derivatives. This was claimed by Pheasant et al., (1979) following studies of FA metabolism in humans suffering from malignancy. As reduced folate monoglutamates are better substrates than FA in the synthesis of folate polyglutamates, this may lead to enhanced synthesis of these derivatives in host tissues.

3. Diminished folate breakdown : The catabolic breakdown of folates has been reported to be reduced in tumour-bearing

rats (Saleh et al., 1981). This was apparent from the reduced urinary excretion of folate catabolites by these rats, compared to normal rats.

Tumour tissue has been reported (Williamson et al., 1970; Weber et al., 1971) to have elevated lactate production (as shown by the elevated lactate/pyruvate ratio) and thus more reducing intracellular conditions. Excessive lactate production can lead to increased lactate in the blood and may thereby raise the lactate/pyruvate ratio in other tissues. Therefore tumour tissue may induce more reducing conditions in the host tissues and it has been suggested (Saleh et al., 1981) that this may diminish the oxidative breakdown of folates and thus favour folate retention.

In these studies, observed differences in the disposition of the folate pool in tumour-bearing rats, compared to normal rats, involved both the liver and the intestine. Interpretation of these effects in the intestine however, is made difficult on account of the absorptive function of this tissue.

The total levels of $5CH_3THF$ in the liver (per gram. wet weight) between 2-6 hours were higher in both groups of tumour-bearing rats compared to normal rats. This observation is unexpected in view of the competition for folate by the tumour mass and suggests an alternative physiological effect of the tumour tissues. Horne and Wagner (1979) reported that the uptake of $5CH_3THF$ into isolated hepatocytes is enhanced in the presence of ethanol. Following further observations that other oxidisable alcohols and a reduced oxygen tension produce the same effect,

it was suggested that this process is mediated by lowered redox potential in the cell. The observations of the present study, that the hepatic levels of $5CH_3THF$ are increased in tumour-bearing rats, may also result from a lowered redox potential in this tissue; in this case however, as a result of lactate production by the tumour.

At 24 hours after FA administration, the liver of both groups of tumour-bearing rats showed enhanced folate incorporation, compared to the corresponding normal animals. This reflected increased levels of folate polyglutamates as these were the major derivatives in this tissue at this time. Similar findings have been reported by Connor and Blair (1979). These observations might be explained in terms of a tumour induced reduction in hepatic folate breakdown and loss or the increased hepatic synthesis of folate polyglutamates due to increased reduction of the administered FA. Alternatively, the apparent tumour induced enhancement of $5CH_3THF$ incorporation into the host liver may favour the synthesis of folate polyglutamates in this tissue; possibly after prior conversion of $5CH_3THF$ to a more suitable substrate.

Saleh et al., (1981) reported a reduced hepatic level of folate at 24 hours after the oral administration of radiolabelled FA in rats bearing the sarcoma Mc103B, compared to normal rats. In this study however, the tumour size was almost 5 times greater than was the case in the present study. A possible explanation for this is that competition for circulating folate by the very large tumour mass was the major tumour effect

influencing the disposition of the hepatic folate pool.

7.4. The long-term disposition of the hepatic folate polyglutamate pool

Following the synthesis of hepatic folate polyglutamates from the administered FA, these species represented the principal folate derivatives up to 22 days after administration. The qualitative pattern of these derivatives (as evident from the initial DEAE-cellulose chromatography) remained very constant and as previously found in the liver, three folate polyglutamates were present; namely folate polyglutamate A, B and C. The continued existence of these three species up to 11 days after FA administration would suggest that these are the natural endogenous folate polyglutamates present in the liver. Furthermore, as the pattern of three folate polyglutamates (A, B and C) had emerged by 10-24 hours after the administration of FA, this suggests that the administered folate had established equilibrium with the endogenous hepatic folate by this time.

The non-detection of dual labelled derivatives corresponding to $10CHOFA(glu)_4$ (A) and $5CH_3THF(glu)_3$ (B) following HPLC analysis of these extracts may have resulted from folate breakdown due to the longer storage time (6-8 months) of these extracts prior to HPLC analysis, than before the initial DE52 chromatography (0-3 months).

The long half life (7.1 days) of hepatic folate indicates that hepatic folate polyglutamates are in slow equilibrium with the extracellular circulating folate. This may emphasise an important role of folate polyglutamates in respect of the intracellular retention of folate. Barford et al., (1977)

administered similar levels of radiolabelled FA to rats and observed a similar decline in hepatic radioactivity ($t\frac{1}{2}$ = 8 days). Following the oral administration of FA to rats, Leslie & Baugh (1974) found that 4% of the administered folate was present in the liver at 24 hours which then declined to 1.9% in 14 days (i.e. $t\frac{1}{2}$ is approximately 14 days). In their study however, the level of administered folate was 10 times greater than used in the present studies.

The present long-term studies have also shown that the incorporation of the administered radiolabelled folate into the hepatic folate polyglutamate pool continues up to 5 days after administration. As the urinary excretion of radiolabelled folate and folate catabolites, an indication of the circulatory levels of these species, was maximal before 24 hours, this suggests a slow release of folate from other body tissues that becomes incorporated into the liver. Similar findings were reported by Barford et al., (1977) however, Leslie & Baugh (1974) found maximal folate levels at 6 hours after FA administration. In the latter studies, the considerably larger dose of administered FA may have saturated the hepatic folate storage capacity in a short period of time.

The extent to which the use of a pulse dose of FA reflects the normal pattern of folate metabolism in rats is unclear. The normal dietary intake of FA (the dietary form of folate in laboratory foodstuff) (containing 0.7ug g^{-1} FA) would not lead to a greater intake than 10 ug per rat, per day. Previous studies (Barford et al., 1977; Connor and Blair, 1979) have

shown that the smaller the pulse dose of FA administered to rats, the greater the proportion that becomes incorporated into the hepatic folate pool. Therefore, it is likely that the normal dietary intake of FA over a 24 hour period would lead to a greater proportion becoming incorporated into the hepatic folate polyglutamate pool than would be observed following a pulse dose of a comparable amount of folate. This illustrates a limitation of the pulse dose procedure.

The exponential decline of the hepatic radioactivity indicates the operation of a first order reaction. This process would therefore appear to be dependent on the concentration of folate in the tissue. A possible explanation for this is the breakdown of hepatic folate and the subsequent loss of folate catabolites in the urine. Further support for a first order folate breakdown is evident from the urinary levels of radiolabelled folate catabolites which were quantitatively maximal from 0-24 hours and then declined over successive days in an exponential manner.

7.5 Folate binding proteins

Marked differences in the levels of protein bound folate were evident in the three tissues investigated. The higher level of folate binding proteins in the liver, compared to the tumour and intestine, may reflect higher levels of folate binding proteins in this tissue. Alternatively, those differences may reflect the variation of folate identity in the tissues.

Folate binding proteins have been widely reported in the literature

(Leslie and Rowe, 1972; Corrocher et al., 1974; Waxman and Schreiber, 1975). Zamierowski and Wagner (1974) reported the highest level of folate binding in the liver, a lesser amount in the kidney but no detectable binding in skeletal muscle or the spleen. Whilst the present studies confirm that folate binding is highest in hepatic tissue, the report (Zamierowski and Wagner, 1974) of a "significant amount" of intestinal folate binding is not supported.

The level of folate binding in tumour tissue has been little investigated, however, Pheasant and Blair (1979) reported no protein bound folate in the Novikoff hepatoma. The present studies have shown similar findings in the case of the Walker 256 carcosarcoma. The bound radioactivity in the latter tumour was predominantly ^{3}H labelled indicating the binding of a p-aminobenzoyl derivative. Whilst this species was not identified in the present study, Caston and Kamen (1979) have reported the binding of p-AB in mammalian tissue and body fluids.

The precise role of folate binding proteins is not fully understood, however, Zamierowski and Wagner (1974) reported the identification of DHF reductase as a folate binding protein.

Folate binding proteins may also be required to sequestrate circulating folate, either to increase intracellular availability or for storage purposes. This would explain the greater incorporation of administered folate into hepatic tissue. The absence of folate binding proteins in the intestine, as apparent from the present studies, may facilitate the transfer

of absorbed folate into the circulation.

7.5. Folate polyglutamate analysis using HPLC

When suitable conditions were employed, HPLC allowed the separation of folate polyglutamates from folate monoglutamates and the individual resolution of folate polyglutamates; therefore, supporting the findings of Stout et al., 1976 and Cashmore et al., 1980. This and the speed of folate polyglutamate analysis using HPLC, confirms that this procedure offers considerable advantages over the more time consuming and less selective conventional chromatographic procedures.

Under conditions of gradient elution using an increasing concentration of Na_2SO_4, anion-exchange HPLC resolved folate polyglutamates in order to increasing number of glutamate residues. This demonstrates the capacity of the glutamate residues to influence binding to the anion-exchange column and presumably reflects an increasing anionic charge as the number of ionised glutamate residues increases. The simple linear relationship observed when the retention time (R_t) is plotted against the glutamate number for the FA, $5CH_3THF$ and 10CHOFA series is further support for an incremental increase in the anionic charge as the number of glutamate residues increases. These results confirm those of Cashmore and co-workers (1980) who observed similar chromatographic properties of folate polyglutamates chromatographed under conditions of gradient elution (increasing phosphate buffer concentration) anion-exchange HPLC. These workers also resolved folate polyglutamtes differing in the number of

glutamate residues using reverse phase gradient elution HPLC. However, in this case, the folate polyglutamates eluted in order of decreasing number of glutamate residues.

The present studies also revealed that certain folate polyglutamates (e.g. $10CHOFA(glu)_5$ and $5CH_3THF(glu)_4$) differing in the state of reduction and substitution of the pteridine ring, or the state of substitution at N10, were either not resolved or poorly resolved. These results serve to illustrate possible ambiguity in folate polyglutamate analysis using a single HPLC system and the necessity for developing more selective chromatographic procedures. The application of HPLC to the analysis of folate polyglutamates has been demonstrated by McGuire et al., (1980) who employed anion-exchange HPLC for the identification of THF polyglutamates synthesised in vitro by rat liver folypolyglutamate synthetase.

7.7. Criticisms of methods and suggestions for future studies

All forms of chromatographic analysis have inherent drawbacks. Thus, whilst able to demonstrate differing chromatographic properties and therefore, the different identities of two compounds, the demonstration of identical chromatographic properties does not prove identical chemical and physical structure. In many areas where the identification of complex biochemicals (often available in only small or trace amounts) is sought, more rigorous chemical analysis is often either not possible or is impracticable for everyday routine assay. These difficulties are characteristic as far as the identification of folate coenzymes is concerned.

With regard to the identification of folate monoglutamates, this situation is somewhat ameliorated. Most folate monoglutamates exhibit characteristic chromatographic properties and are susceptible to complete resolution in certain systems. Moreover, there are only a limited number of these species. In the case of folate polyglutamates of which the chromatographic properties are complex, individual resolution difficult and the number of theoretical possibilities large; the identification of these derivatives is considerably more problematic.

In the present study, the identification of $5CH_3THF$ and FA is not ambiguous on account of the characteristic chromatographic properties of these species. However, the identification of folate polyglutamate B as $5CH_3THF(glu)_3$ is less certain in view of the large number of folate polyglutamates (not available as authentic markers in this work) with possibly identical chromatographic properties as $5CH_3THF(glu)_3$.

Certain features of the chromatographic behaviour of folate polyglutamates under conditions of gradient elution anion-exchange HPLC may allow a more simple systematic approach to the preliminary identification of these derivatives. In this respect, the good linear relationship between the retention time and the number of glutamate residues, allows interpolation and possibly, extrapolation to predict the retention times of these species. Therefore, if the experimentally determined and the theoretically predicted retention times of many classes of folate polyglutamates were determined in several similar systems, this may reduce the ambiguity associated with the chromatographic identification of these derivatives. In addition, this

may also allow a more accurate approach to the choice of chromatographic markers employed for identification purposes.

The observed day-to-day or week-to-week variability in the retention time of folate markers chromatographed under conditions of gradient elution HPLC, may result from the considerable sensitivity of anion-exchange HPLC to minor differences in the running conditions. A more reproducible system may be achieved with the use of reverse-phase HPLC which has been reported to be a very effective alternative to anion-exchange HPLC (Cashmore et al., 1980).

In the present study, quantitation of radiolabelled folate derivatives was made difficult due to overlap or incomplete resolution of the peaks. This was particularly the case following DE52 chromatography A, which in most cases resulted in the incomplete resolution of folate polyglutamates A and B, and the incomplete resolution of a ^{3}H labelled species which co-chromatographed with folate polyglutamate A. Accurate quantitation of these derivatives will necessitate improved conditions of resolution and this may be achieved with the further development of HPLC.

The proposed identity of folate polyglutamate C as that of a THF polyglutamate may allow further investigation of identity by a combination of both chemical and chromatographic means. THF (and presumably THF polyglutamates) reacts non-enzymatically with formaldehyde in low concentration to give $5,10CH_2$ THF (Blakley, 1969) which could then be reduced (using $NaBH_4$) to $5CH_3$THF. Therefore, folate polyglutamate C may be susceptible to conversion to the corresponding $5CH_3$THF

polyglutamate and identified chromatographically with the appropriate folate marker. Though folate polyglutamate C or the chemically derived $5,10CH_2THF$ derivative could themselves be identified with the appropriate folate marker, THF and $5,10CH_2THF$ are considerably less stable than $5CH_3THF$ (Blakley, 1969). Therefore, conversion of folate polyglutamate C to the more stable $5CH_3THF$ derivative and the use of the more stable $5CH_3THF$ polyglutamate markers, may represent a more satisfactory approach

A limitation the present studies stems from the use of only two animals for each time period for much of the present studies of folate metabolism. This prevented a more satisfactory statistical analysis of the results; this being particularly relevant to a more definitive interpretation of tumour effects on host tissue folate metabolism. Further investigations of these effects should therefore be undertaken using more animals for each time period.

The present studies indicated that prolonged storage of folate extracts (with an initial ascorbate content of 2% w/v) may cause changes in the identity of the folate coenzymes. More accurate quantitative studies might therefore be improved if the storage time prior to analysis is both reduced and standardised.

Studies of tumour-bearing animals raise questions regarding applicability to the human situation. Human tumours are normally slow growing and therefore the use of the Walker 256 carcosarcoma, a very rapidly growing tumour, may not be appropriate to an understanding of human tumours.

In view of the slower growth rate of the sarcoma Mcl03B, this tumour may be of greater value.

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