Folate Metabolism in Normal and Tumour Bearing Mammals

A thesis

submitted for the degree of

Doctor of Philosophy

By Michael J. Connor

in the Department of Chemistry

University of Aston in Birmingham

SUMMARY

Folate Metabolism in Normal and Tumour Bearing Mammals

by Michael Joseph Connor

a thesis submitted for the degree of
Doctor of Philosophy
June 1979

The whole animal metabolism and physiological disposition of folate derivatives has been studied in normal and tumour bearing rodents. Within 48 h of administration of radioactive folate tissue radioactivity was incorporated largely into high molecular weight folate conjugates. The major derivative was isolated from rat liver and identified as 10-formylfolate-γ-tetraglutamate by spectral, microbiological, chemical and chromatographic techniques. A minor derivative exhibited similar properties and was assigned the structure 10-formylfolate-γ-triglutamate. Similar conjugates were found in normal intestine and in the tumours and livers of Walker 256 tumour bearing rats. Urine collected 0-48 h after administration of folic acid, 10-formylfolate or 10-formylfolate-γ-tetraglutamate contained several metabolites including both intact folates and folate catabolites. The major catabolite was identified as p-acetamidobenzoate by chromatography and reverse isotope dilution analysis and a reduced pteridine and folate derived CO_2 and urea also characterized. The relative amounts of intact folate and folate catabolites varied with time and dose. Walker 256 tumour bearing rats excreted less intact folate and had markedly increased hepatic uptake, suggestive of tumour induced folate deficiency. A novel urinary pteridine derivative was excreted confirming the value of the folate pool as a potential source of tumour markers. A preliminary investigation of folate metabolism in male, female and immune-suppressed female mice was also made.

The results presented support the existence of two metabolically distinct folate pools. The folate monoglutamates constitute the first pool, effectively functioning as a transport and short term storage form for the synthesis of the second pool - the folate polyglutamates - the active coenzymes. The folate in both pools is catabolised to simpler derivatives; the folate monoglutamates to p-acetamidobenzoate, probably by intestinal metabolism during enterohepatic circulation, and the folate polyglutamates to p-acetamidobenzoyl-L-glutamate and the unidentified pteridine *in situ* in the cells.

Key Words

Folate catabolism; folate metabolism; 10-formylfolate; Walker 256 tumour

This work was carried out from October 1975 to September 1978 in the Department of Chemistry, in the University of Aston in Birmingham. It has been done independently and has not been submitted for any other degree.

M. Honnor.

ACKNOWLEDGEMENTS

I would like to thank all those who have helped me in this work, especially Prof. J.A. Blair for his supervision, patience and encouragement. My thanks also to the other members of the Aston Folate Group for many interesting discussions and much useful advice, the Science Research Council for financial support and finally, Mrs. P. Trotter for her excellent typing.

To my mother and father

CONTENTS

FIGURES

TABLES

CHAPTER 1

INTRODUCTION

Folate coenzymes act as one carbon carriers in diverse areas of mammalian metabolism (reviewed by Blakley, 1969; Hoffbrand, 1976; Rowe, 1978). The active compounds are reduced, to the 7,8 dihydro or 5,6,7,8 tetrahydro levels, and often substituted (N5 and N10 positions) derivatives of folic acid (I). Compounds of this type such as 5-methyltetrahydrofolate (5 MeTHF) (II) and 10-formyltetrahydrofolate (10 CHOTHF) (III) make up the bulk of urinary and plasma folate. Within the tissues folate conjugates occur. These are probably poly-γ-glutamate derivates by analogy with yeast folate (Pfiffner et al, 1946) although detailed data and an adequate characterization of their function are lacking in mammalian systems.

A simplified scheme showing the major folate requiring reactions and interconversion routes in the mammal is illustrated in Figure 1.1. Since the mammal cannot synthesise folate de novo the in vivo fate of supplementary or dietary folate will reflect both the nutritive status of the organism and the equilibrium between the various competing folate requiring reactions.

Both purine and pyrimidine biosynthesis require folate coenzymes. Carbon atoms 8 and 2 of the purine ring are thought to be derived by the donation of the one carbon unit from the bridge carbon group of 5, 10-methenyltetrahydrofolate (5,10CH=THF) (IV) to 5'-phosphoribosyl glycinamide and the donation of the one carbon group of 10 CHOTHF to 5'-phosphoribosyl-4- carboxamide -5-aminoimidazole respectively (illustrated in Figure 1.2). Both of these reactions liberate tetrahydrofolate (THF) (V) which can re-accept one carbon units and thus be recycled.

Recently the role of 5,10 CH=THF in purine biosynthesis has been questioned. Using purified glycinamide ribotide transformylase (EC 2.1.2.2) from Escherichia coli Dev and Harvey (1978 a) have shown that the required coenzyme is 10 CHOTHF. They suggest that the

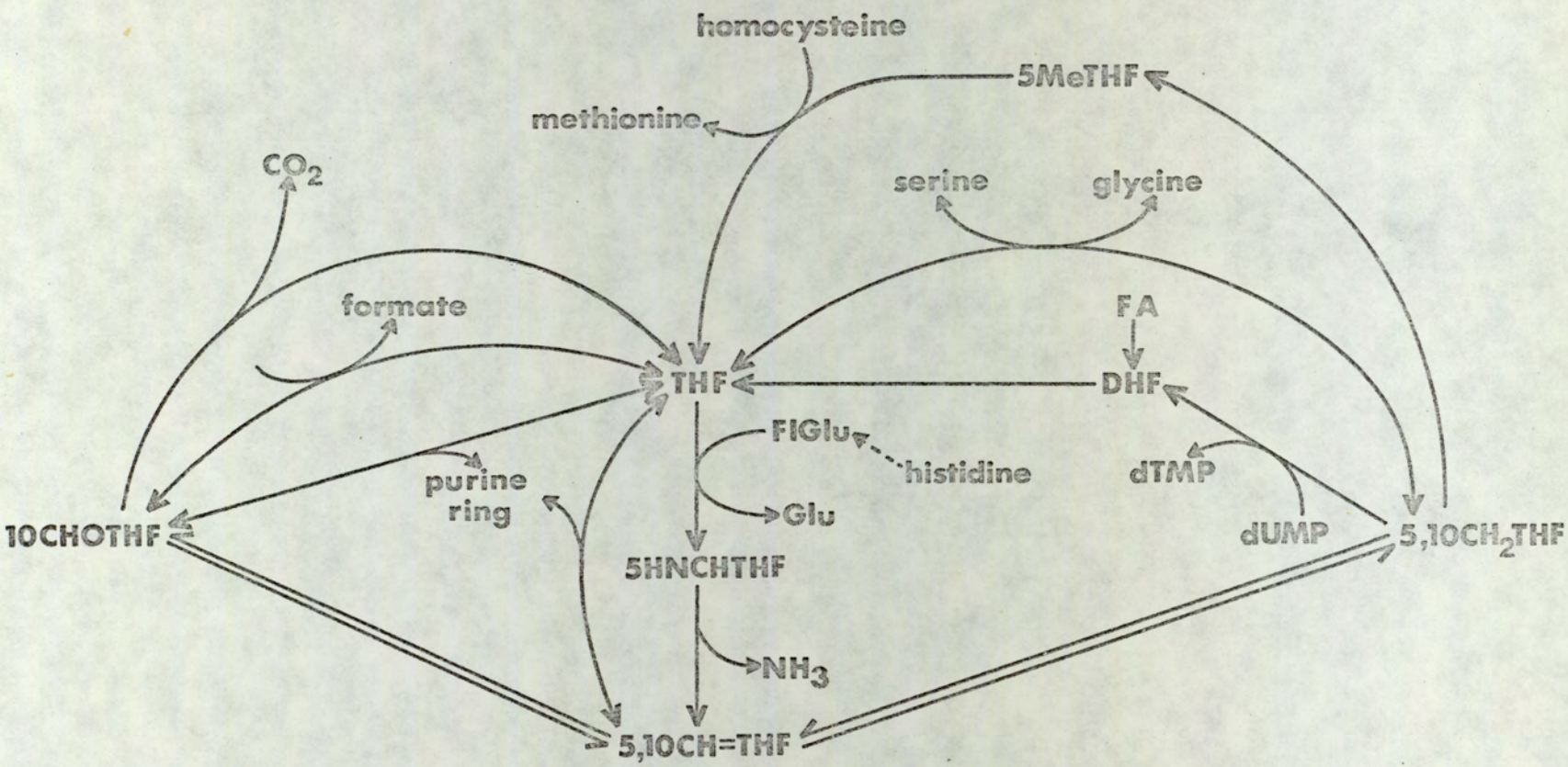
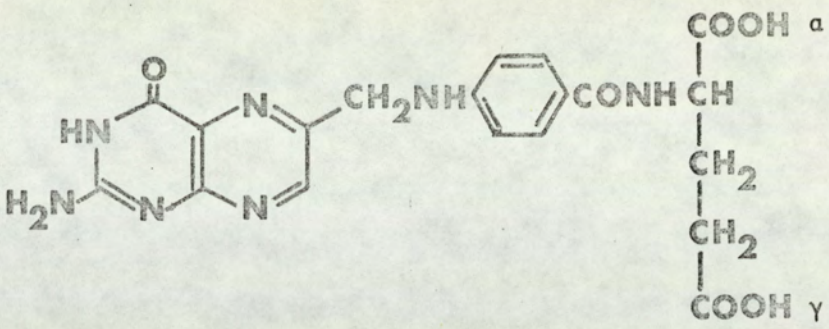
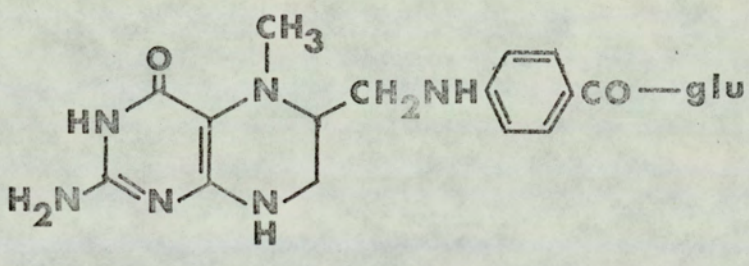


Figure 1.1 The major folate interconversion pathways.

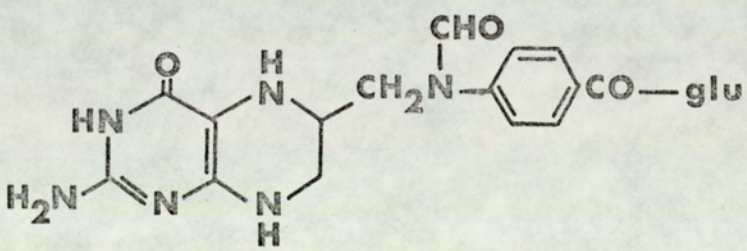
The figure illustrates the important folate interconversion routes in the mammal emphasising the equilibrium between 5,10 CH_2THF and 10 CHOTHF. Individual reactions are described in more detail in the text.



I Folic acid



II 5-Methyltetrahydrofolic acid

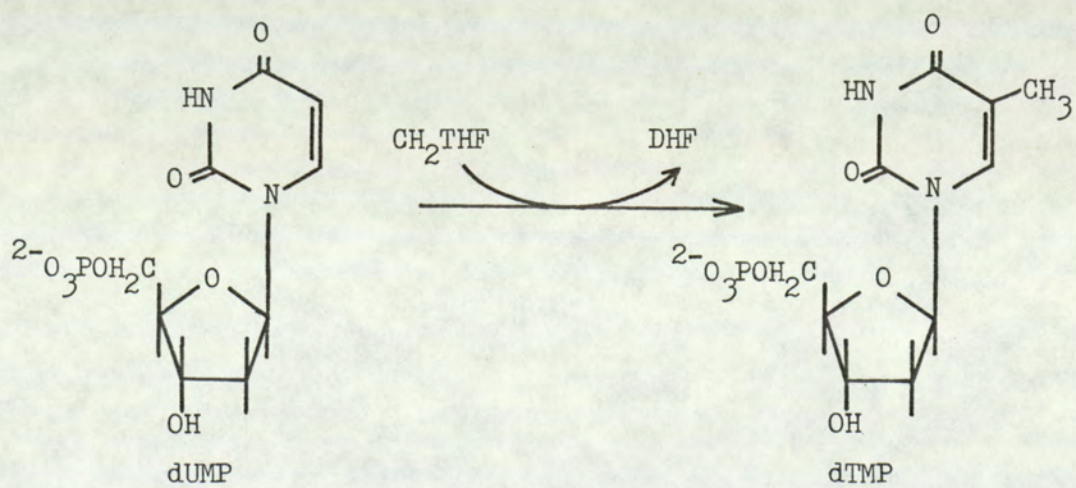


III 10-formyltetrahydrofolic acid

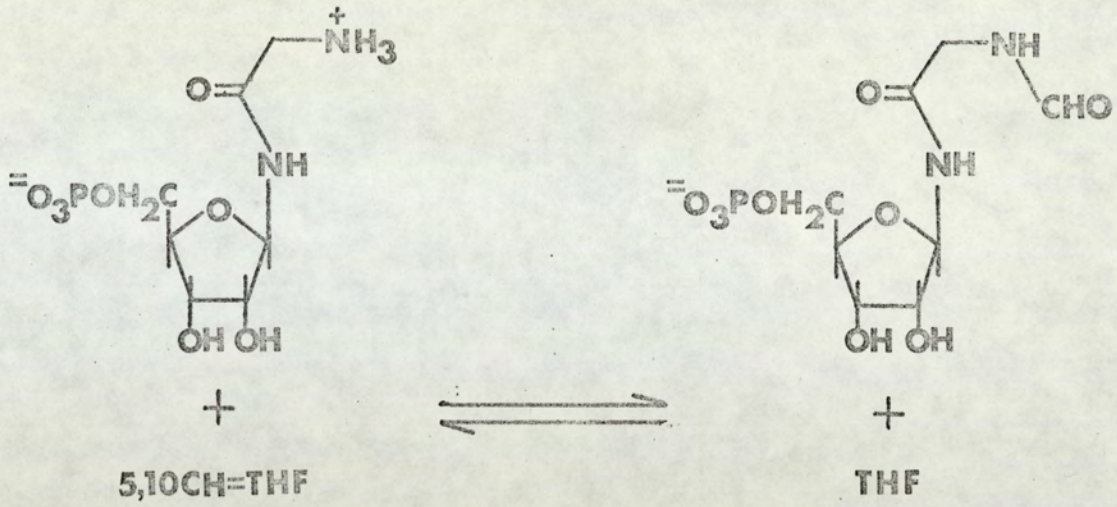
earlier studies on pigeon liver and bacterial extracts in which the pathway was originally elucidated either contained contaminant 5,10 CH=THF cyclohydrolase (EC 3.5.4.9), an enzyme which converts 5,10 CH=THF to 10 CHOTHF, in their preparations or that reactions were investigated in tris-HCl buffers at pH 7.8, 5,10 CH=THF being rapidly chemically hydrolysed to 10 CHOTHF at this pH (Beavon and Blair, 1972). 5,10 CH=THF probably occurs in the cell only in transitory amounts during the conversion of 5, 10 CH_2THF to 10 CHOTHF.

It is interesting to note that in E. coli (Dev and Harvey, 1978 b), in pig liver (Tan et al, 1977) and in sheep liver (Paukert et al, 1976) 5,10 CH_2THF dehydrogenase and 5,10CH=THF cyclohydrolase activities are contained on a single multifunctional polypeptide. In the pig and sheep liver 10 CHOTHF synthetase (EC 6.3.4.3) activity, catalysing the direct formylation of THF to 10 CHOTHF using formate as the one carbon unit, was also combined. The E. coli enzyme undergoes allosteric inhibition by 10 CHOTHF for both activities although whether this occurswith the mammalian enzymes is unknown.

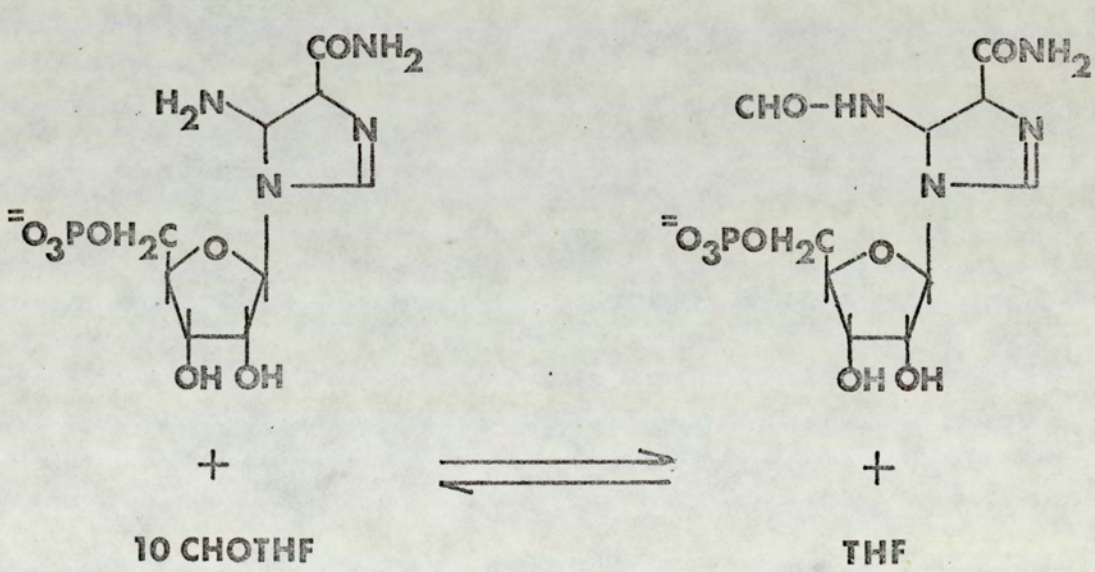
A second N5-N10 bridged folate is involved in pyrimidine anabolism. Deoxyuridylate (dUMP) is methylated with the one carbon group of 5,10 methylenetetrahydrofolate (5,10 CH_2THF) (VI) yielding deoxythymidylate (dTMP) and the partially oxidized folate derivative 7,8 dihydrofolate (DHF) (VII), by the enzyme thymidylate synthetase (EC 2.1.1.b):



dUMP dTMP

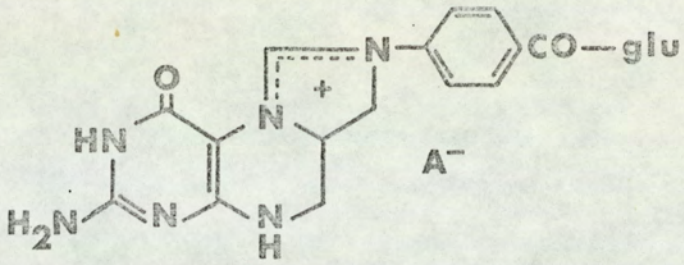


(a) Generation of C8 of the purine ring : donation of the one carbon group of 5,10 CH=THF to 5' phosphoribosylglycinamide catalysed by 5,10 CH=THF : 2-amino-N-ribosylacetamide-5'-phosphate transformylase

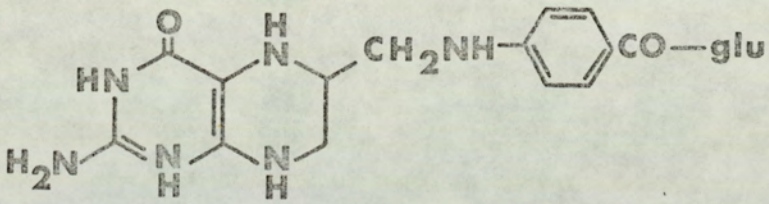


(b) Generation of C2 of the purine ring : donation of the formyl group of 10 CHOTHF to 5'-phosphoribosyl-4-carboxamide-5-aminoimidazole catalysed by 10 CHOTHF : 5-amino-1-ribosyl-4-imidazole carboxamide-5'-phosphate transformylase

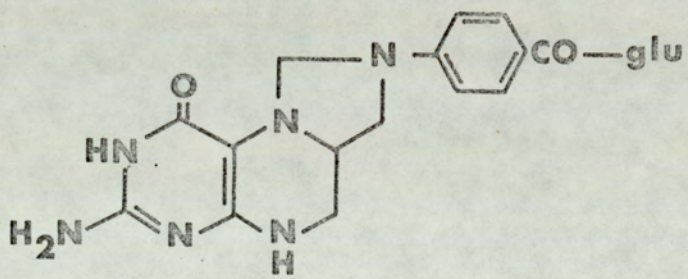
Figure 1.2 Folate dependent steps in the biosynthesis of the purine ring



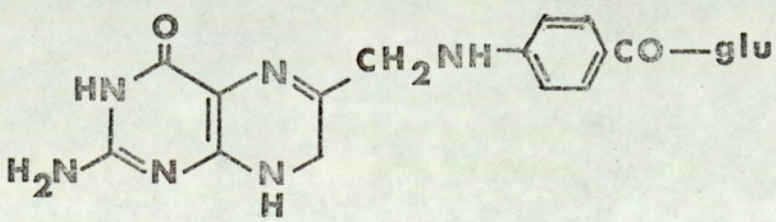
IV 5,10-Methenyltetrahydrofolic acid



V 5,6,7,8-Tetrahydrofolic acid



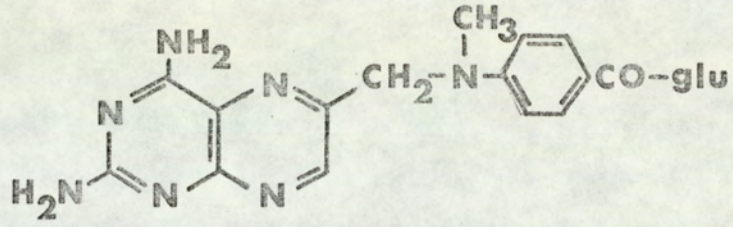
VI 5,10-Methylenetetrahydrofolic acid



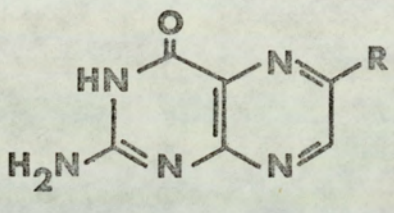
VII 7,8-Dihydrofolic acid

Pharmacologically this is one of the most important folate requiring reactions. Once generated DHF can only re-enter the folate pool if it is reduced to THF. The enzyme responsible, dihydrofolate reductase (5,6,7,8, - tetrahydrofolate : $NADP^+$ oxidoreductase; EC 1.5.1.3) is readily inhibited by 4-amino-4-deoxy folic acid analogues (Bertino et al, 1964) and is the target site for the drug methotrexate (MTX) (VIII). DHF is chemicallylabile and readily undergoes oxidation yielding fragmentation products which are metabolically inactive with regard to the folate pool. The major pteridine products obtained include xanthopterin (IX), 7,8-dihydroxanthopterin and dihydropterin-6-aldehyde in amounts dependent upon the reaction conditions (Whiteley et al, 1968; Chippel and Scrimgeour, 1970).

Xanthopterin was isolated from human urine by Koschara as early as 1936 although it was likely to have been generated from reduced pteridine precursors during the analytical process employed and probably has no metabolic function (see Blair, 1958b). More recently 7,8-dihydroxanthopterin, which is readily oxidized in air to xanthopterin, has been found in the urine of patients suffering from atypical phenylketonuria (Watson et al, 1977) although in this case the large amounts excreted (about 3 mg L^{-1}) suggest its origin is probably by decomposition of the 6-alkylpterin tetrahydrobiopterin (XI) which is synthesised de novo in the mammal from guanosine triphosphate (GTP) in comparatively large amounts. The latter authors also reported dihydroxanthopterin in the urine of patients undergoing methotrexate therapy. Since MTX inhibits DHF reductase dihydroxanthopterin could have been produced by DHF accumulation and subsequent decomposition. Again however this finding may be due to disruption of tetrahydrobiopterin metabolism since quinonoid dihydropterin reductase (EC 1.6.99.7) the enzyme which regenerates tetrahydrobiopterin is also inhibited by MTX (Goodfriend and Kaufman, 1961) and a build up of

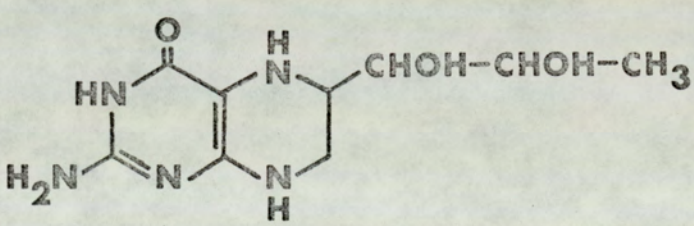


VIII Methotrexate

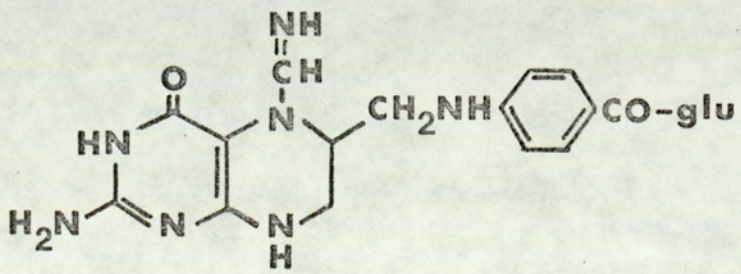


IX R = OH; Xanthopterin

X R = CHO; Pterin-6-aldehyde



XI Tetrahydrobiopterin



XII 5-Formiminotetrahydrofolate

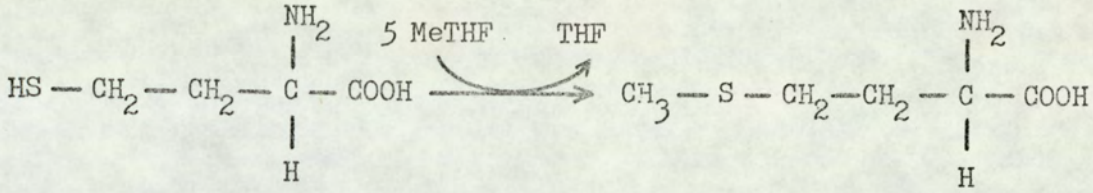
Crithidia fasciculata active substances (i.e. biopterin like materials) have been observed in the serum and urine of patients receiving MTX therapy (Leeming et al, 1976). The only known direct overlap between folate and tetrahydrobiopterin metabolism involves DHF reductase, since this is probably the enzyme which catalyses the reduction of 7,8-dihydrobiopterin in the de novo synthesis of tetrahydrobiopterin (Spector et al, 1978). The recent proposal that quinonoid dihydropteridine reductase may be important in vivo in the reduction of transitory quinonoid folate species (Pollock and Kaufman, 1978) has yet to be confirmed.

Dihydropterin-6-aldehyde can undergo aerial oxidation to pterin-6-aldehyde (X) (Mengel et al, 1977). Chromatographic and spectroscopic evidence for the latter compound in the urine of patients with various oncogenic lesions has been reported (Halpern et al, 1977). That pterin-6-aldehyde was derived from folate was supported both in vitro where radioactive pterin-6-aldehyde accumulated in the incubation medium of cultured human fibroblasts when [2 - ^{14}C] folic acid was present, and in vivo since although pterin-6-aldehyde was absent from normal human urine it was present in the urine of normal subjects 24 hours after the administration of large non-physiological doses of folic acid. Both these latter observations could be artefactual. The well known photolytic and oxidative degradations of folic acid preparations and solutions (reviewed by Blakley, 1969) probably proceed via pterin-6-aldehyde formation (Lowry et al, 1949) and this is a likely source of the compound. In two later studies a compound with similar properties to that reported by Halpern et al (1977) was found in about 50% of urine samples from normal subjects (Dinovo et al, 1978) and the identity of the compound as pterin-6-aldehyde reappraised and assigned the structure 6-hydroxymethylpterin (Stea et al, 1978). Other more detailed reports in man (Fukushima and Shiota, 1972; Nixon and Bertino,

1972) and in the rat (Beavon and Blair, 1975; Murphy et al, 1978) using radiotracers either failed to observe or to characterise pterin-6-aldehyde or 6-hydroxymethylpterin as folic acid catabolites.

The regulation of DHF levels is of considerable importance to the cell. To minimise loss of folate by chemical degradation it is important that DHF be recycled rapidly. Measurements using Erlich ascites tumour cells show that after exposure to 10^{-5} M ^{3}H-DHF only THF could be found within the cells; intracellular DHF is so low as to be undetectable ($< 10^{-8}$ M) (Goldman, 1977). Since the K_m for DHF is below 10^{-6} M the interaction between DHF and DHF reductase is essentially first order within the cell and it is possible to inhibit the reductase by up to 95% without affecting cellular growth rates (Jackson and Harrap, 1973). This probably explains why so few tumours respond to traditional methotrexate therapy and the greater success of high dose regimens. In the latter case sufficient unbound methotrexate is present in the cell to achieve cessation of THF synthesis either by being available for exchange with enzyme bound methotrexate (Jackson et al, 1977) or by the inhibition of any additional dihydrofolate reductases with reduced affinity for methotrexate (Goldman, 1977). Tumour cell lines have been shown to undergo mutation developing methotrexate resistance both by increasing the absolute concentration of DHF reductase and by changing its kinetic properties (Jackson and Niethammer, 1977). Recently the first known example of selective multiplication of genes in mammalian cells was demonstrated by Alt et al (1978) in the DHF reductase gene of a strain of murine leukaemia cells.

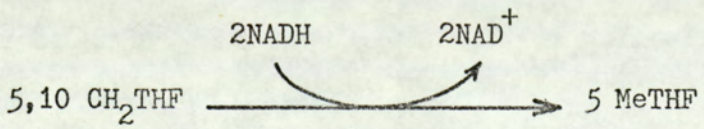
Folate coenzymes are involved in the metabolism of several amino-acids. 5 MeTHF, the major plasma folate, denotes its one carbon group to homocysteine forming methionine



Homocysteine Methionine

in a reaction catalysed by a cobalamin (B_{12}) requiring enzyme, 5 MeTHF-homocysteine methyltransferase (EC 2.1.1.13) (henceforth referred to as methionine synthetase). The existence of a B_{12} independent methyltransferase as found in E. coli (see Blakley, 1969) has never been established in the mammal although its presence as a minor pathway has not been eliminated (see Rowe, 1978). Following the clinical finding that alleviation of the haematological (but not neurological) disorders found in B_{12} deficiency occurred after treatment with folic acid the "methyl trap" hypothesis was developed. This assumes that in the absence of B_{12} THF cannot be regenerated from 5 MeTHF since the cobalamin dependent methionine synthetase reaction does not operate; folate metabolised to 5 MeTHF becomes trapped and lost to the folate pool (Noronha and Silverman, 1962; Herbert and Zalusky, 1962).

5 MeTHF is biosynthesised from 5,10 CH_2THF by the enzyme 5,10 CH_2THF reductase (EC 1.1.1.68) using NADH as the cofactor



As the equilibrium position *in vitro* is shifted far to the right the reaction is considered to be irreversible *in vivo*. The mammalian enzyme has been partially purified and found to be inhibited by S-adenosylmethionine at physiological concentrations (Kutzbach and Stokstad, 1971). Since S-adenosylmethionine is synthesised from methionine and methionine from 5 MeTHF then 5,10 CH_2THF reductase may be under allosteric modulation. The nature of the irreversibility of the reduction, important in providing a biochemical rationale for the methyl trap hypothesis, is still under some criticism. For example, Thorndike and Beck (1977) demonstrated the production of ^{14}C-formaldehyde from [5 - ^{14}C] MeTHF in normal and leukaemic leukocytes, accountable only by postulating reoxidation of 5 MeTHF back to 5,10 CH_2THF; they thus suggest that THF can be regenerated from 5 MeTHF by an alternative route to the B_{12} dependent methionine synthetase reaction. For a more detailed appraisal of the methyl trap hypothesis the reader is referred to Rowe (1978) and Krebs *et al* (1976).

Although methionine can be synthesised from homocysteine in the mammal it is generally considered as an essential aminoacid. This reflects the relatively high requirement for methionine compared to its rate of synthesis and the distribution of homocysteine between the alternate pathways of remethylation to methionine or degradation via cystathionine (Finkelstein and Mudd, 1967). Homocysteine can be methylated by two routes: either by betaine homocysteine transmethylation or using 5 MeTHF (*vide supra*), the latter pathway probably being more important *in vivo* (Finkelstein *et al*, 1971). It has been suggested that the 5 MeTHF transmethylation reaction forms a rather elegant regulatory system for one carbon metabolism. Administration of oral methionine loads to the human causes a rapid fall in plasma folate (*L. casei* activity) reversible by folate supplements (Connor *et al*, 1978) and this is presumably due to repressed 5 MeTHF synthesis.

Krebs et al (1976) suggest that in liver the regulatory control rests in the 10 CHOTHF concentration. At high methionine concentration the level of 5,10 CH_2THF is relatively increased (due to inhibition of 5,10 CH_2THF reductase by S-adenosylmethionine) and those folates in equilibrium with it will also increase. Since the cellular concentration of 10 CHOTHF is below the K_m for 10 CHOTHF dehydrogenase (EC 1.5.1.6) the enzyme catalysing the release of CO_2 and THF from 10 CHOTHF, an increased loss of one carbon groups by oxidation will occur. The release of homocysteine derived from methionine will remove available 5 MeTHF, again liberating THF, and any surplus homocysteine will enter an alternate pathway via cystathionine. In the contrary situation of a relatively low methionine concentration one carbon units are preserved since S-adenosylmethionine inhibition of 5,10 CH_2THF reductase is diminished and 5 MeTHF is generated at the expense of 5,10 CH_2THF (and thus 10 CHOTHF) and loss of one carbon units by oxidation is lowered. Other workers suggest that homocysteine besides regulating the release of THF from 5 MeTHF (Farnworth and Hill, 1977) can act as a stimulator of both 5,10 CH_2THF reductase and methionine synthetase activities in vivo (Osifo, 1978). The maintenance of homocysteine levels is emphasised by the occurrence of lethal homocystinuria and homocystinaemia in human subjects with genetically reduced 5,10 CH_2THF reductase activity, although they have comparatively normal methionine levels (Wong et al, 1977).

Interconversion of the aminoacids serine and glycine and the degradation of histidine provide the major source of one carbon units for THF. Serine hydroxymethyltransferase (EC 2.1.2.1) catalyses the reaction.

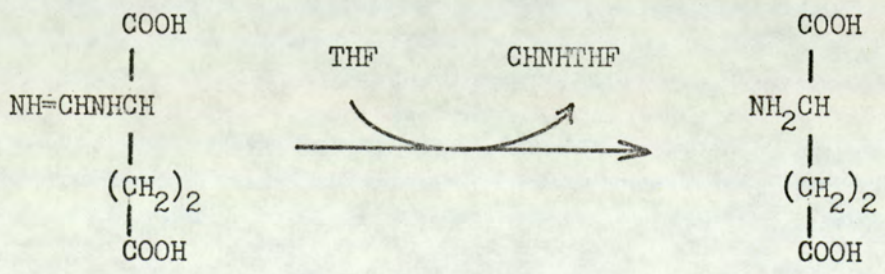
$$\text{serine} + \text{THF} \rightleftharpoons \text{5,10 } CH_2\text{THF} + \text{glycine}$$

since 5,10 CH_2THF is generated or utilized by this reaction there are 3 major routes available to this derivative apart from

reduction to 5 MeTHF.

A finely balanced control system exists regulating serine production by the above reaction. In mitochondria the glycine/serine level regulates both the production of free THF and via 5,10 CH_2THF to 10 CHOTHF the amount of CO_2 released by 10 CHOTHF dehydrogenase (Lewis et al, 1978). Transmethylation of glycine with the methyl group from S-adenosylmethionine yields sarcosine (N-methylglycine). The latter authors demonstrated that under appropriate conditions both 5,10[$^{14}CH_2$]THF and 10[^{14}CHO]THF could be generated from [$^{14}CH_3$]-sarcosine and the one carbon group can in turn appear as [3-^{14}C]-serine, ^{14}C formate and ^{14}C carbon dioxide though the mechanism of transfer of the methyl group to 5,10 CH_2THF is unknown. At high glycine levels serine and sarcosine production are enhanced; the transfer of the methyl group from sarcosine to 5,10 CH_2THF increases 10 CHOTHF production and thus one carbon group oxidation to CO_2; also by loss of S-adenosylmethionine due to sarcosine biosynthesis inhibition of 5,10 CH_2THF reductase is reduced and methionine levels are maintained; the constant recycling occurring until equilibrium is reached.

Formiminoglutamic acid (FIGlu) derived from histidine catabolism is a major source of one carbon units. Formiminotransferase (EC 2.1.2.5) catalyses the transfer of the formimino group from FlGlu to THF and the rather unstable product 5-formiminotetrahydrofolate (CHNHTHF) (XII) is rapidly hydrolysed to 5,10 CH=THF.



FlGlu Glutamic acid

The rate of flux through this pathway is such that the presence of FIGlu in urine has been used to diagnose folate deficiency (Zalusky and Herbert, 1962).

Folates are involved in several other less well characterised reactions. Probably the most important is the metabolism and role of the folate conjugates found in the tissues. In most of the reactions described above both folate polyglutamates and monoglutamates can participate. Kinetic studies *in vitro* indicate that folate polyglutamates serve as better substrates in several reactions and from this it has been inferred that folate polyglutamates fulfill the coenzyme role *in vivo* (reviewed by Hoffbrand, 1976; Rowe, 1978). The failure of a mutant strain of Chinese hamster ovary cells to grow in the absence of exogenous glycine, thymidine and purines, and evidence that this is due to deletion of the enzyme system which synthesises polyglutamate derivatives has further strengthened this suggestion (McBurney and Whitmore, 1974; Taylor and Hanna, 1977). Whether this is due to an absolute requirement for polyglutamate forms as coenzymes or is due to the failure to convert diffusible folate monoglutamates into non-diffusible folate polyglutamates and thus retain folate in the cell is still to be established (see Moran *et al*, 1976). The non-diffusible nature of the folate polyglutamates was the origin of the idea that they constituted a pool of stored folate. Although the folate monoglutamates are rapidly interconverted *in vivo* and can thus function as coenzymes (Blair, 1975) evidence suggests that conversion of retained folates back to folate monoglutamates is relatively slow (Barford *et al*, 1977 a) and that folate polyglutamates are synthesised from folate monoglutamates even in folate depletion (Poirier, 1973). The controversy as to the role of the folate polyglutamates remains to be resolved as does an adequate characterisation of the naturally occurring tissue derivatives. The latter point will be dealt with in

more detail later.

Both the synthesis and degradation of the folate polyglutamate derivatives in the mammal remains obscure. Folate conjugases (glutamate carboxypeptidase, EC 3.4.12.10) form a ubiquitous group of enzymes found in both the cell and the plasma. Within the cell they are mainly lysosomal (Shin et al, 1976) although some activity is found in rat liver mitochondria (Wang et al, 1967). The function of conjugase remains unknown although in the intestine it probably releases folate monoglutamates from the folate polyglutamates in food stuffs since the latter are poorly absorbed intact (Rosenberg and Godwin, 1971), and the lysosomal enzyme may release folate monoglutamates from folate polyglutamates at cell death. Conjugase activity in the rat uterus varies with the ovarian cycle (Krumdieck et al, 1976).

Attempts have been made to prepare folate polyglutamate synthesising systems in vitro from mammalian tissue although these have so far proved capable of producing only short chained (up to two additional glutamate residues) derivatives (Spronk, 1973; d'Urso Scott and Makulu, 1973). The specificity of mammalian pteroyl polyglutamate synthetase, the ligase which synthesises folate polyglutamates, is unclear. Spronk (1973) found that only THF was a substrate in a crude preparation of rat liver although recent work using an apparently homogenous purified pteroyl polyglutamate synthetase from the same source demonstrated that 10 CHOTHF and 5,10 CH_2THF were conjugated almost as well as THF (McGuire et al, 1979). In view of the formation of methotrexate polyglutamates in the livers of patients treated with methotrexate (Jacobs et al, 1977) the limitation to a specific substrate in vivo would appear unlikely. 5 MeTHF, the major serum folate, is not available unless it undergoes prior loss of the methyl group. The latter observation has been proposed to explain both the altered polyglutamate to monoglutamate ratio in the red blood cells of pernicious

anaemia sufferers, where the "methyl trap"is operating, and the macrocytic anaemia associated with this condition, this being due to impaired purine and pyrimidine metabolism caused by the lack of the appropriate (polyglutamyl) folate coenzymes (Lavoie et al, 1974; Perry and Chanarin, 1977).

Recent reports suggest that folate derivatives serve as coenzymes or regulators of uroporphyrinogen I synthetase (EC 4.3.1.8), an enzyme involved in haem synthesis (Piper and Van Lier, 1977). Folates competitively inhibit glutamate dehydrogenase (EC 1.4.1.2) (White et al, 1976) and this may be related to the homologies in the primary structure of DHF reductase and glutamate dehydrogenase. Since the concentration of folate required to inhibit glutamate dehydrogenase is higher than is normally found in vivo White et al suggest that conversely the binding of folate by such a plentiful protein (5% of total mitochondrial protein) could regulate the level of free folate. Folates also inhibit and bind to ribonuclease A (Sawada et al, 1977) and this could presumably regulate free folate in a similar manner. Protein binding of folate in vivo may occur on a considerable scale. At least four endogenous hepatic folate binding proteins have been found although only one of these has been identified as a folate metabolising enzyme (DHF reductase) (Zamierowski and Wagner, 1974).

1.2 Methods of Analysis of Folates and Pteridines

Folate derivatives occur naturally in low concentration (10^{-5} to 10^{-9} M), are chemically labile undergoing a variety of complex degradation reactions, show a great diversity of structure and generally present intractable difficulties towards physical methods of analysis. The chemistry of folates is controversial, complex and often misunderstood. These factors make analysis of the various derivatives both difficult and open to misinterpretation. Before proceeding with further discussion of their metabolism and the rationale of this project it is pertinent to examine the methods of analysis which have and are being employed in this field.

1.2.1 Methods of Detection

Early studies relied heavily on the use of microbiological assay methods and these are still in routine clinical use. The organisms employed were found in some instances only to respond to tissue extracts after prolonged autolysis or treatment with pancreatic or intestinal extracts. This was attributed to the existence of conjugated folate in tissues which after enzymic hydrolysis became microbiologically active.

There are three commonly used bacteria in folate assays: Streptococcus faecalis, Lactobacillus casei and Pediococcus cerevisiae (formerly known as Leuconostoc citrovorum). Each organism responds to several folate derivatives but since these are not the same for each bacterium crude deductions can be inferred about the particular folates in a sample by the difference in growth rates. Each bacterium is assumed to respond to different folates to the same extent, e.g. 1 mole of folic acid and 1 mole of 5 MeTHF give the same growth response for L. casei. However the latter conclusion is not strictly correct, despite its widespread assumption, since different folate derivatives elicit a differential response (Pollock and Kaufman, 1978).

In view of the rapidity with which the folate monoglutamates are interconverted *in vivo* (Nixon *et al*, 1973) tissue autolysis or the use of impure enzyme sources could lead to marked errors in interpretation of microbiological assay data. Both *L. casei* and *S. faecalis* respond to folate polyglutamate derivatives, at least to the diglutamate level and probably to the triglutamate level for *L. casei*, since incubation of yeast extracts with chicken pancreas conjugase yields derivatives active for both organisms although the product of this enzyme is a folate diglutamate (R. Leeming, personal communication). Many examples of the "over interpretation" of microbiological assay data exist in the literature. The major serum folate was believed to be a folate triglutamate derivative since it was active for *L. casei* but not for *S.faecalis* but later identified as 5 MeTHF (Herbert *et al*, 1962). *L. casei* has been used to measure pteroic acid absorption in man, although *L. casei* does not respond to this derivative (Brown *et al*, 1973).

Several folates (though neither 5 MeTHF nor 10 CHOTHF) are fluorescent and this has been used to assay folates (Uyeda and Rabinowitz, 1963). Fluorescence has been useful in the study of insect and animal pigments although in the mammal extensive concentration and purification is required. Absorbance in the ultraviolet region is marked for all pteridine compounds (ε = 15,000 - 20,000) but is not specific enough except to differentiate pure compounds although it is useful in the identification of chromatographic markers. A useful list of U.V. spectral data is given by Blakley (1969). Other physical methods of analysis lack the required sensitivity (e.g. nuclear magnetic resonance) or are complicated by the physical and chemical properties of pteridines. Thus their extreme involatility renders elemental analysis and mass spectrometry technically difficult, although conversion to volatile degradation forms may facilitate this (Hachey *et al*, 1978).

The most important and widespread technique depends upon the use of radiolabelled tracers. Both tritium and carbon -14 labelled folic acid are commercially available. The use of tritiated folic acid raises problems of the specificity of labelling (Blair, 1975). Analysis of commercial folic acid (Amersham/Searle) by Zakrewski et al (1970) demonstrated both considerable labelling at the C9 position and the presence of ^{3}H in the pteridine nucleus (presumably C7), although it is only recently that the commercial source has revised its description of [3', 5', 9(n) - ^{3}H] folic acid to [3', 5', 7, 9 - ^{3}H] folic acid. Batch variations have given a range between 15% and 60% of the tritium at the C9 position and this is important when considering in vivo studies. Tritiated water derived from tritiated folates has been reported in in vivo studies (Calvert et al, 1977; Connor et al, 1979) and in vitro (Moran et al, 1976).

The use of carbon -14 labelled folate diminishes the problem of non-specific exchange reactions (Blair, 1975) although it is available only at lower specific radioactivities. The use of [2-^{14}C] folic acid has been criticised since labelling the pterin ring complicates the differentiation between folate metabolites and pterin degradation products (Murphy et al, 1976). The use of dual labels in metabolism studies offers more potential and has been employed to investigate both one-carbon group turnover (Nixon and Bertino, 1972; Nixon et al, 1973) and the fate of the folate skeleton (Barford et al, 1977 b; Connor et al, 1977) in vivo.

Biochemical assays are available but these lack the sensitivity required to analyse raw tissue samples. FIGlu excretion, mentioned earlier, has been assayed to determine folate deficiency, but FIGlu excretion is only marked in pronounced folate depletion and is preceded by the onset of clinical manifestations which are readily observable. Total folate can also be determined using radioimmuno-

assay methods although these are non-specific and thus limited in metabolism studies.

1.2.2 Methods of Identification

Individual folate derivatives can be characterised by differential microbiological assay (videsupra) but this is only acceptable as confirmatory evidence in qualitative analysis. Microbiological assay of the folate in body fluids, after the administration of oral doses of folate, cannot differentiate between folate derived from the administered dose and that derived by displacement from the body pool (Johns and Plenderleith, 1963).

The use of radioactive tracers combined with chromatographic separation of metabolites gives more precise information, albeit with the drawbacks associated with pulse labelling and the limitations of the chromatographic techniques employed. The experimental simplicity and the ease of handling of samples make column chromatography the preferred and all too often only, method used in the identification of metabolites. Separation of folate derivatives by DEAE-cellulose ion-exchange (Silverman et al, 1961) is the most widespread technique in use today. The early claims that folate polyglutamates elute at higher ionic strength than the folate monoglutamates and can thus be separated by ion-exchange has been extensively criticised in recent times (Kisliuk et al, 1974; Baugh et al, 1974; Barford et al, 1977 a) although authors still erroneously rely on shifts to lower ionic strength of folate derivatives treated with conjugase preparations as identification of folate polyglutamates (e.g. Reed et al, 1977). The separation of individual folate monoglutamates is not marked (Kisliuk et al, 1974) and the absence of cochromatography, and thus non-identity, is often the only acceptable evidence. Shin et al (1972) used Sephadex gel filtration to separate high and low molecular weight derivatives. Whilst gel filtration is useful for separating long chain polygluta-

mate derivatives and protein bound folate from folate monoglutamates care must be taken in the interpretation of chromatographic data. Folates and pteridines do not elute from Sephadex gels according to any simple rules and undergo non-specific binding. Thus the 5 MeTHF degradation product 4aOH5MeTHF elutes in the 900 dalton region and could be confused with folate triglutamates. d'Urso Scott et al (1974) described the build up of a metabolite with the elution properties of a folate diglutamate in an isolated cell system designed to study pteroylpolyglutamate synthesis but correctly identified this as 10-formylfolate (10 CHOFA). The eccentric behaviour of folate derivatives can thus lead to misidentification if characterisation is limited to simple column chromatography.

Several methods of chemical degradation of folate polyglutamates have been devised to facilitate analysis of tissue folates. These rely on "oxidative" or "reductive" cleavage of the C9-N10 bond and chromatographic identification of the resultant p-aminobenzoyl-L-glutamate derivatives produced in an attempt to simplify the problems encountered in the chromatography of intact folates caused by the different states of reduction and substitution of the various naturally occurring compounds. There are several theoretical and practical problems associated with the methods in current use. Oxidative cleavage using alkaline or neutral potassium permanganate has been widely used (Houlihan and Scott , 1972; Brown et al, 1974; Reed et al, 1977) based on the established cleavage reaction of folic acid (Whittle et al, 1947; Zakrewski et al, 1970). The failure of folates other than folic acid to undergo cleavage under these conditions is now well documented (Gapski et al, 1971; Lewis and Rowe, 1977; Maruyama et al, 1978). Maruyama et al (1978) found p-aminobenzoyl-L-glutamate itself to be unstable under these conditions.

The alternative cleavage method uses Zn/HCl reduction and is

again based on a known reaction of folic acid (Hutchings et al, 1947). Baugh et al (1974) extracted folate from tissues using 5% TCA in 8M-urea to effect both denaturation of endogenous enzymes and efficient recovery of folate. The authors described this as a "relatively mild" and "nearly quantitative" extraction although recent reports by them suggest that cyano derivatives of intact folates may be formed under the extraction conditions (Francis et al, 1977). The reduction reaction was characterised by Baugh et al,(1974) using synthetic folic acid polyglutamates and, as with the oxidative cleavage reaction, is again a specific reaction of folic acid since the major folate derivatives 5 MeTHF, 10 CHOTHF, 5,10 CH_2THF and THF do not undergo reductive cleavage (Baugh et al, 1979; Lewis and Rowe, 1979). Thus both the reductive and oxidative cleavage methods are invalid on chemical grounds and are inapplicable, at least in their present form, to the study of endogenous tissue folates.

Both reductive and oxidative cleavage rely on the use of a radiotracer, with the isotope incorporated in the p-aminobenzoyl-L-glutamate moiety, and ion-exchange separation of the fragments obtained. Baugh et al (1974) claimed that formyl-folates cleaved to methylaminobenzoyl-L-glutamate derivatives and as these did not coelute exactly with the standard p-amino-benzoyl-L-glutamate markers allowed considerable flexibility in the interpretation of chromatographic data. Houlihan and Scott (1972), who rely on the use of tritiated folic acid tracers, summarily claimed that the pterin derivatives obtained in cleavage reactions eluted at the start of the gradient on ion-exchange chromatography and thus did not interfere with the elution profile of the p-aminobenzoyl-L-glutamate derivatives. The latter observation is not borne out by the present study nor by earlier workers (Zakrewski et al 1970) since the oxidation product pterin-6-COOH elutes at a higher ionic strength than p-aminobenzoyl-L-glutamate. The use of tritium

labelled tracers in such experiments could lead to serious error in view of the variation in the extent of labelling of the benzene ring (discussed above). A more important limitation of cleavage methods is the total lack of evidence provided on the nature of one carbon substitution or the state of reduction of the pteridine moiety of the different folate polyglutamates present.

Identification of tissue folate by non-destructive techniques is largely dependent upon the use of folate "conjugase" preparations to liberate folate monoglutamates, more readily assayed by microbiological and chromatographic methods, from folate polyglutamate forms. Hog kidney conjugase and chicken pancreas conjugase (Bird et al, 1965) have been partially purified and are widely used. Only recently has a folate conjugase, from chicken liver, been purified to homogeneity (Rao and Noronha, 1977 a) and characterised (Rao and Noronha, 1977 b). Authors are frequently not so strict in their use of purified folate conjugase and caution is advised in interpreting the results from studies where such crude preparations as dilute serum or plasma are employed (e.g. Reed et al, 1977).

Paper and thin layer chromatography (t.l.c.) and electrophoresis have been applied with some success in the field. Pterins and the more stable folates are readily characterised although aerial oxidation of labile derivatives is often unavoidable. Chromatography of pure derivatives is relatively simple although direct analysis of tissue and urine samples by t.l.c. requires considerable work up and concentration before sufficient material is available for detection and to avoid the problems associated with excessive salt deposition on the origin (Beavon and Blair, 1975). It has been proved possible to identify such materials as dihydroxanthopterin in neat urine (Watson et al, 1977) using paper chromatography and electrophoresis. Electrophoresis will separate folate monoglutamates from folate polyglutamates

(Connor et al, 1977) and has been used in the identification of the products of conjugase activity on synthetic substrates (Rosenberg and Neumann, 1974).

Recent publications suggest that high performance liquid chromatography (HPLC) may prove a useful tool in folate analysis, but this technique is still in the developmental stage (Stout et al, 1976; Arly-Nelson et al, 1977; Chapman et al, 1978) and the drawbacks of both incomplete separation and the relatively small sample sizes used, which lead to problems in detection, are still to be overcome.

1.3 Rationale for the Present Investigations

Folate derivatives are essential components in normal metabolism and are required for cell growth and propagation. This project concerns the fate *in vivo* of administered folate derivatives in the mammal and the effect of tumours on this metabolism.

Several previous investigations have revealed changes in the folate profile between slow and rapidly dividing tissues. Sotobayashi *et al* (1966) observed an apparent increase in the concentration of 10 CHOTHF relative to 5 MeTHF in tumours compared to normal liver. Barford and Blair (1978) reported a similar shift in the urine of tumour bearing rats compared to normal rat urine. Folate deficiency is a common clinical finding in patients suffering from a wide spectrum of tumours (Blakley, 1969) and has also been observed in animal models (Poirier, 1973). Changes in the ratio of long to short chained folate polyglutamates have been observed in regenerating liver (Whitehead, 1973) and analogously in *E. coli* after viral infection (Nakamura and Kozloff, 1978). An abnormal metabolite of folic acid, possibly pterin-6-aldehyde, has been found in the urine of cancer patients and also claimed in the urine of rats bearing implanted tumours (Halpern *et al*, 1977). Unfortunately in the majority of these and other studies inadequate characterisation of the folate species found and problems associated with the methods of assay used reduce the value of the conclusions inferred.

The present study is directed towards further elucidation of the fate of folate derivatives in the normal mammal and where possible by the application of rigid or more rigorous chemical and biological analysis to the identification of the different derivatives present in tissues and body fluids and provide the groundwork for the development of more definitive assay methods. Changes in whole body metabolism of folate induced by implanted proliferating tissue (the Walker 256

carco-sarcinoma) and a comparison of tumour and hepatic folate is also made. The metabolism of both folic acid and 10 CHOFA is examined, the latter derivative being an important dietary folate but the subject of physiological and nutritional controversy (see Chapter 5).

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Reagents

The following materials were obtained commercially from their respective sources; folic acid, xanthopterin, pterin, lumazine and isoxanthopterin from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.); p-aminohippuric acid, p-aminobenzoyl-L-glutamate, d,l dithiothreitol,β-glucuronidase and alkaline phosphatase from the Sigma Chemical Co. Ltd. (London, U.K.); p-acetamidobenzoic acid and p-aminobenzoic acid from the Aldrich Chemical Co. Ltd. (Wembley, Middlesex, U.K.); 5 MeTHF from Eprova Research Laboratories (Basle, Switzerland).

Other standard pteridines were gifts and donations from the following individuals and organisations; various lumazine derivatives from Prof. W. Pfleiderer (Universitat Konstanz, West Germany); methotrexate and leucovorin (5 CHOTHF) from Lederle Laboratories Division (Cyanamid of Great Britain Ltd., London); 6-methylpterin, neopterin and reduced biopterin from Roche Products Ltd. (Welwyn Garden City, U.K.); pterin-6-sulphonic acid from Prof. H. Woods (University of Strathclyde, Glasgow, U.K.); pteroic acid and tritiated pteroyl-γ-heptaglutamate from Prof. I.D. Rosenberg (University of Chicago, Illinois, U.S.A.); p-acetamidohippuric acid from Dr. S.P. James (Dept. Biochemistry, Birmingham University, Birmingham, U.K.). Other chemicals and general laboratory reagents used were of Analar grade.

The following folate and pteridine derivatives were chemically synthesised by the methods described below.

10-formylfolic acid (10 CHOFA)

This was prepared by the direct formylation of folic acid (Blakley, 1959). Folic acid (4 g) was dissolved in 160 ml of formic acid (90%) and the solution stored in the dark at room temperature for two days. The solution was then poured into an excess of diethyl ether (500 ml). The creamy white precipitate was harvested by filtra-

tion, washed several times with ether and dried by suction. The yield of 10 CHOFA was 4.03 g (95%) and gave a single band on t.l.c. and gel filtration. U.V. spectroscopy gave λmax = 254 nm, 327 nm (pH1); λmax = 245 nm, 265 nm, 355 nm (pH7); λmax = 259 nm, 370 nm (pH13).

[2-^{14}C]&[3', 5', 9(n) - ^{3}H] 10-formyl-folic acid

This was prepared by a microscale version of the previous preparation. A solution of [2-^{14}C] folic acid (50 µCi; 58.2 mCimM^{-1}; Batch 20) and [3', 5', 9(n) - ^{3}H] folic acid (250 µCi; 500 mCimM^{-1}, Batch 39) was made up in 0.6 ml of formic acid (98%). After storing the solution in the dark at room temperature for 48 hours the excess formic acid was removed by freeze drying. Chromatography of the product on Sephadex G15 gave a single radioactive peak at fraction 21 (folic acid elutes at fraction 36). Ultraviolet absorbance and microbiological assay with L.casei and S.faecalis showed that a quantitative conversion to 10 CHOFA had occurred. To minimise radiochemical degradation the prepared material was stored in aliquots (0.14µmoles) in the deep freeze (-20°C) until used. Repeated Sephadex G15 chromatography revealed that no detectable degradation occurred under these conditions even over a 6 month period.

[10-^{14}C][3', 5' 9(n) - ^{3}H] 10-formyl-folic acid

Formic acid (0.1 ml; 90%) was added to a vial containing 1 mCi of ^{14}C-formic acid (60.7 mCimM^{-1}; batch 34). After mixing well the solution was added to a vial containing [3', 5', 9(n) - ^{3}H] folic acid (250 µCi; 500 mCimM^{-1}; batch 39). The resultant solution was then left in the dark at room temperature for 48 hours. Excess formic acid was then removed by freeze drying using a cold finger containing solid KOH pellets to trap unconsumed ^{14}C-formic acid. The 10 CHOFA product was then purified using Sephadex G15, DEAE-cellulose ion exchange and back on to Sephadex G15 to remove several carbon-14 labelled impurities. After purification the overall yield of tritium as 10 CHOFA was 91%; the yield of carbon-14 as 10 CHOFA was poor

probably because of loss of volatile ^{14}C-formic acid in the initial stages. The specific radioactivities of the product were tritium 500 $mCimM^{-1}$ and carbon-14 0.2 $mCimM^{-1}$. The product was characterised as 10 CHOFA by the chromatographic and U.V. spectral assays described above.

10-formyl-7,8-dihydrofolate (10 CHODHF)

10 CHODHF was prepared by dithionite reduction of 10 CHOFA as suggested by Friedkin et al (1962). 10 CHOFA (0.3 g) was dissolved in 1M-mercaptoethanol (25 ml) by the addition of conc. NaOH to pH 7.5. Sodium dithionite (2 g) was then added with stirring. After 30 min the reaction mixture was acidified with a few drops of conc.HCl and left in the fridge for 1 hour. The bright daffodil-yellow precipitate was harvested by centrifugation and washed twice with ice-cold 1M-mercaptoethanol (10 ml). The precipitate was then recovered and freeze dried yielding 0.13 g of product. Assessment of purity and product identity was difficult since the properties of 10 CHODHF are not dealt with in the literature. 10 CHODHF was unstable in neutral solution ($t_{1/2}$ = 11 min in 0.05 M-sodium phosphate buffer, pH7) and decomposed during chromatography at pH7 to 10 CHOFA in quantitative yield. However U.V. absorbance studies revealed that 10 CHODHF was stable in acid solution, giving a single band of 10 CHODHF on DEAE-cellulose ion exchange chromatography at pH2.

U.V. spectroscopy gave λmax = 363 nm; ε = 15,330 (pH2); immediately after neutralization to pH7 this gave λmax = 268 nm, 366 nm.

10-formyltetrahydrofolate (10 CHOTHF) & 5,10-methenyltetrahydrofolate (5,10 CH=THF)

5,10 CH=THF was prepared by acid isomerization of 5 CHOTHF and 10 CHOTHF in turn prepared from 5,10 CH=THF. 5 CHOTHF (100 mg) was dissolved in 0.1 M-HCl (20 ml) containing mercaptoethanol (1% v/v) and

left to isomerize in the dark at room temperature for 3 hours. The product (5,10 CH=THF) was then recovered by freeze drying; U.V. spectroscopy gave λmax = 355 nm (pH1). 10 CHOTHF was synthesised from 5,10 CH=THF by dissolving the latter in tris buffer pH8 containing mercaptoethanol and freeze drying as described by Rowe (1971). The 10 CHOTHF produced gave three peaks on ion exchange chromatography corresponding to 10 CHOTHF (50%), 10 CHOFA (40%) and 5 CHOTHF (5%); 10 CHOTHF could not be re-chromatographed without some oxidation to 10 CHOFA. U.V. spectroscopy of the neutral 10 CHOTHF peak gave λmax = 260 nm (pH7) and on acidification gave λmax = 355 nm (pH1) due to 5,10 CH=THF formation.

5-Methyl-5,6-dihydrofolate (5 MeDHF) and 4a-hydroxy 5-Methyl-tetrahydrofolate (4aOH5MeTHF)

These two compounds were synthesised from 5 MeTHF by the literature method of Gapski et al (1971), and purified by DEAE-cellulose chromatography.

7,8-dihydrofolic acid (DHF)

DHF was synthesised from folic acid by the method of Futterman (1963). Folic acid (0.2 g) was dissolved in water (20 ml) with a small volume of 0.1M-NaCl. Sodium ascorbate (50 ml, 10% w/v in water) was then added and the solution adjusted to pH6 with HCl (2 M). Sodium dithionite (2 g) was added and the solution left to stand at 4°C for 15 min. After acidification to pH 2.8 with HCl (2 M) the creamy white precipitate was harvested by centrifugation. It was washed twice with 20 ml 0.5% HCl and once with 50 ml acetone to remove ascorbate and then dried under vacuum and stored at -15°C until required. Purity estimated from the U.V. absorbance (λmax = 282 nm; pH7) was between 80-90% but this decreased rapidly on storage.

p-acetamidobenzoyl-L-glutamate

This was prepared by the method of Baker et al (1964). p-Amino-

benzoyl-L-glutamate (1.0 g) was dissolved in 10 ml aqueous acetic acid (50% v/v) and acetic anhydride (1.5 ml) added. After standing in the dark at room temperature overnight the reaction solution was diluted to 20 ml with distilled water, cooled (4°C) for several hours, and the precipitate recovered by centrifugation. After washing with ice-cold water the product was recrystallized twice from boiling water yielding small white needles (0.82 g). Elemental analysis gave C = 54.8%, N = 8.1%, H = 5.3% (expected C = 54.5, N = 9.1%, H = 5.22%). Nuclear magnetic resonance spectroscopy revealed the presence of a singlet (i.e. CH_3-CO-) at τ = 7.5 absent from the starting material.

pterin-6-aldehyde (pterin-6-CHO)

Pterin-6-CHO was synthesised from folic acid by the original sulphite cleavage method of Waller et al (1950). Folic acid (6 g) was dissolved in water (750 ml) with a few drops of c.NaOH. Sodium sulphite (50 ml) was added and the mixture left to stir overnight at 80°C. The following day charcoal (50 g) was added to the deep blue reaction mixture and it was then heated to boiling and filtered. The filtrate was treated with dilute iodine solution until a slight excess of iodine persisted as indicated by starch/iodide paper. After cooling for several hours at 4°C the dirty brown precipitate was filtered off. This precipitate was purified by repeatedly being dissolved in dilute NaOH and reprecipitated with HCl. The dark but crystalline product was dried in air at 50°C. Thin layer chromatography in 0.1 M-sodium phosphate buffer at pH7 gave two blue fluorescent bands visible under U.V. light corresponding to pterin-6-CHO and its oxidation product pterin-6-COOH.

pterin-6-carboxylic acid (pterin-6-COOH) and 2,4-diamino pteridine-6-carboxylic acid (2,4,-NH_2-pterin-6-COOH)

Pterin-6-COOH was prepared by permanganate oxidation of folic acid (Zakrewski, et al (1970). To a solution of folic acid (1 g) in 20 ml of

NaOH (1 M) was added 0.5 ml aliquots of potassium permanganate (4% w/v in water) until the solution remained a dark green colour (about 25 ml required). This solution was left to stir at room temperature for 1 hour. The brown manganese dioxide was removed by filtration and the filtrate was treated with HCl to pH 4.7. After stirring the mixture was filtered and the precipitate washed with 0.1 M -HCl. The product was then dried in air at 50°C. Chromatography on Sephadex G15 gave a single U.V. absorbing peak, λ max pHi = 234 nm, 260 nm, 310 nm (lit. 234 nm, 260 nm, 309 nm), pH13 = 262 nm,(290 nm),365 nm (lit. 262 nm, 365 nm). 2,4-Diaminopteridine-6-carboxylic acid was prepared in an identical manner but starting with methotrexate.

dihydroxanthopterin

Dihydroxanthopterin was synthesised by the method of Stocks-Wilson (1971) based on the general method of Albert and Matsuura (1962). Xanthopterin (0.5 g) was dissolved in 200 ml tris buffer (0.1 M) at pH 7.8. A slurry of sodium borohydride (1.14 g) in water (10 ml) was added with constant stirring. After standing for 20 min at room temperature 6 ml of 50% acetic acid was added slowly to adjust the pH to between pH7 and pH8. The pink/buff precipitate was harvested by centrifugation. After washing several times with acetic acid (0.1%) it was dried in a vacuum desiccator overnight at room temperature.

2.2 Chromatography and Electrophoresis

i) Ion Exchange Chromatography

Diethylaminoethyl (DEAE) cellulose (DE52, Whatman Ltd. Maidstone, Kent, U.K.) (80 g) was washed with distilled water and equilibrated in 0.05 M-phosphate buffer, pH7 containing dithiothreitol (5 mg %) until the washings were of constant ionic strength and at pH7. After decanting off the fines and degassing the prepared DE52 was packed into glass columns (normally 2 cm x 50 cm, occasionally small colums 1 cm x 20 cm were used) plugged with glass wool. Samples (5-50 ml) and

appropriate standards were diluted to the conductivity of the starting buffer with water before loading on to columns. Standard linear gradients (0 - 1.2 M-NaCl in starting buffer) were eluted automatically using an LKB Ultra Grad attached to a peristaltic pump (LKB Instruments, Croydon, Surrey, U.K.). The eluant was passed through two detectors in series; a purpose built scintillation flow cell for detecting radioactivity (Nuclear Enterprises Ltd., Edinburgh, U.K.) and a U.V. monitor (LKB Uvicord II, LKB Instruments). Fractions (5 ml) were collected using an LKB Ultrarac fraction collector (LKB Instruments). The gradient, usually eluted over eight hours, was determined by measuring the conductivities of every 10th fraction with a Mullard conductivity cell.

Identical procedures were employed when using DEAE-Sephadex A25 (Pharmacia AB, Uppsala, Sweden) except that this material does not generate fines and was eluted using 0 - 1.5 M-NaCl in starting buffer. The elution position of several important folate, pterin and p-aminobenzoic acid derivatives are given in table 2.1.

ii) Gel Filtration

Sephadex G10, G15, G75 and G150 were obtained from Pharmacia. A slurry of the gels was prepared in phosphate buffer, pH7 containing dithiothreitol (DTT) (5 mg %) and they were left to swell for the appropriate times. After degassing, the slurries were packed into 2 cm x 60 cm perspex columns (Wright Scientific Ltd., Surrey, U.K.) and allowed to pack under pressure. After loading samples and standards (5 - 30 ml) elution was achieved using 0.05 M- buffer for Sephadex G10, G15 and G75 and 0.01 M- buffer for Sephadex G150. The eluant was examined and collected as above. A summary of the elution pattern of relevant folates, pteridines and p-aminobenzoic acid derivatives may be found in table 2.1.

Table 2.1 The elution properties of folates, pteridines and p-aminobenzoic acid derivatives on Sephadex-G15 gel filtration and DEAE-cellulose ion exchange chromatography

Compound	Elution position	
	Sephadex-G15 fraction (Kav)	DEAE-cellulose molarity NaCl
Folic acid	37 (1.79)	0.95
pteroylheptaglutamate	11 (0.13)	0.60
10 CHOFA	21 (0.77)	0.55
10 CHOTHF	18 (0.58)	0.45
5 CHOTHF	28 (1.22)	0.59
5 MeTHF	37 (1.79)	0.66
5 MeDHF	18 (0.58)	-
4aOH5MeTHF	16 (0.45)	0.40
DHF	56 (3.01)	-
Methotrexate	60 (3.27)	-
pteroic acid	85 (4.87)	-
Xanthopterin	21* (0.77)	0.32
dihydroxanthopterin	76 (4.29)	-
isoxanthopterin	45 (2.31)	-
pterin	35 (1.67)	0.24
pterin-6-CHO	43 (2.18)	0.61
pterin-6-COOH	35 (1.67)	0.73
6-Hydroxymethylpterin	32 (1.47)	-
6-methylpterin	44 (2.24)	-
6-methyltetrahydropterin	36 (1.73)	-
pterin-6-sulphonic acid	34 (1.60)	-
D-neopterin	28 (1.22)	-
lumazine	30 (1.35)	-
6-COOH-lumazine	27 (1.15)	-
6-oxolumazine	34 (1.60)	-
p-aminobenzoic acid	35 (1.67)	0.40
p-acetamidobenzoic acid	36 (1.73)	0.45
p-aminobenzoyl-L-glutamate	18 (0.58)	0.40
p-acetamidobenzoyl-L-glutamate	20 (0.66)	0.45
p-aminohippuric acid	33 (1.51)	-
p-acetamidohippuric acid	27 (1.15)	0.35
^3H_2O	21 (0.76)	0

Elution conditions are described in the text

* the elution position of xanthopterin is concentration dependent possibly due to changes in the hydration state (see Blakley, 1969)

iii) Paper Chromatography and t.l.c.

Paper chromatography was performed by the descending method in a glass tank equilibrated with the relevant solvent using Whatman 3 MM or 5 MM paper and chromatograms were run overnight in the dark. Thin layer chromatography (t.l.c.) was performed in small glass tanks lined with filter paper to facilitate saturation with the solvent and to minimise photolysis of light labile derivatives, using prepared cellulose t.l.c. plates (MN 300 UV or MN 300 "Polygram" sheets, Macherey-Nagel). The chromatography solvents used are given in the text. Samples and standards were applied as spots or streaks using glass micropipettes and where multiple applications were required samples were dried in situ with a stream of cold air. Standards were observed as dark absorbing or fluorescing spots by viewing under U.V. light at 254 nm or 355 mn.

iv) Electrophoresis

Electrophoresis was performed with a Shandon Southern flat bed tank (Shandon Southern Products, Runcorn, U.K.) and cellulose acetate or paper (Whatman No. 1) strips (2.5 x 16 cm). Separation of folate derivatives using paper was found to be superior to cellulose acetate. Typical runs were performed at pH7 in 0.05 M-sodium phosphate buffer containing dithiothreitol (DTT) (5 mg per 100 ml w/v) at constant voltage (20 Vcm^{-1}) for 60-90 min. Electrophoresis at pH4 (0.1 M-sodium acetate buffer) gave poorer and at pH 8.8 (0.1 M-sodium barbitone buffer) similar separations.

2.3 Animals

Experiments were usually conducted on male Wistar rats (200 g) (Scientific Products Farms, Canterbury). Tumour bearing and control Wistar rats were obtained from the Chester Beatty Research Institute, Fulham Road, London. Normal mice (20 g) were bred in the Department of Pharmacy, University of Aston; normal and thymus deprived CBA/LAC

female mice were gifts from the Chester Beatty Research Institute.

Both prior to and during experimentation animals were kept at 21°C in a sealed room having a fixed 12 hour dark 12 hour light cycle and allowed free access to food (Breeding Diet, Heygates Ltd.).

Metabolism studies were carried out by housing single rats in cages designed for the separate collection of faeces and urine (Jencons Metabowls; Jencons (Scientific) Ltd., Hemel Hempstead, Herts., U.K.). Where required air was pumped through the cages continuously and the effluent passed through a cold trap and a bubbler containing 5M-KOH (20 ml) to collect respired water and carbon dioxide respectively. Mice were housed in pairs in Jencons Metabowl Minor metabolism cages. Urine samples except where stated were collected into 10 ml of 0.05 M-sodium phosphate buffer pH7 containing sodium ascorbate (2% w/v).

Rats and mice were dosed orally by stomach intubation using specially prepared steel dosing needles. Where possible conscious animals were dosed although in some cases rats were restrained by light ether anaesthesia. Administered compounds were dissolved in 0.05 M-sodium phosphate buffer at pH7 containing sodium ascorbate (2% w/v). The volumes administered did not exceed 0.5 ml for the rats and 0.1 ml for the mice.

Animal experiments were terminated either by stunning rats with a blow on the head followed by surgical opening of the thorax and by cervical dislocation when using mice or by the use of ether. After death tissues were removed either for freeze drying for direct determination of radioactivity or for extraction for the qualitative examination of the retained radioactivity.

2.4 Measurement of Radioactivity

Prepared samples were counted in a Nuclear Enterprises liquid-scintillation counter type NE8310 (Nuclear Enterprises Ltd., Edinburgh).

Aqueous samples, e.g. column eluants and urine samples, were made up to 1 ml with water and 10 ml of a scintillation cocktail added composed of toluene (1 litre) and Fisons emulsifier mix No. 1 (Fisons, Loughborough, Leics., U.K.) (500 ml) in which was dissolved 2,5-diphenyl-oxazole (PPO) (5 g) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) (0.1 g). Radioactivity on paper chromatograms and electrophoresis strips was either eluted with water and treated as above or was counted directly by cutting up the strips into appropriately sized pieces and placing them in to vials to which was added 10 ml of toluene (1 litre) in which was dissolved PPO (5 g) and POPOP (0.1 g). Cellulose powder was scraped from t.l.c. plates and counted in a similar manner. It should be noted that whilst the counting of carbon-14 is unhampered by the presence of cellulose or paper the lower energy beta particle of tritium is and this may lead to quantitative errors in the estimation of this isotope.

Samples were counted for 10 min or 10,000 counts. Corrections for quenching were made using the external standard ratio method related to a quench curve drawn up using increasing concentrations of 2,4-dichloro-phenol-indophenol against a standard of each isotope used. The carbon-14 and tritium of dual labelled samples were counted simultaneously using narrow window settings for each isotope, with due allowance made for the overlap of isotopes using either the external standard ratio or the channels ratio methods.

Freeze dried tissue and faecal samples were determined by combustion using a Beckman Biological Materials Oxidizer. Tritium was trapped as tritiated water in a dry ice/methanol cold trap and counted in 10 ml of Fisons tritium absorber "H". Carbon-14 was trapped as carbon dioxide in 15 ml of Fisons absorber "P".

2.5 Determination of Metabolites

Preliminary information on the identity of the radiolabelled species in tissue extracts or urine following radiolabelled folate administration

was obtained by co-chromatography of samplesand authentic standards on DEAE-cellulose and Sephadex-G15. The distinctive behaviour of folic acid on DEAE-cellulose (table 2.1) was such that further identification criteria were not pursued. 5 MeTHF elutes in a position free from overlap with other intact folates in urine samples on DEAE-cellulose but could be confused with folate conjugates in the tissues,although the two are resolvable on Sephadex-G15. During re-chromatography both authentic 5 MeTHF and labelled 5 MeTHF in samples underwent distinctive chemical decomposition, however since the specific radioactivity of the 5 MeTHF and breakdown products was constant such spontaneous degradation formed confirmatory evidence for their coidentity. 10 CHOTHF was identified by its elution behaviour on column chromatography and by its oxidation to yield 10 CHOFA or acid isomerisation to 5 CHOTHF, both derivatives having different chromatographic properties from the parent compound. Other metabolites were characterised as described in the relevant chapters.

Metabolites were quantified by estimating the total radioactivity associated with marker compounds. Where ever possible quantitation was performed on homogenous peaks free from overlap with other metabolites. In the case of unidentified derivatives e.g. folate S where markers were unavailable quantitation was hampered by problems of decomposition during handling and poor resolution, making quantitation less precise than for the known metabolites. The use of a dual-labelled tracer facilitated metabolite identification since folate catabolites could be more readily discerned due to their isotope imbalance.

2.6 Miscellaneous

Microbiological assay with L. casei, P. cerevisiae and C. fasciculata were determined in the Department of Haematology at the General Hospital, Birmingham by established procedures under the supervision of Mr. R. Leeming. Bioautograms of t.l.c. and electrophoresis strips were also developed in that department.

Subcellular fractionations of tissues were performed with the assistance of Dr. R.J. Cook in this laboratory.

U.V. spectroscopy was carried out on a Pye-Unicam SP 1700 spectrometer and fluorescence spectra determined with an Amino-Bowman Spectrofluorimeter.

CHAPTER 3

THE METABOLISM OF [2-^{14}C] FOLIC ACID IN THE NORMAL RAT AND IN THE RAT BEARING THE WALKER 256 CARCOSARCINOMA

Disorders of pteridine metabolism have long been associated with neoplastic disease. Reduced plasma folate, abnormal rates of clearance of injected folic acid and elevated FIGlu excretion after histidine loading suggest that implicit folate deficiency may occur in a wide variety of patients with cancerous lesions (Chanarin et al, 1958; Rose, 1966; Magnus, 1967). These observations combined with the impressive success of the antifolate drug methotrexate as an anti-tumour agent have contributed to the notion that this derangement of the folate pool is due primarily to an abnormal requirement for folate coenzymes induced by increased cellular proliferation. Although other supportive evidence exists for this (see Blakley, 1969) there have been few direct studies and a detailed appreciation of the mechanism by which tumours interfere with folate metabolism in vivo is lacking.

This chapter describes a preliminary comparative study of the metabolism of [2-^{14}C] folic acid by normal rats and rats bearing the Walker 256 carcosarcinoma. Folic acid itself has been used as a model compound in metabolism studies almost since its original synthesis by the Lederle group (Angier et al, 1946). Early studies relied heavily on the use of differential microbiological assay and many of these are reviewed by Blakley (1969). Tritiated folic acid was first synthesised and used by Johns and coworkers (1961) where it was found to undergo rapid clearance from the plasma in man following i.v. administration presumably due to tissue uptake and metabolism. Chromatography of urine samples revealed largely unchanged folic acid with smaller amounts of folinic acid (5-formyltetrahydrofolate) and p-aminobenzoyl-L-glutamate and pteridines, the latter two probably arising by chemical decomposition during collection in situ since antioxidants were not incorporated. More recent studies, some using [2-^{14}C] folic acid, have shown similar although more extensive metabolism with the principal urinary and plasma metabolite being 5 MeTHF (Blair and Dransfield,

1971; Murphy et al, 1976; Retief et al, 1976; Barford et al, 1978). The relative chemical stability of folic acid compared to the reduced coenzyme forms and its ready entry into the folate pool make it an ideal starting point for metabolism studies.

In the mammal folic acid probably enters the pool via reduction to DHF and THF using the enzyme dihydrofolate reductase (tetrahydrofolate : $NADP^+$ oxidoreductase, EC 1.5.1.3). Thus prior administration of the dihydrofolate reductase inhibitor methotrexate to rats results in an increased urinary excretion of unmetabolised folic acid and decreased tissue retention (Barford et al, 1977a) due to failure of reduction and entry of folic acid into the body pool. An alternative route for reduction which utilizes pyruvate and coenzyme A to produce DHF from folic acid (but not THF) found in bacteria (Wright and Anderson, 1957) has not been recorded in the mammal.

Except in special conditions where it may arise through the chemical degradation of reduced precursors folic acid does not occur naturally. Foodstuffs normally lack folic acid although it is pertinent to note that the feeding pellets used to maintain laboratory animals contain it as an additive. Some preparatory vitamin pills contain folic acid although this is discouraged since it may mask incipient pernicious anaemia in man. It is established clinical procedure for folic acid supplementation to be given to women during pregnancy where mild folate deficiency may develop.

Folic acid is in effect a "foreign compound" and as such undergoes certain reactions, both chemical and biological, peculiar to itself. For example, large doses of folic acid produce kidney hypertrophy in the rat (Oertel, 1975) possibly by stimulating DNA synthesis (Kokolis, 1975). It also inhibits several folate requiring enzymes including formiminotransferase (Itoh, 1975) and folic acid polyglutamates are potent inhibitors of thymidylate synthetase (Kisliuk et al, 1974).

After oral administration folic acid is rapidly absorbed across the intestine although the exact mechanism of transfer remains controversial. Active transport of folic acid has been reported (Burgen and Goldberg, 1962; Hepner, 1969) and a folic acid specific binding protein of unknown function has been isolated from the intestinal brush border (Leslie and Rowe, 1972). Work in this laboratory (Blair and Matty, 1974) and more recently elsewhere (Kesavan and Noronha, 1978) suggests that transport is effected by simple diffusion of the neutral non-ionized species formed in an actively secreted acid microlayer at the surface of the gut. Once absorbed both unchanged and metabolised folic acid are found in the plasma and appear fairly quickly in the urine (Blair, 1975). Thus oral doses of radiolabelled folic acid are readily converted into the active coenzymes and [2-^{14}C] folic acid can be used to label the body folate pools.

In this study the metabolic handling and entry of [2-^{14}C] folic acid into the body folate pools in normal and tumour bearing rats is examined by chromatographic analysis of urine, voided within 48 hours of an oral dose, and examination of various tissue extracts for analysis of the retained radioactivity.

Materials and Methods

Normal (4)* and Walker 256 tumour bearing (4) male rats were dosed orally with [2-^{14}C] folic acid (specific activity 54.3m Ci mM^{-1}; 81.4 µg per kg). Urine samples were collected over a 48 hour period into 10 ml of 2% ascorbic acid (pH 4.5) (0-6 h urine) or 10 ml of 2% sodium ascorbate in phosphate buffer at pH7, and examined by Sephadex gel filtration and in some cases by DE52 ion exchange chromatography. At the end of the experiment the rats were killed and the livers and intestine quickly removed, washed in ice cold saline and extracted

* Urine samples were collected from 2 normal rats only.

in one of the following ways:

a) Chopped and homogenised in 0.05 M-phosphate buffer, pH7 containing sodium ascorbate (2% w/v). The homogenate was then placed into a water bath (95°C) for 10 minutes, cooled, centrifuged and the supernatant recovered and combined with the washings of the pellet. (After Bird et al, 1965).

b) Chopped and dropped into 2 volumes of boiling 0.05 M-sodium phosphate buffer, pH7 containing sodium ascorbate (2%) for 10 minutes. After cooling the broth was homogenised and centrifuged to recover the supernatant. (After Shin et al, 1972).

c) Chopped and homogenised in cold 0.05 M-sodium phosphate buffer pH7 containing sodium ascorbate (2% w/v). Cold trichloroacetic acid (TCA) (10% w/v) was then added and after centrifuging the supernatant was recovered.

d) Chopped and homogenised for 30 seconds in cold isotonic sucrose in 0.01 M-sodium phosphate buffer, pH7 containing mercaptoethanol (2% v/v) and sodium ascorbate (2% w/v). The homogenate was then centrifuged and the supernatant retained. (After Corrocher et al, 1974).

All extracts were stored frozen until required.

Results

The distribution of radioactivity in the tissues, faeces and urine is summarised in table 3.1. Both qualitatively and quantitatively urinary excretion of radioactivity was similar between the two groups. Sephadex G15 chromatography of urine samples is illustrated in figures 3.1 and 3.2. The 0-6 h urine of both groups were identical. An early eluting peak (figure 3.1 (a)) chromatographed in the same place as 5-formyltetrahydrofolate (5 CHOTHF). 5 CHOTHF fulfills no known metabolic function and was probably derived by acid isomerisation of 10 CHOTHF during collection since the 0-6 h urine was collected into

ascorbic acid (pH 4.5). The second major peak represents largely unmetabolised folic acid and 5 MeTHF, these two derivatives cochromatographing in this system. The 6-24 h urines were also similar. An early eluting peak cochromatographed with authentic 10 CHOFA, the urine on this occasion being collected at pH7. 10 CHOFA was probably derived *in situ* by air oxidation of the labile coenzyme form 10 CHOTHF, 10 CHOFA itself has no known metabolic role. The slight mismatches between the peaks (figure 3.1 (b)) are not significant and arise due to small unavoidable variations in running conditions. That 10 CHOFA was produced during collection rather than *in vivo* is suggested by its absence from the 0-6 h samples collected under conditions which favoured the cyclisation of 10 CHOTHF to the (acid) stable 5,10 CH=THF. The remaining radioactivity consists of two incompletely resolved components. The first elutes at about fraction 36, the elution position of 5 MeTHF and folic acid, the second component peaking at about fraction 41/42 being well clear of the common folate monoglutamate standards available. Later chapters will show that this latter compound, which increases in time relative to the other urinary species, is not an intact folate but a pteridine derivative (pteridine P) derived by a previously undescribed folate cleavage reaction. In the 24-48 h urine samples pteridine P is the major labelled component, although small amounts of formylfolates and 5 MeTHF are also present. Table 3.2 summarises the distribution of radioactivity between the 3 components resolved by Sephadex G15 chromatography. DE52 ion exchange chromatography of the 0-6 h urine from normal animals resolved the radioactivity into 4 components the major being folic acid (60%) with smaller amounts of 5 MeTHF (6%), formylfolate derivatives (23%) and an unknown (7%) eluting between the formyl and methyl derivatives. No further analysis of urine by ion exchange was performed.

fig 3.2

Analysis of the tissue radioactivity extracted by different

methods demonstrated that the bulk of the retained radioactivity after 48 h, in both the liver and intestine, was a high molecular weight form. Sephadex G15 or Sephadex G10 chromatography of hot ascorbate extracts (method b) gave the bulk of the radioactivity eluting near to the void volume (see figure 3.3). No differences between the hot liver extracts from livers derived from normal and tumour bearing animals were observed and in both cases about 70% of the radioactivity eluted near to the void volume and thus behaved as high molecular weight derivatives (69% normals, 72% tumour bearing). Hot extracts of intestine gave similar high molecular weight peaks (71% radioactivity) (see figure 3.3 (b)). Estimation of the molecular weight of these radioactive species using their elution positions on the column gave between 900-1,000 daltons. Other peaks present were not identified conclusively but eluted in the region of the formylfolates with no 5 MeTHF being apparent. Occasionally small amounts of a low molecular weight derivative appeared (about fraction 32) which was probably pterin (2-amino-4-hydroxy-pteridine) derived by chemical degradation. DE52 ion exchange chromatography of "hot" liver extracts gave a major peak (71% radioactivity) corresponding to the high molecular weight peak on gel filtration and smaller amounts of early eluting material some of which may be 10 CHOTHF. The high molecular weight peak eluted in a similar position to 5 CHOTHF, just before 5 MeTHF and well before folic acid and thus did not elute at higher ionic strength than the folate monoglutamates, behaviour which has been claimed for the more polar polyglutamate derivatives. Sephadex G15 gel filtration of a liver extract obtained by homogenisation and TCA precipitation (method C) of a normal liver gave a high molecular weight peak (66% radioactivity) similar to that of the hot extraction method and again 5 MeTHF was absent. DE52 ion exchange chromatography was also similar (see figure 3.4 (a) (b)).

Non-protein destructive extraction of liver (Method D) gave extracts which on gel filtration gave peaks at the void volume on Sephadex G15 (figure 3.5 (a)). Sephadex G75 gel filtration resolved this into 2 components one close to the void volume and associated with protein and the other in the region of the high molecular weight derivatives, folates and pterins (the latter 3 are poorly resolved on G75). The amount of folate bound to protein decreased with storage time and was probably affected by freeze thawing. Similar extracts prepared without mercaptoethanol in the extraction buffer on Sephadex G15 chromatography gave diminished recoveries of protein bound folate and relatively more of the low molecular weight derivatives eluting in a similar position to 10 CHOTHF. Whether the presence of mercaptoethanol stabilizes the binding of folate to protein or simply reduces the formation of oxidation products which are less firmly bound to protein is unknown. Since in this experiment no attempt was made to prepare high speed supernatants contamination by lysosomal enzymes would no doubt result in both a steady loss of supernatant enzyme activity and an increase in the amount of folate conjugases etc., this probably explains the former observations. Thus endogenous folate would appear to be largely protein bound with the protein itself being associated with folate metabolising enzymes. When the protein bound peak from Sephadex G15 was removed and boiled to remove the protein rechromatography gave a 40:60 ratio of high molecular weight to low molecular weight forms indicating that folate metabolism and degradation were occuring. Whether the binding proteins themselves or co-eluting enzymes are responsible for this degradation is unknown. Although there are several literature references to hepatic folate binding protein (Corrocher et al, 1974; Zamierowski and Wagner, 1974) with the possible exception of dihydrofolate reductase these are unidentified. Figure 3.5 (b) shows that there are 2 protein bound peaks

a major (32% radioactivity) eluting at the void volume (i.e. molecular weight 70,000 daltons) and a minor (9% radioactivity) eluting at fraction 16 corresponding to a much lower molecular weight protein when the extract shown in figure 3.5 (a) was subjected to Sephadex G75 gel filtration. DHF reductase is a likely candidate for this protein (molecular weight 20,000-30,000 Daltons).

An extract of liver prepared by method (a) on Sephadex G15 chromatography gave a small peak (3% radioactivity) close to the void volume, some 10 CHOFA (15%) with the bulk of the radioactivity peaking at about fraction 30 (60% radioactivity). The identity of this material is unknown although it may be 5 MeTHF or possibly pterin. The small recovery of high molecular weight folate in this case is probably due to a failure to inactivate the endogenous conjugases sufficiently rapidly to prevent hydrolysis during extraction. Whilst limited degradation during the homogenisation itself may have occurred, the relatively low rate of heating gave ample time for degradation by the fast acting lysosomal "conjugases" and this was probably the more important factor. This effect would probably have been reduced if the homogenisation had been performed in isotonic sucrose (as in method (d)) to minimise microsomal rupture.

Discussion

Following an oral dose of [2-^{14}C] folic acid several labelled compounds occur in the urine of both normal and tumour bearing rats, including 10-formylfolate derivatives, 5 MeTHF, folic acid and a previously undescribed unknown folate degradation product (pteridine P). Over a 48 h period the relative proportions of the compounds changed, the unidentified metabolite increasingly dominating urine samples.

Earlier studies in this laboratory suggested that urinary excretion of metabolites is initially complex but that urine samples become increasingly dominated by the derivative described in the literature

as 4aOH5MeTHF (Barford and Blair, 1975). Other workers using high specific radioactivity tritiated folic acid and longer time periods of up to 14 days have suggested that the dominant long term urinary species are in fact degradation products such as p-aminobenzoyl-L-glutamate and its derivative p-acetamido-benzoyl-L-glutamate (Murphy et al, 1976; Murphy et al, 1978). The compound ascribed the structure 4aOH5MeTHF was first described by Gapski et al (1971) as a product of peroxide oxidation of 5 MeTHF and has also been found in studies of 5 MeTHF oxidation where the active species was molecular oxygen (Blair et al, 1975). The degradation mechanism probably proceeds via the formation of 5 MeDHF although both the actual structure of the product (i.e. 4aOH5MeTHF) and the mechanism by which it is formed are controversial. Although Barford and Blair (1975) examined urine by Sephadex G15 chromatography they failed to report an unknown metabolite eluting after 5 MeTHF and folic acid. Confusion of pteridine P, which dominates the 24-48 h urine samples in the present study, with 4aOH5MeTHF by Barford and Blair is extremely unlikely in view of the very different elution behaviour of the two compounds from Sephadex G15. It may be possible that those conditions which would favour the degradation of 5 MeTHF to 4aOH5MeTHF, i.e. artefact formation, would also allow the oxidation of pteridine P to one or more of the simpler oxidized pterins. These latter compounds are often very insoluble in aqueous solution at pH7 and may thus have precipitated out and been lost during urine collection. In the present study adequate protection by the rigorous incorporation of the antioxidants ascorbate and dithiothreitol would have effectively prevented such artefact formation. Confirmation of the metabolites reported here has also been demonstrated by concomitant studies in this laboratory using dual labelled folic acid (A.E. Pheasant, M.J. Connor and J.A. Blair, unpublished observations).

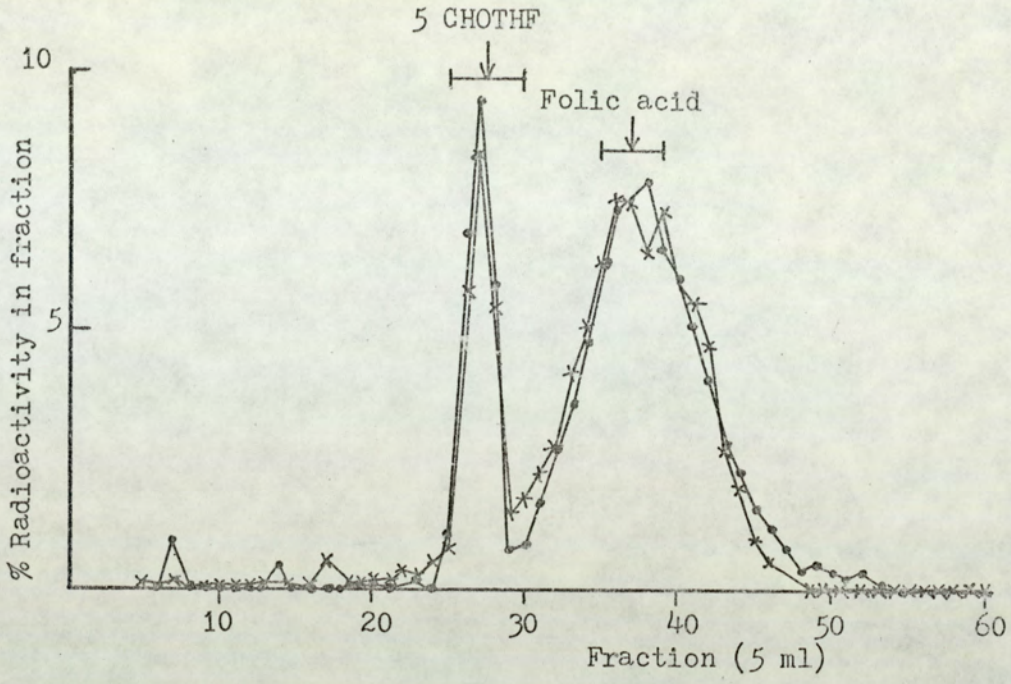
The nature of the identity of the retained tissue folates has

been the subject of controversy (Barford et al, 1977a). In the present study the use of hot ascorbate extraction or TCA treatment to rapidly remove protein combined with Sephadex G15 gel filtration demonstrates that high molecular weight derivatives constitute the bulk of the retained radioactivity with smaller amounts of formyl folate derivatives. Labelled 5 MeTHF was apparently absent from liver extracts although this has been claimed in the literature as a major hepatic folate, (Bird et al, 1965) albeit largely on the basis of microbiological assay.

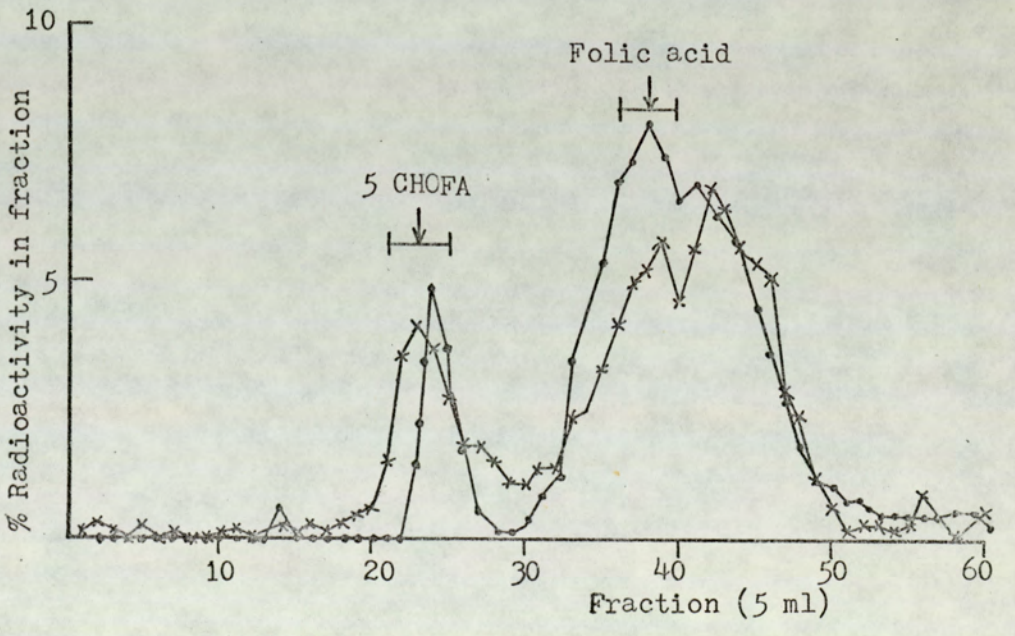
Largely by analogy to yeast folates there are many claims in the literature that the retained tissue folates are glutamic acid conjugates (see Chapter 1) although definitive evidence for this was lacking in the mammal. Biosynthesis of high molecular weight folate is relatively slow compared to the rapid interconversion of the folate monoglutamates and it is only 4-6 h after adminstration that measurable amounts of high molecular weight derivatives may be found in the tissues (Corrocher et al, 1972 ; Hillman et al, 1977). It is conceivable therefore that the observed changes in the urinary excretion pattern with time reflects changes in the tissue folate i.e. initial entry of folic acid into the folate monoglutamate pool gives rise to folate monoglutamates in the urine, the high molecular weight derivatives formed later giving rise to different metabolites and ultimately to metabolite "P". Identification of both the retained tissue folates and the urinary metabolites is evidently important in understanding these changes and will be reported in subsequent chapters.

Decreased faecal and urinary radioactivity and slightly raised hepatic folate levels in the tumour bearing rats may be indicative of the tumour induced folate deficiency described earlier. Slight differences in the urinary distribution of metabolites are evident with excretion of both the unknown pteridine P and the folic acid/5 MeTHF level being reduced (73% and 90% controls respectively) and formylfolate

excretion being raised (114% controls) in tumour bearing rats. Whilst the small number of animals used in the study reduces the overall significance of the differences observed some general deductions may be drawn. The reduced urinary folic acid/5 MeTHF level of the tumour bearing rats and their decreased faecal excretion are suggestive of tumour induced folate deficiency. The slight elevation in the formyl-folate level in tumour bearing rat urine is in agreement with the hypothesis advanced by Blair (1975) that the ratio of 5 MeTHF to 10 CHOTHF is decreased in diseased states, although precise quantitation of the urinary 5 MeTHF level is required for confirmation of this.



(a) 0-6 h urine



(b) 6-24 h urine

Figure 3.1 Sephadex G15 chromatography of urine samples (15 ml) from normal (·-·-·-·) and tumour bearing (-x-x-x-) rats collected 0-6 h and 6-24 h after the administration of [2-^{14}C] folic acid.

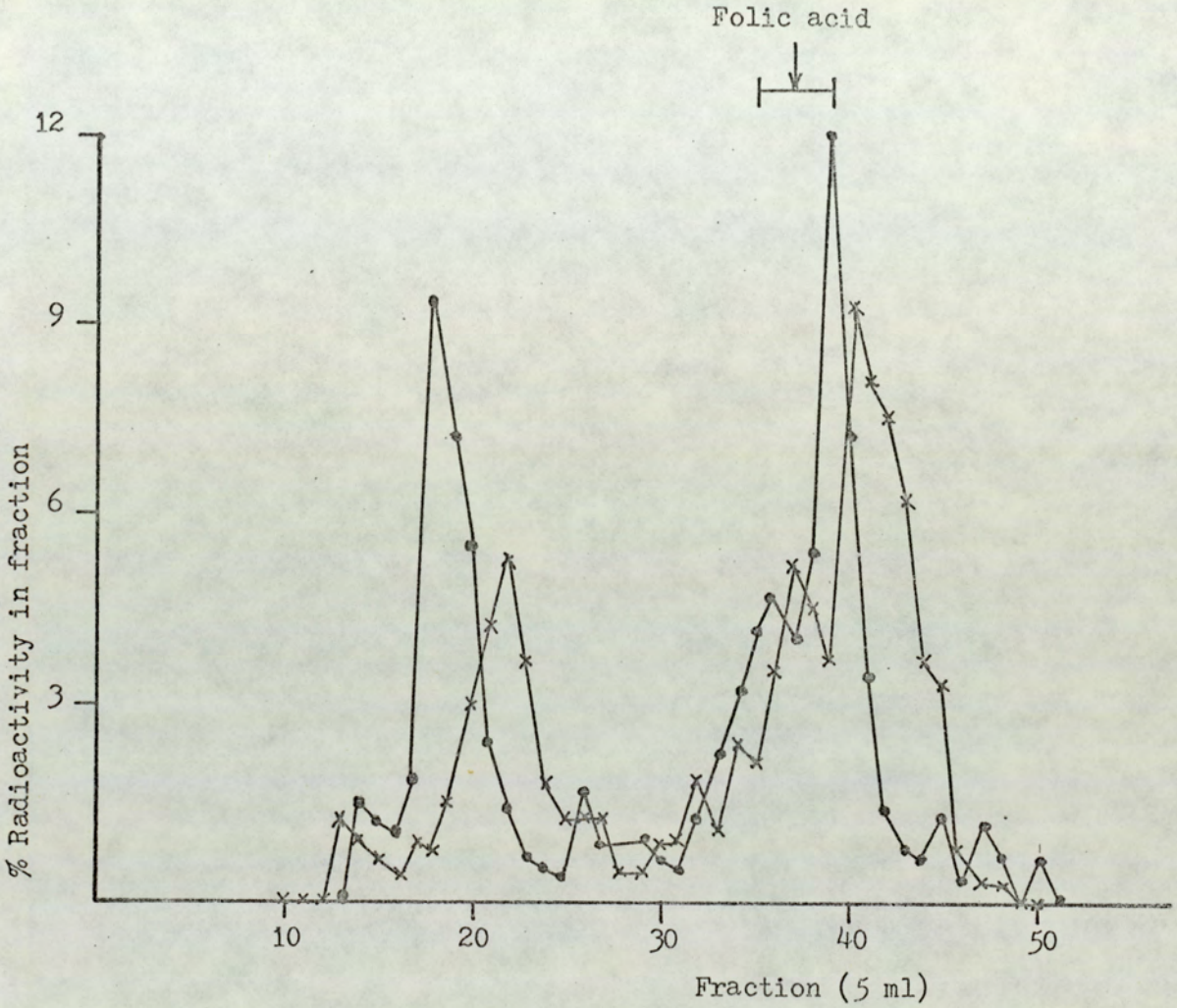


Figure 3.2 Sephadex G15 chromatography of urine samples (15 ml) from normal(•-•-•-•-•) and tumour bearing (-x-x-x-) rats collected 24-48 h after the administration of [2-^{14}C] folic acid

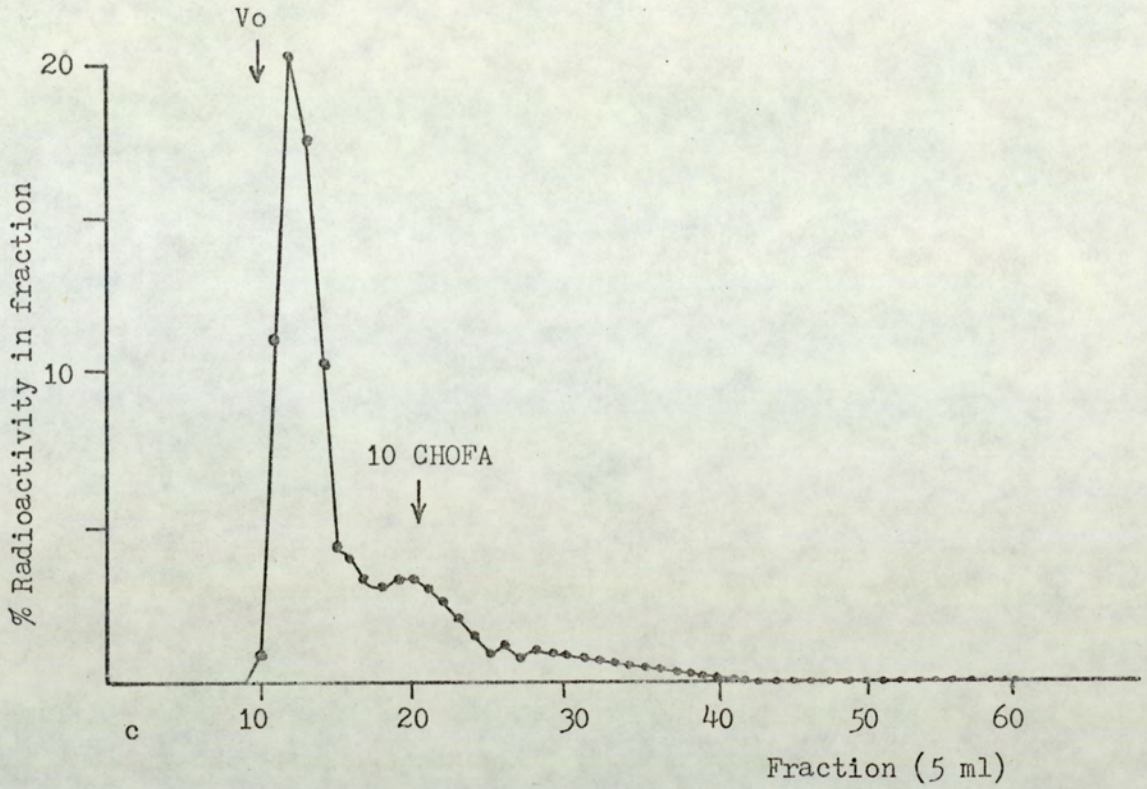


Figure 3.3 (a) Sephadex G15 chromatography of a hot extract (method b) of normal rat liver. Livers from tumour bearing rats gave similar chromatograms.

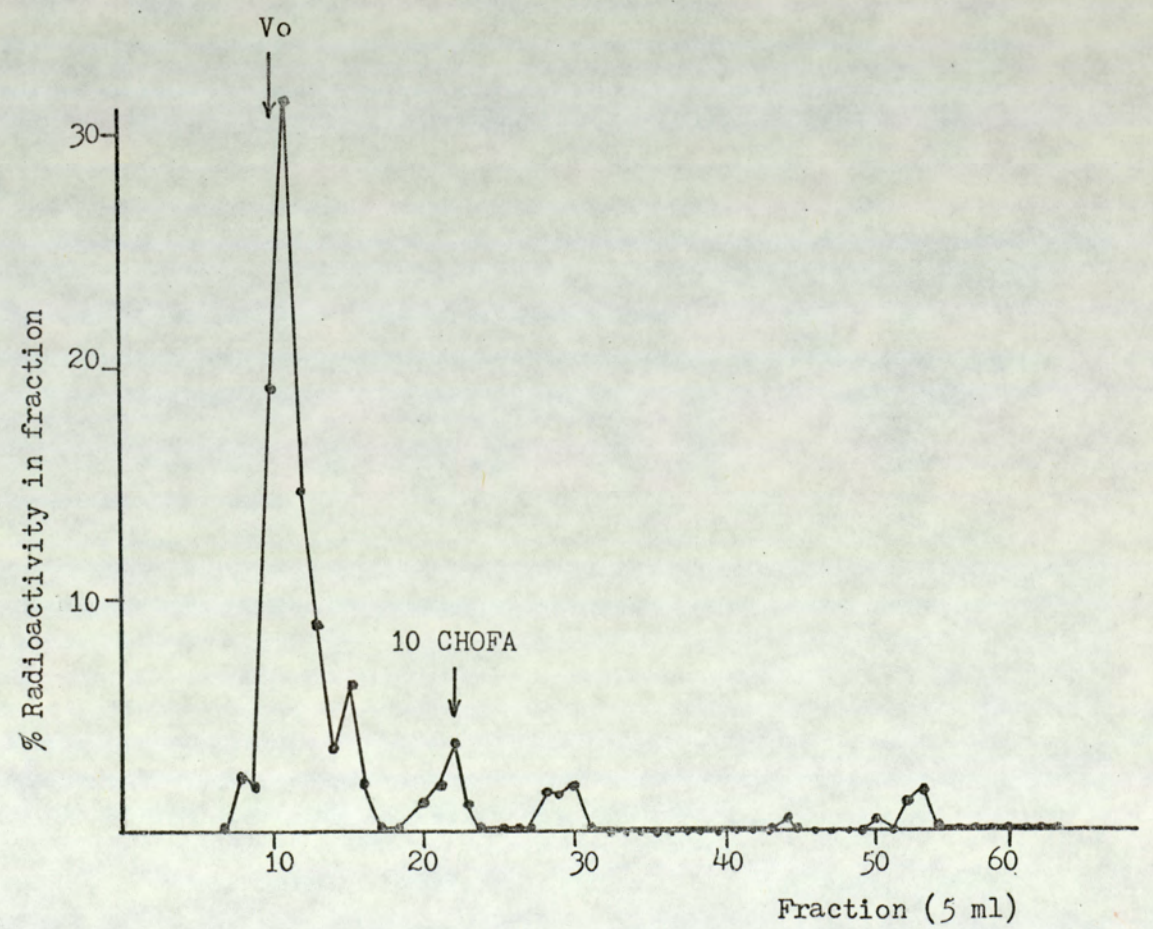
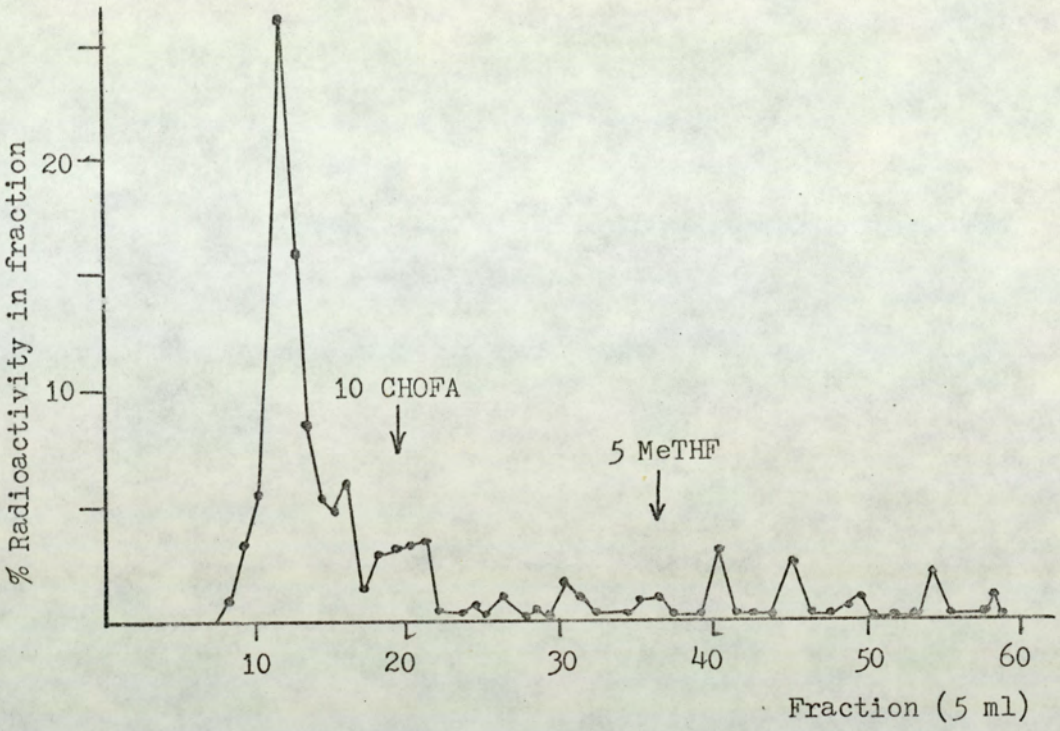
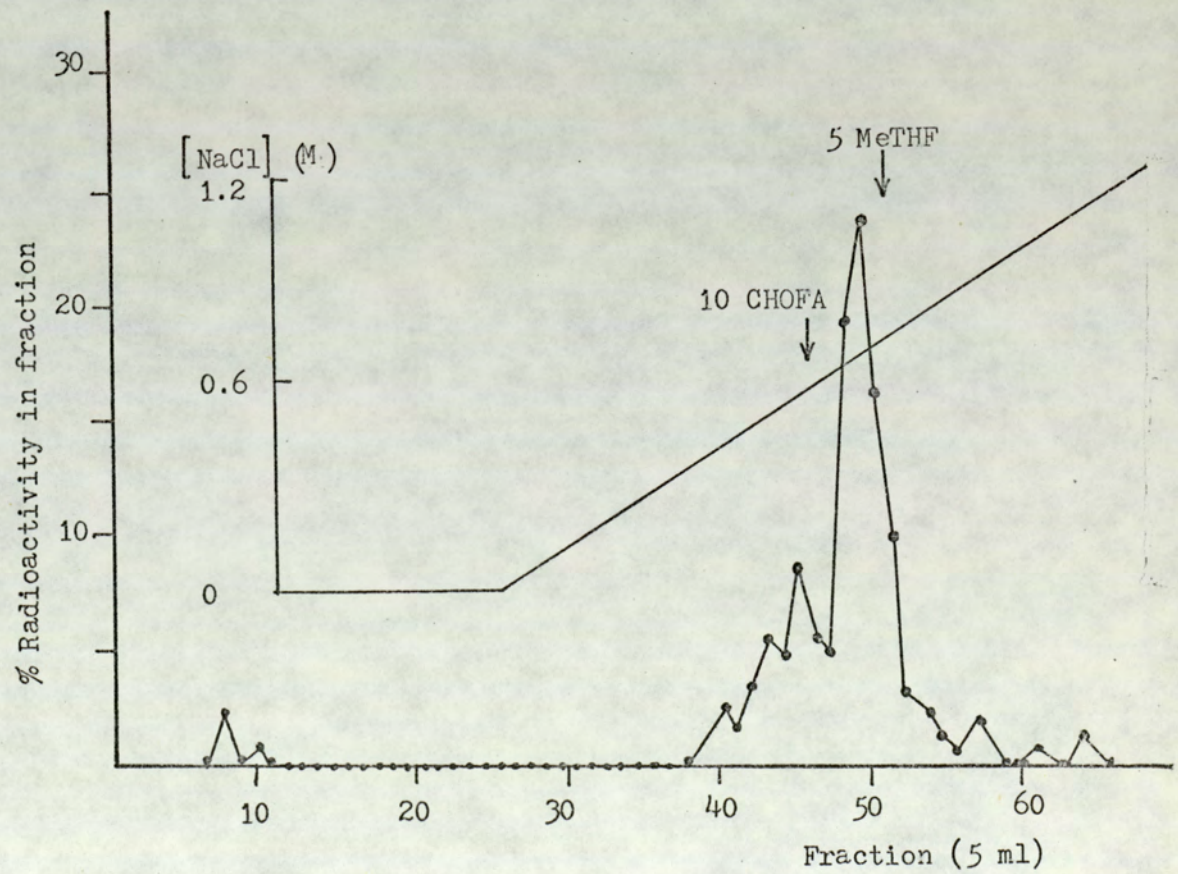


Figure 3.3 (b) Sephadex G10 chromatography of a hot extract (method b) of the intestine from a normal rat.

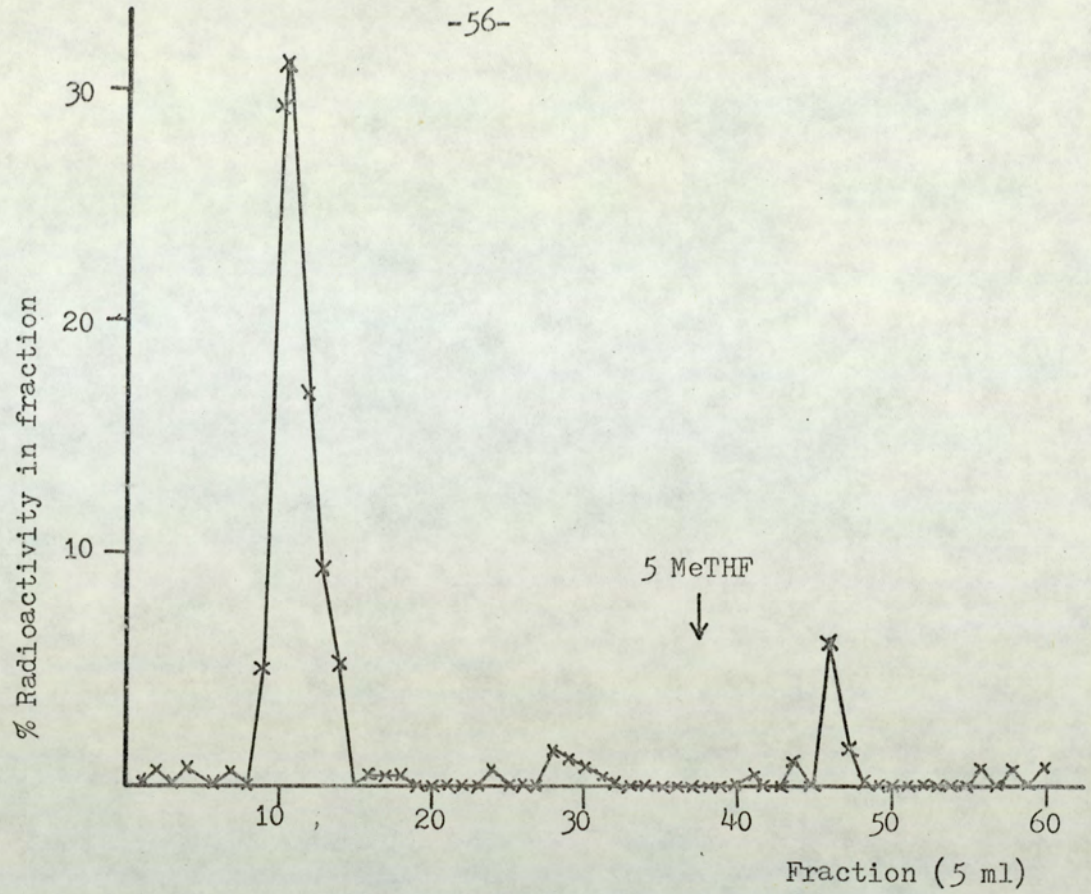


(a) Sephadex G15 chromatography

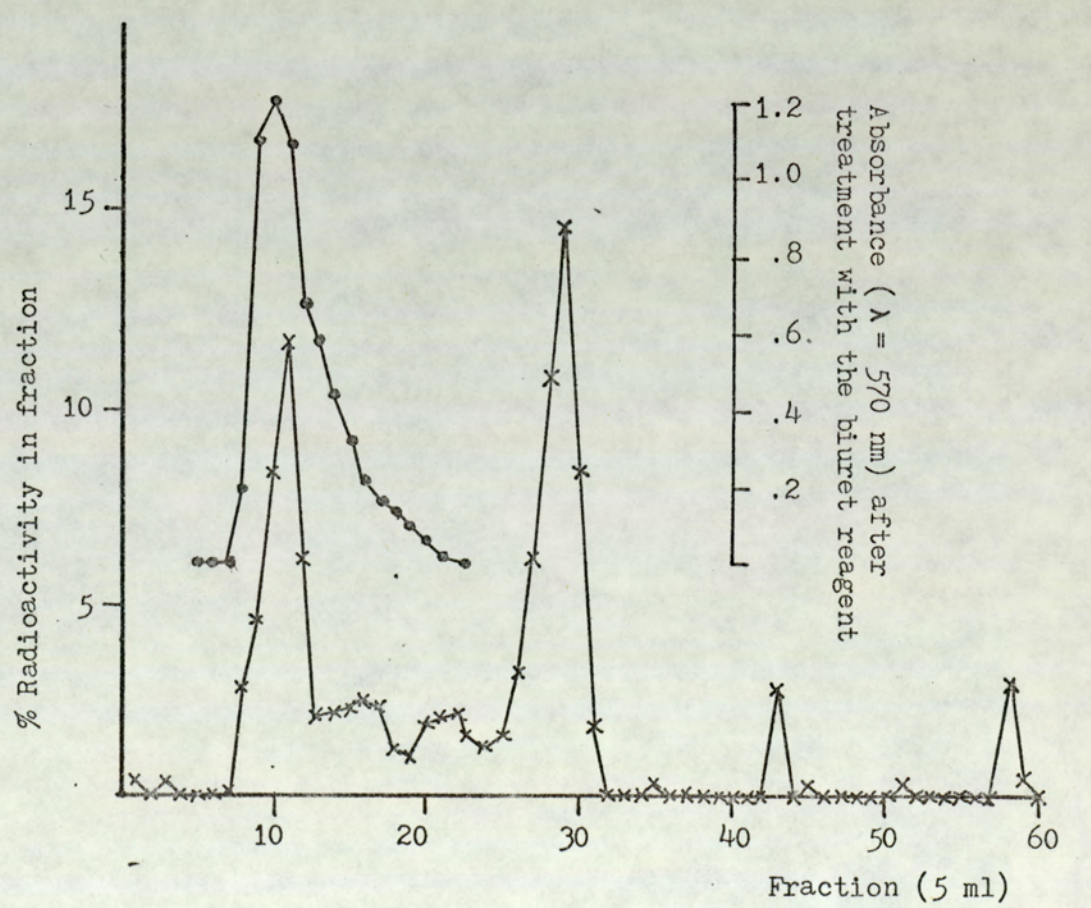


(b) DEAE-cellulose ion exchange chromatography

Figure 3.4 Sephadex G15 and DEAE-cellulose chromatography of an extract (method c) of the liver of a tumour bearing rat.



(a) Sephadex G15 chromatography



(b) Sephadex G75 chromatography

Figure 3.5 Sephadex G15 and Sephadex G75 chromatography of a cold extract (method D) of normal rat liver.

Table 3.1 The distribution of radioactivity in the tissues, urine and faeces of normal and Walker 256 tumour bearing rats 48 h after an oral dose of [2-^{14}C] folic acid (81.4 $\mu g kg^{-1}$).

		Normal		Tumour Bearing	
		Wet weight	% Dose	Wet weight	% Dose
Liver		9.3 g	10.2	8.9 g	11.7
Intestine		5.2 g	1.9	-	-
Tumour		-	-	8.1 g	2.1
Faeces			25.3		19.2
Urine	0- 6 hr		10.6		12.9
	6-24 hr		12.7		8.3
	24-48 hr		2.6		2.8
	Total		25.9		24.0
Total			63.3		57.0

Table 3.2 Summary of the distribution of urinary radioactivity amongst the component metabolites resolved by Sephadex G15 chromatography, 0-48 h after the administration of [2-^{14}C] folic acid to normal and tumour bearing rats.

Normal Rats

Urine Sample	Fractions	Components	% Sample	% Dose
0- 6 h	19-29	Formylfolates	23.7	2.52
	30-48	Folic acid & 5 MeTHF	71.7	7.62
6-24 h	22-29	Formylfolates	11.8	1.50
	30-39	Folic acid & 5 MeTHF	45.0	5.71
	40-51	Unknown "P"	39.6	5.03
24-48 h	17-25	Formylfolates	30.8	.79
	31-37	Folic acid & 5 MeTHF	21.2	.54
	38-48	Unknown "P"	37.5	.96
Total		Formylfolates		4.81
		Folic acid & 5 MeTHF		13.87
		Unknown "P"		5.99

Tumour Bearing Rats

0- 6 h	19-29	Formylfolates	23.8	3.08
	30-47	Folic acid & 5 MeTHF	69.4	8.97
6-24 h	18-29	Formylfolates	21.1	1.76
	30-40	Folic acid & 5 MeTHF	33.9	2.82
	41-51	Unknown "P"	38.9	3.24
24-48 h	17-25	Formylfolates	23.8	0.66
	31-39	Folic acid & 5 MeTHF	27.5	0.76
	40-48	Unknown "P"	40.7	1.12
Total		Formylfolates		5.5
		Folic acid & 5 MeTHF		12.55
		Unknown "P"		4.36

CHAPTER 4

THE EXTRACTION, PURIFICATION, ISOLATION AND CHARACTERIZATION OF THE HIGH MOLECULAR WEIGHT FOLATE OF RAT LIVER

In the previous chapter it was demonstrated that 48 h after a dose of [2-^{14}C] folic acid to rats the retained radioactivity in the tissues behaved as a high molecular weight species on gel filtration. Similar observations have been reported in the literature (Shin et al, 1972; Barford et al, 1977a). Although these chromatographic studies have established that the retained folates are not folate monoglutamates their precise chemical stucture has remained debatable (see Blair, 1975).

The best documented characterization of a naturally occuring high molecular weight folate derivative is that of a crystalline folic acid conjugate isolated from yeast (Pfiffner et al, 1946). The conjugate on chemical analysis assayed as folic acid conjugated with an additional six glutamic acid residues. Since the compound isolated was a folic acid conjugate and folic acid is not naturally occurring at least some degradation of the molecule must have occurred during isolation, although this remains the most extensively characterised folate conjugate. Wright and her coworkers (1955) isolated a variety of folate derivatives, some apparently conjugated with glycine and alanine as well as glutamic acid, from the microbe Clostridium aerobactor but these findings were not supported by Curthoys et al (1972) who found only triglutamate conjugates in the related species C. acidi-urici.

Attempts to isolate the folates of mammalian tissues in the 1950's revealed the presence of 5 CHOTHF (Keresztesy and Silverman, 1951), 10 CHOFA (Silverman et al, 1954) and of 5 MeTHF (Donaldson and Keresztesy, 1959) all from horse liver. The techniques employed in these studies involved prolonged extraction processes and autolysis giving ample opportunity for the interconversion and degradation of the various folate coenzymes present. The introduction of hot extraction methods to rapidly inactivate endogenous folate conjugases and other folate metabolising enzymes combined with the incorporation

of antioxidants allowed the demonstration of tissue folate conjugates using differential microbiological assay (Bird et al, 1965). However the failure to use purified conjugase in the assays and the absence of any direct structural evidence of the derivatives present leaves such information very much open to interpretation. The technical problems surrounding more recent chromatographic studies using ion exchange combined with radioactive tracers have been dealt with in Chapter 1.

The experiments described in this Chapter concern the isolation of high molecular weight folate conjugate to allow the unambiguous determination of the folate species found in rat liver 48 h after the administration of radiolabelled folic acid. The Chapter is divided into two sections; section 1 describes the purification and isolation and section 2 the properties and characterization of this high molecular weight folate.

Methods

To minimize enzymic degradation during extraction the high molecular weight derivatives were extracted from freshly excised tissues with boiling ascorbate at neutral pH. This technique was shown in the previous Chapter to allow the liberation of high molecular weight derivatives from the proteins by which they are bound, to minimize enzymic degradation and also allows the rapid processing of large numbers of rat livers.

The extraction and purification procedure is illustrated in figure 4.1. In an initial study hepatic folate was purified using a ^{14}C tracer (extraction A). A second more extensive purification to homogeneity was attempted using a mixture of ^{14}C and ^{3}H labelled tracers (extraction B). After extraction the high molecular weight folate was purified by column chromatography and the major component of purified extract B isolated by paper chromatography.

i) Preparation of Crude Extracts

Extraction A

The livers (500g) from 60 male Wistar rats (200-250 g) (starved for the previous 24 h) were removed, washed in cold isotonic saline, chopped and dropped into 3 volumes of boiling 0.05 M-phosphate buffer pH7 containing sodium ascorbate (2% w/v) and dithiothreitol (5 mg per 100 ml). After 10 minutes at this temperature (95°-100°C) the extracts were pooled and quickly frozen. The livers of two rats dosed orally 48 h earlier with [2-^{14}C] folic acid (100 µg per kg body weight, 3µ Ci ^{14}C per rat) were treated similarly to provide the labelled tracer.

The pooled frozen extracts were thawed and homogenised at 4°C in a Moulinex blender and then centrifuged at top speed in a bench centrifuge. The supernatant was harvested and combined with the washings obtained by resuspending the pellet in buffer and recentrifuging giving a final volume of 400 ml. TCA (40 g) was then added to remove residual soluble protein and the acidified extract recentrifuged. The supernatant was recovered and adjusted to pH7 with NaOH. The neutral supernatant (c.600 ml) was then freeze dried to reduce the volume. The freeze dried residue was dissolved in 150 ml of 0.01 M-phosphate buffer, pH7 containing dithiothreitol (5 mg per 100 ml) and filtered through Whatman No. 1 paper at 4°C to remove gelatinous phosphate salts (this inorganic debris contained no measurable radioactivity). The extract (filtrate) was retained for partial purification by column chromatography.

Extraction B

The procedure described for extraction A was adhered to with the following differences.

1) 100 male Wistar rats (150-200 g), (liver weights 8.0 g) fed food and water ad libitum were the source of the liver.

2) The labelled tracer was provided by dosing, 48 h earlier, 24

rats with [2-^{14}C] folic acid (specific activity 58.2 mCimMol^{-1}) (2 μCi per rat; 102 μgkg^{-1}) and 13 rats with [3', 5', 9(n) - ^{3}H] folic acid (specific activity 500 mCi mMol^{-1}) (5 μCi per rat; 30μg per kg body weight). The use of a dual labelled tracer was considered essential to demonstrate the integrity of the folate structure in the high molecular weight derivatives, to check on decomposition during extraction and to facilitate characterization and metabolism studies.

ii) Purification of the Extract by Column Chromatography

The steps are outlined in Figure 4.1. Column chromatography was performed as described in Chapter 2.

1) Sephadex G15 Gel Filtration

Aliquots of extract (20 ml) were processed on these columns to remove low molecular weight radioactive derivatives, unlabelled impurities and to desalt. Fractions containing labelled high molecular weight folate i.e. eluting near to the void volume (Vo) were pooled and freeze dried for ion exchange chromatography.

2) DEAE-Cellulose and DEAE-Sephadex Ion Exchange Chromatography

The freeze dried fractions from (1) were sequentially chromatographed on both anion exchange media to remove most of the remaining u.v. absorbing impurities. Ion exchange resolved the high molecular weight folate peak into a major and minor component. Only partial resolution was obtained on DEAE-cellulose but an almost complete separation was achieved on DEAE-sephadex and a major (BI) and a minor (BII) derivative were obtained from the purification of extract B. Although at least two components were observed during the purification of extract A these were incompletely resolved and since this was in effect a preliminary study they were treated as a single component.

3) Sephadex G15 Gel Filtration

Radioactive fractions from (2) were desalted by a second Sephadex G15 column, but eluted this time using distilled water. The eluted

radioactivity was freeze dried yielding a white powder which on elemental analysis was found to be largely inorganic phosphate salts. The yields of this powder are

Extract A = 67.1 mg

Extract BI = 651.5 mg

BII = 388.1 mg

U.V. spectral analysis indicated that a major U.V. absorbing impurity was still present (λmax = 261 nm at pH1 and pH7, λmax = 263 nm at pH13).

iii) The Isolation of High Molecular Weight Folate by Paper Chromatography

The freeze dried desalted extract (BI) was dissolved in distilled water and subjected to paper chromatography (Whatman 3MM) developing with propanol: acetic acid: water (200:1:99 vol/vol). Under U.V. light a dark absorbing band (Rf = .01) with the U.V. spectrum of the impurity mentioned above and an intense blue fluorescent band (Rf = .14) were visible. Radioactivity was associated with the blue fluorescence only. An attempt to purify extract BII by a similar method failed since this derivative chromatographed near to the origin and was not resolved from the U.V.absorbing impurity.

Results

The recoveries of high molecular weight folate obtained during the purification of extracts A and B are summarised in tables 4.1 and 4.2 respectively. Recoveries were estimated by determining the radioactivity and, in the case of extract B, by microbiological assay (L. casei before and after chicken pancreas conjugase treatment) and are expressed as percentages of the crude extract. Accidental loss of material reduced the overall yields especially during the purification of extract A (table 4.1). Except for the initial Sephadex G15 chromatography where low molecular weight radioactive components present in the extract were removed recovery of radioactivity was satisfactory,

the relatively small losses being attributable to selection of those fractions from any one chromatographic run which contained significant amounts of radioactivity to reduce the level of impurities eluting in similar positions and partly to slight decomposition during purification. Both the latter problems could probably be overcome by purifying smaller quantities of extract and thus reducing the number of chromatographic runs required. The numbers of these required for the present extractions were considerable (see tables 4.1 and 4.2) considerably prolonging the purification time.

Recovery of microbiological activity during purification of extract B did not parallel recovery of radioactivity, microbiological activity being lost at a faster rate. The ratio of L. casei activity before and after conjugase treatment increased during purification. Both the latter points suggest that considerable loss of unlabelled folate was occurring and although some of the lost activity could be due to degradation to non-microbiologically active forms or increasing concentration of an unknown inhibitor most of this lost activity is probably due to the removal of unconjugated folate and low molecular weight conjugates during the purification. Other factors which may influence the microbiological assay data include the problem of the complex mixture of ready made nutrients in mammalian liver which could markedly exaggerate the growth response of L. casei and thus the microbiological activity in the initial stages may be erroneously enhanced; also since microbiological assay is very sensitive the necessary dilutions required etc. could have introduced considerable error.

Results from extraction B suggest that the amount of BI in the extract was 7-8 times greater than BII. Assuming that the ratio of BI to BII was constant and that no other derivatives were lost during the purification then BI corresponds to 64.4% ^{3}H and 64.2% ^{14}C and BII to 8.6% ^{3}H and 7.8% ^{14}C in the crude extract. Assuming that the high

molecular weight peak obtained in the initial Sephadex G15 gel filtration represents all the high molecular weight folate (i.e. 100%) in the extract and that no degradation occured during extraction and gel filtration then BI corresponds to 88.2% ^{3}H and 89.1% ^{14}C and BII to 11.8% ^{3}H and 10.9% ^{14}C of the high molecular weight folate respectively.

The relative distribution of isotopes in the extract and in the residual precipitates is summarised in table 4.3. About 17% of the liver radioactivity was unextractable by the procedure used. The nature of this material is unknown but may represent folate bound or absorbed irreversibly on to protein or possibly to folate encapsulated in unruptured organelles and trapped in the cellular debris.

Tables 4.4 and 4.5 contain a summary of the recovery of microbiological activity and the specific radioactivities of the high molecular weight folate. The quantities of high molecular weight folate extracted during extraction A and B are comparable (2.36 nM folate g^{-1} liver and 2.25 nM folate g^{-1} liver respectively) and the extraction procedure was thus reproducible. Considerable dilution of label has occured (table 4.5) during metabolism (150 fold in A; 22 fold in B). Direct comparison of the specific radioactivities of the recovered high molecular weight folate from the two extractions are invalid since although the estimated total hepatic high molecular weight folate levels were similar a larger amount of radioactivity was dosed to the rats used for extraction B. If we assume that total conversion of the dosed radioactivity into the high molecular weight product occurred we can determine the total product (X) by the equation

$$X = \frac{A \times a}{Ap} - a$$

where A = specific activity folic acid dosed
Ap = specific activity metabolite
a = total folic acid dosed (μM)

Since the total recovery of radioactivity as BI and BII is known (4.13 μCi ^{14}C; 8.86 μCi ^{3}H and 0.5 μCi ^{14}C; 1.19 μCi ^{3}H respectively) the fractional incorporation can be calculated and the total hepatic

levels of BI and BII determined as 1.90 nmol g^{-1} liver (using ^{14}C) and 1.98 nmol g^{-1} liver (using ^{3}H) for BI and 0.24 nmol g^{-1} liver (using ^{14}C) and 0.25 nmol g^{-1} liver (using ^{3}H) for BII. The total high molecular weight derivatives (I & II) calculated by this method (2.14 nmol g^{-1} using ^{14}C and 2.23 nmol g^{-1} using ^{3}H) is similar to that determined from the recovery of microbiological activity. Similar calculations using the data derived from extraction A gave a total (I & II) value of 1.96 nmol g^{-1}. The similar values for total high molecular weight folate found in the two extractions indicate that in each case folic acid was metabolised by the liver to conjugated forms to the same extent and in a similar manner. The close agreement in value of the specific radioactivities of BI and BII indicate that they are very closely related, one possibly being a chemical degradation product of the other since even very closely related metabolites would not be expected to have such similar specific radioactivities.

The Identification and Characterisation of Rat Liver High Molecular Weight Folate

The purified folate conjugates were subjected to a variety of physical, chemical and biological assays and these are reported here. The gift of chemically synthesised tritiated pteroylheptaglutamate (i.e. folic acid hexaglutamate - folic acid with 6 additional glutamate residues) from Professor I.D. Rosenberg allowed a comparative study of the chromatographic properties of the [2-^{14}C] labelled material (extract A) with an authentic folate polyglutamate. Detailed analysis of the dual labelled materials from extract B was performed to demonstrate their homogeneity and the first unambiguous assignment of the chemical structure of a high molecular weight folate from mammalian tissue.

a) Differential Microbiological Assay

Samples of purified extracts were assayed for microbiological activity with L. casei, S. faecalis and P. cerevisiae before and after

treatment with a "conjugase" preparation obtained by partial purification of a commercial chicken pancreas preparation (Difco). Samples were pretreated by incubating aliquots at 37°C and pH7 for 1 hour in 0.2% sodium ascorbate to which was added conjugase (0.1 ml per 10 ml solution) where required. Conjugase activity of the pancreas extract was checked by determining the release of folate activity from an aqueous yeast extract. Folate activity was determined by comparing the growth response of each bacterium to standard curves drawn up using folic acid standards in the case of L. casei and S. faecalis and 5 CHOTHF for the P. cerevisiae assay.

The results (table 4.6) are expressed for each sample relative to the value obtained for L. casei after conjugase treatment (100%) since this was found in practice to be consistently the highest value and the most reliable figure for estimating 10 CHOFA concentrations. Identical responses were obtained when the conjugates in extracts BI and BII were isolated by paper chromatography. Since chicken pancreas conjugase was used the P. cerevisiae values may be underestimated as this organism responds poorly to folylglutamates and folyldiglutamates which have been claimed as the products of action of conjugase from this source.

It can be seen that for all extracts considerable elevation of microbiological activity was obtained on incubation with "conjugase", consistent with the release of folate from an inactive conjugate, and a similar response to the synthetic folate polyglutamate was found. Microbiological assay data is reviewed and tabulated by Blakley (1969). The consistently high S. faecalis values after conjugase treatment combined with the literature data suggest that the liver conjugates in all cases are unlikely to be derivatives of 5 MeTHF.

b) A Comparison of some of the Chromatographic and Microbiological Properties of the Liver Extract (A) and Authentic Pteroylheptaglutamate

Pteroylheptaglutamate and extract A were treated with a partially

purified chicken pancreas "conjugase" preparation and a preparation of rat intestinal "conjugase". The former enzyme hydrolyses folate polyglutamate substrates ultimately to pteroyldiglutamates (Dabrowska et al, 1949) whilst the latter supposedly lib erates monoglutamates (Rosenberg and Neumann, 1974). The products of conjugase treatment were analysed by Sephadex G15, DE52 ion exchange chromatography and microbiological assay.

Methods

Chicken pancreas conjugase purified from a commercial chicken pancreas extract (Difco Laboratories) was provided by Mr. R. Leeming (General Hospital, Birmingham). The source of rat intestinal conjugase was a simple extract of rat intestines obtained in the following manner: the small intestines were removed from two rats and washed and cleaned in cold isotonic saline. A 20% homogenate (w/v) was prepared by chopping the intestine in 4 volumes of 0.1 M-phosphate buffer, pH6 containing 2% sodium ascorbate and homogenising. The homogenate was centrifuged (bench centrifuge) and the supernatant harvested and stored frozen in aliquots. 14.9 g of rat intestine yielded 70 ml of supernatant. The extract retained conjugase activity when stored under these conditions for at least 6 months.

The enzyme incubations performed are tabulated below. Assays were carried out in 0.1 M-sodium phosphate buffer, pH6 containing 0.2% sodium ascorbate in conical flasks loosely stoppered with cotton wool plugs and the reactions allowed to proceed for two hours at 37°C in a shaking water bath, in a total volume of 10 ml. After two hours the flasks were rapidly frozen and stored at -15°C until required. $Pteglu_7$ and the liver extract were incubated both together and separately with conjugase to check on the presence of natural conjugase inhibitors, as have been reported in yeast extracts (Rosenberg and Godwin, 1971), which may have copurified with the liver extract. A control in which the substrates were incubated

in the absence of conjugase was performed to ascertain any spontaneous chemical degradation.

Flask	$PteGlu_7$	Liver Extract	Intestinal Conjugase	Chicken Pancreas Conjugase
1	+	-	+	-
2	-	+	+	-
3	+	-	-	+
4	-	+	-	+
5	+	+	-	+
6	+	+	+	-
7	+	+	-	-

+ = added; - = not added

Results

The results of the conjugase assays are summarised in table 4.7 using data derived from Sephadex G15 chromatography. No differences were observed when $pteglu_7$ and the liver extract were incubated separately or together and no significant breakdown of either derivative occurred when they were incubated without enzyme. Sephadex G15 gel filtration of the products obtained from incubations 5, 6 and 7 is illustrated in figures 4.2 and 4.3. Rat intestinal conjugase hydrolysed $pteglu_7$ to folic acid (> 86% of the radioactivity) and the liver extract to a material which behaved as 10 CHOFA (> 65% radioactivity). Incubation with chicken pancreas conjugase gave different products and for both substrates these eluted earlier from Sephadex G15 columns i.e. at a lower k_{av}. This would indicate that they had higher molecular weights than the folic acid and 10 CHOFA produced from rat intestinal conjugase action and is consistent with the production of pteroyldiglutamate derivatives as the product of action of chicken pancreas conjugase.

The good yield of folic acid as the product of rat intestinal conjugase action on pteglu$_7$ indicates that little conversion to other folate forms due to contaminant folate metabolising enzymes occurred during the incubation. Thus 10 CHOFA, the apparent product obtained when the liver extract was used as a substrate for rat intestinal conjugase would appear to be a major component of the liver high molecular weight folate.

c) A Detailed Study of the Folate Moiety Released from the Isolated High Molecular Weight Derivative (BI) by Rat Intestinal Conjugase

The paper chromatography purified BI was treated with the rat intestinal conjugase preparation described above. BI (35 nM) was incubated in a shaking water bath for 1 hour at 37°C with 1 ml of rat intestinal conjugase preparation in 0.1 M-phosphate buffer, pH7 in a total volume of 10 ml. The reaction mixture was then concentrated by freeze drying. The product was analysed by Sephadex G15 gel filtration, DEAE-cellulose ion exchange, thin layer chromatography, thin layer gel filtration and electrophoresis and compared to authentic 10 CHOFA and other folate monoglutamate markers. The results are summarised in table 4.8 where the liver extract BI and the product obtained when BI was incubated with conjugase are compared to 10 CHOFA.

Fluorescent and absorbing spots on chromatograms were viewed under U.V. light (λ = 366 nm or 254 nm) and bioautograms and electrophoresis performed as described in the following section.

In all systems tested the product of conjugase action on BI was inseparable from 10 CHOFA. The diversity of the systems used, dependent upon a variety of different physical properties strongly supports the coidentity of 10 CHOFA and the product liberated by conjugase action. Assuming that the observations made in the previous section that the activity of other folate metabolising enzymes in the conjugase preparation is minimal then the liver extract BI is a conjugate of 10 CHOFA.

d) The Behaviour of Liver Folate Conjugate and Standard Folate Monoglutamates on Electrophoresis

The electrophoretic mobility of the purified and isolated liver extract (BI) was examined on paper (Whatman No. 1) and cellulose acetate strips (table 4.9). Since better separation of folic acid and BI was found on paper than on cellulose acetate, and also the advantage of a higher sample capacity, most of the electrophoresis was performed on paper. The reason for the difference in mobility of folic acid on the two support systems used is unknown although it may be due to nonspecific interactions with the cellulose fibres of the paper, perhaps a similar phenomenon to the behaviour of folic acid on DEAE-cellulose from which it is much harder to elute than the other folate monoglutamates.

A single dual-labelled radioactive peak corresponding to the folate conjugate was found on all occasions and was associated with a band visible under U.V. light as a blue fluorescence. The electrophoretic behaviour of the column purified BI and the paper chromatographic isolated BI were identical although the U.V. absorbing impurity (recorded earlier) was present in the former sample as an absorbing spot moving slower than the radioactivity and associated blue fluorescence. The absence of any U.V. absorbing or fluorescent areas other than that associated with radioactivity on electrophoresis of the paper chromatographic isolated BI demonstrates the considerable homogeneity of this sample. Bioautography (performed by Mr. R.J. Leeming, General Hospital) confirmed this homogeneity where it was found that the maximal growth of L. casei grown on agar plates in which developed electrophoresis strips (previously sprayed with chicken pancreas conjugase) were embedded was in the same region as the radioactivity.

The more rapid migration of the liver folate conjugate towards the anode than the folate monoglutamate standards used suggests that at pH7 the folate conjugate carries a higher negative charge. There is a very limited literature on the electrophoretic properties of folate

although enhanced mobilities over folate monoglutamates have been reported for folate polyglutamate derivatives (Rosenberg and Neumann, 1974).

e) Fluorescence and Ultraviolet Absorbance Spectra

It was observed that the isolated liver folate BI possessed a blue fluorescence visible on paper chromatograms under U.V. light. The U.V. spectrum of BI was determined after elution from the paper chromatogram at pH1 (0.1 M-HCl) pH7 (0.1 M-phosphate buffer) and pH13 (0.1 M-NaOH) and compared to that of 10 CHOFA (figure 4.4). The fluorescence spectra were determined under similar conditions and the emission and excitation spectra of the liver folate and 10 CHOFA are illustrated (figures 4.5 and 4.6). The spectral data are summarised in table 4.10.

The U.V. spectra of 10 CHOFA and BI are similar although the peaks at longer wavelength are less well defined for the liver folate. When eluting such small amounts of material from paper chromatograms it is difficult to avoid contamination from impurities present in even well-washed papers and the background absorbance is always high even when reference blanks are eluted from similar areas of the paper. Such non-specific absorbtion will evidently be most troublesome when considering peaks of low absorbance and this probably explains the poor peak definition referred to.

In general folates and pteridines whilst showing markedly high extinction coefficients (c. 20,000) tend to have rather broad peaks and few distinctive points. 10 CHOFA is unusual in that it has two rather broad overlapping peaks at pH7 (λ max = 247 nm and 268 nm) but a single maximum in this region at pH1 and pH13. A similar pattern of overlapping peaks is present in the liver extract.

The emission and excitation spectra of BI and 10 CHOFA are again similar and there is a good peak match at each pH (table 4.10).

Examination of the excitation spectra of 10 CHOFA at pH7 and pH13 revealed that a 2nd minor peak (λ max = 285 nm) was present, although this is not recorded in the literature. Many oxidised pterin and folate derivatives show excitation maxima between 350-380 nm, whilst that of p-aminobenzoyl-L-glutamate is lower at about 310 nm (Uyeda and Rabinowitz, 1963). The minor excitation maximum could thus represent the contribution of the p-aminobenzoyl-L-glutamate moiety to the spectrum. This is supported by the apparent absence of the peak in acid since at low pH the U.V. absorbance of p-aminobenzoyl-L-glutamate is markedly different from that at neutral and alkaline pH. The excitation spectrum of the liver extract shows a similar minor peak to that of 10 CHOFA at pH7 and pH13, absent at pH1 but this has been shifted to a slightly lower wavelength (i.e. higher energy) (270 nm at pH13; 280 nm at pH7) and there is an increased transmission relative to the peak at 370 nm. Thus the ratio of % transmission at 370 nm to that at 285 nm is 3.8 and 3.9 at pH7 and pH13 respectively for 10 CHOFA whilst the ratio of % transmission at 370 nm to that at 280 nm (pH7) or 270 nm (pH13) is 2.1 and 2.9 respectively for BI. If the assumption that the minor peak is largely due to the p-aminobenzoyl-L-glutamate moiety whilst that at 370 nm is due to the pterin moiety is correct then it would appear that conjugation of the folate molecule in the liver has involved changes remote from the pterin chromophore but close to the p-aminobenzoyl-L-glutamate chromophore and thus supports the glutamic acid residue as the site for conjugation.

Attempts were made to extimate the folate content of the liver extracts by a fluorimetric assay using 10 CHOFA as a standard. It was found that over the range 0-13 $\mu M\ L^{-1}$ 10 CHOFA at pH7 (Excitation wavelength = 370 nm; emission wavelength = 450 nm) the fluorescence was directly dependent upon the concentration. The purified extracts BI and BII assayed as 7.3 $\mu M\ L^{-1}$ (10.7 $\mu M\ L^{-1}$ by microbiological

assay) and 0.3 μM L^{-1} (0.75 μM L^{-1} by microbiological assay) respectively and the paper chromatography isolated BI as 2.4 μM L^{-1} (12 μM L^{-1} by microbiological assay). The cause of the apparent quenching effect is unknown but may be partly due to the elution of "background" in the latter case since fluorescence is very sensitive to impurities.

f) Aminoacid and Pentose Analysis

Samples of BI before and after paper chromatography were acid hydrolysed and the aminoacids analysed with an autoanalyser (performed by Dr. Fox, Analytical Services, Birmingham University). The results are summarised in table 4.11 where glutamic acid content is related to the folate content as estimated by U.V., fluorescence and microbiological methods.

Qualitative aminoacid analysis revealed that several aminoacids were present in the column purified extract in comparable concentration to folate. Paper chromatography effectively removed the alanine and glycine from BI, except for a small amount of glycine (5 moles of glutamate per mole of glycine at this stage), the latter probably persisting as a residual impurity.

The most accurate assessment of the folate content of BI is the microbiological assay since this is least affected by impurities. The U.V. spectral estimation is probably an overestimate since this was performed on paper eluted samples in which the absorbance due to the folate may be enhanced by non-specific absorbance. The fluorescence assay probably gave the least accurate value for the folate concentration due to the quenching effect commented on previously. The aminoacid analysis thus suggests that BI is a folate conjugate containing 5 moles of glutamic acid per mole of folate.

The pentose sugar content of BI was assayed using the Drury method (Drury, 1948) since folate associated pentose has been reported in purified bacterial extracts (Wright, 1955). No pentose was detected.

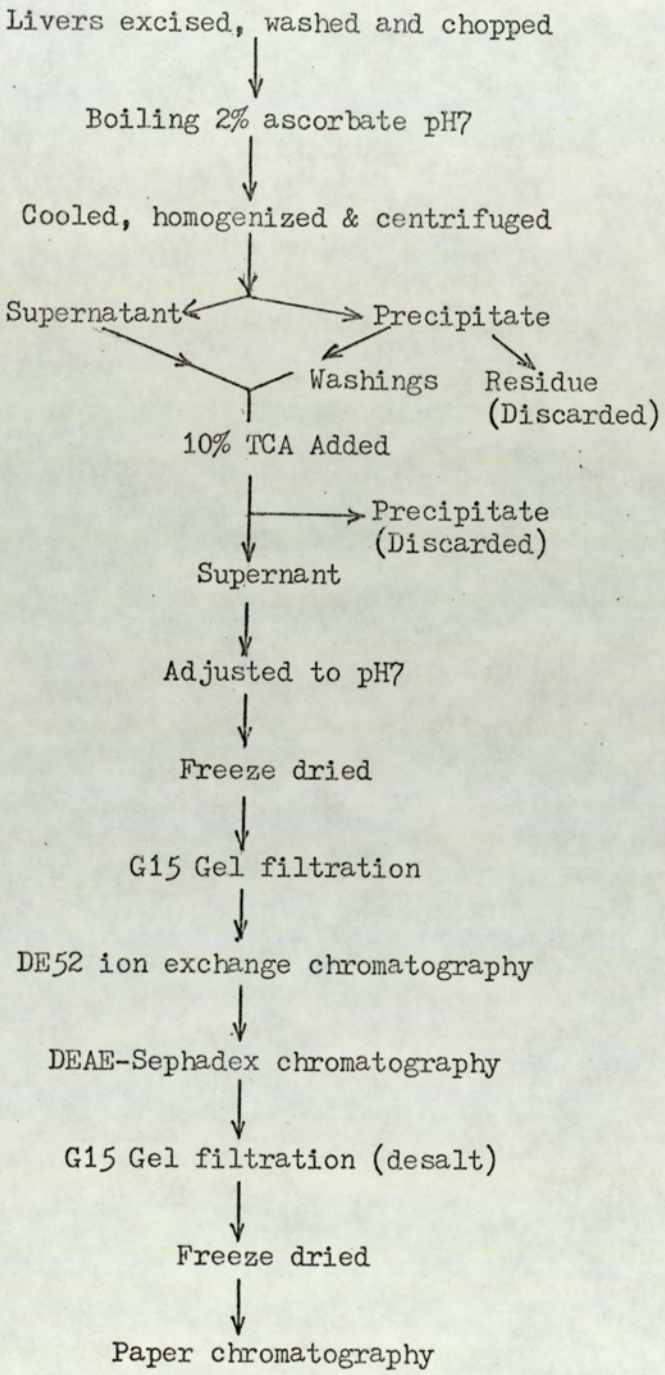
Summary and Conclusion

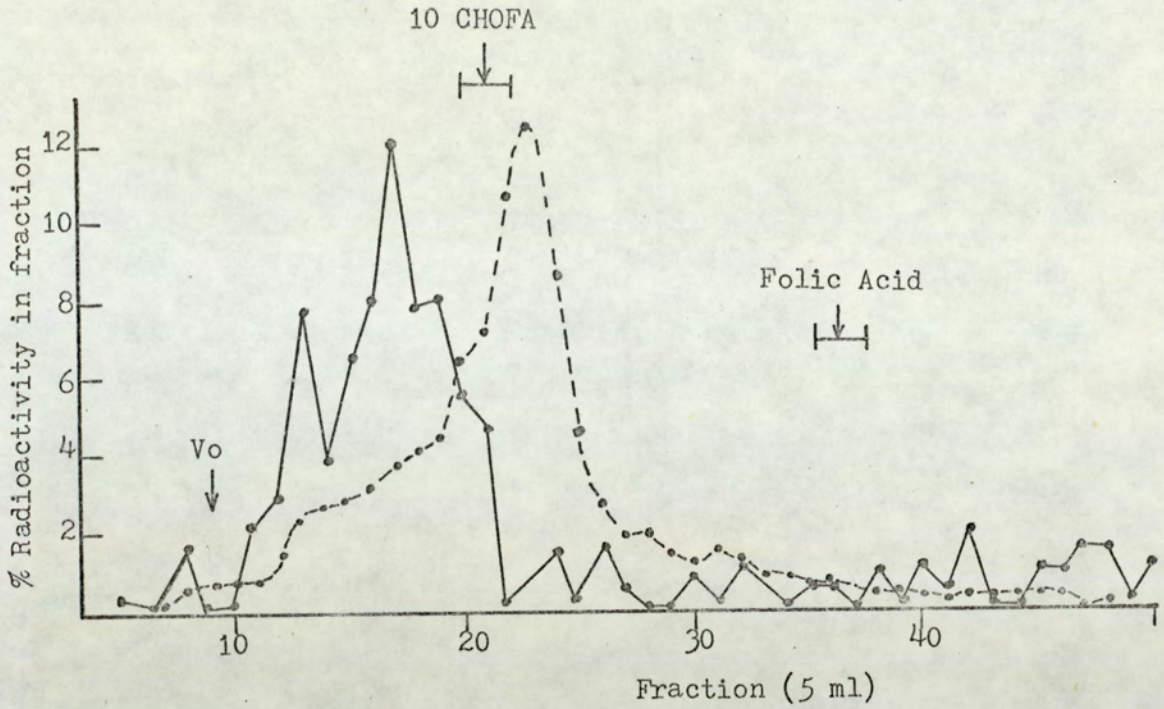
The high molecular weight folate found in rat liver 48 h after the administration of labelled folate has been purified and the principal component isolated as an homogenous material. The folate moiety has been identified as 10 CHOFA by a combination of enzymic, chromatographic, electrophoretic, microbiological and spectral analyses. The isolated conjugate on aminoacid analysis was found to contain a minimum of 5 glutamic acid residues, i.e. was a polyglutamate conjugate. The proposed structure is presented in figure 4.7, i.e. 10 formylfolate-tetraglutamate (10 CHOFA$(glu)_4$). The molecular weight of this compound (985 daltons) is in the range 900-1000 daltons calculable from its Sephadex G15 elution position.

A minor derivative (BII) was also found in liver extracts. This compound which was active for L. casei and S. faecalis but not P. cerevisiae after conjugase treatment, was fluorescent and eluted from Sephadex G15 slightly later than 10 CHOFA$(glu)_4$. Although not definitive this evidence suggests that BII is also a 10-formylfolate conjugate, but in view of its lower molecular weight (on the basis of gel filtration) is conjugated with a lower molecular weight side chain. From the very similar specific radioactivities it was concluded earlier that BI and BII are very closely related, one probably being derived directly from the other possibly by chemical degradation. The side chain is therefore probably again a polyglutamate, in this case being composed of an extra three glutamate residues. The choice of a total of four glutamate residues is in keeping with the microbiological response since L. casei is known to respond to folates containing three glutamate residues or less without prior conjugase treatment (Blakley, 1969), whereas BII required conjugase treatment before it was active. BII is thus tentatively identified as 10-formylfolatetriglutamate (10 CHOFA$(glu)_3$) (molecular weight 856 daltons) (see figure 4.7)

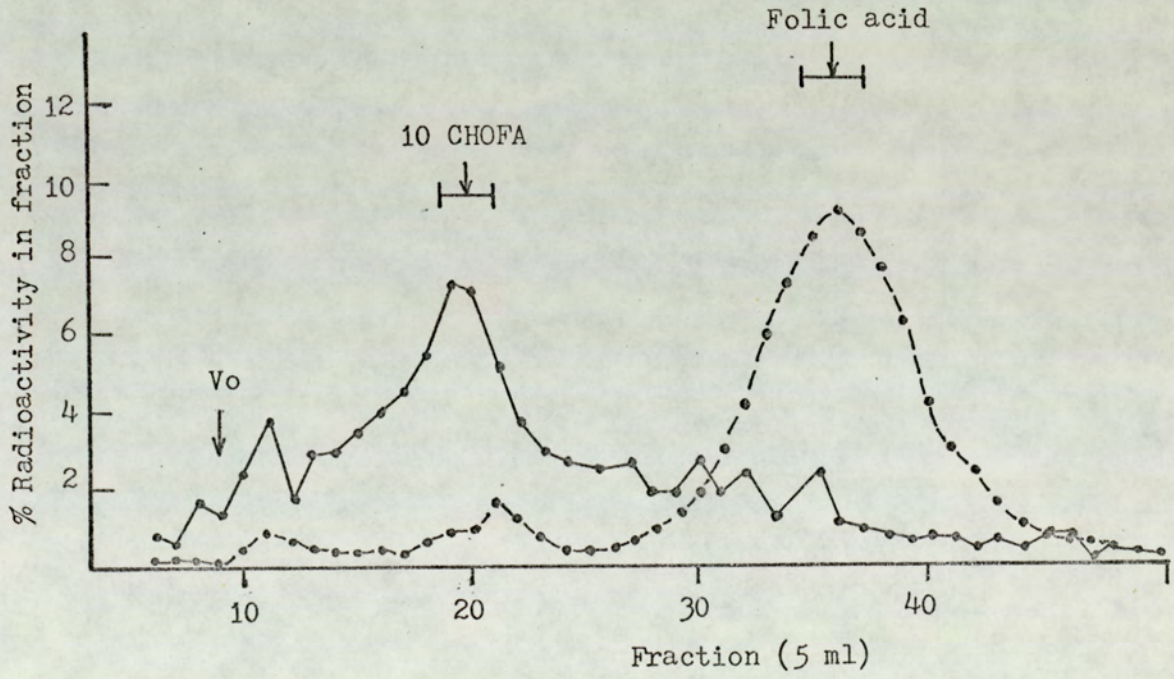
The structure illustrated portrays the folate conjugates as containing a chain of gamma linked glutamate residues. The structure of the side chain of naturally occurring folate polyglutamates has never been determined absolutely. The unique gamma linkage was originally ascribed by extrapolation from data derived using crude conjugase preparations and various synthetic substrates. In these studies partially purified conjugase preparations were tested with conjugates containing different side chain linkages; folate conjugates containing the gamma linkage were hydrolysed more extensively and at faster rates (twofold to tenfold faster) than those containing alpha linkages (Dabrowska et al, 1949; Kazanko et al, 1948; Rowe et al, 1975). In the present study the liver polyglutamates BI and BII were substrates for preparations of folate conjugases, preparations which also hydrolysed authentic pteroylheptaglutamate, a chemically synthesised conjugate containing the gamma linkage in its side chain. Although contamination of the rat intestinal conjugase preparation with non-specific carboxypeptidases would explain the concomitant release of 10 CHOFA from 10 CHOFA$(glu)_4$ and folic acid from pteroylheptaglutamate even if the former polyglutamate derivative contained alpha not gamma linked glutamate residues this cannot hold for the chicken pancreas conjugase preparation since the products contained from both the natural and synthetic polyglutamates were diglutamate and not monoglutamate derivatives, and therefore the products of a specific enzyme activity. The data thus imply that the amide bond between the first two glutamate residues, and probably the remaining amide bonds, are the same for both the liver polyglutamate and the synthetic pteroylheptaglutamate, i.e. that the liver polyglutamate contains the gamma linkage.

Figure 4.1 A summary of the steps employed in the extraction and purification of liver high molecular weight folate.





(a) Chromatography of the products of chicken pancreas conjugase activity



(b) Chromatography of the products of rat intestinal conjugase activity

Figure 4.2 Sephadex G15 chromatography of the products of action of chicken pancreas and rat intestinal conjugase preparations on a mixture of hepatic high molecular weight folate (liver extract A) (—•—•—•—) (labelled with ^{14}C) and pteroyl heptaglutamate (---•---•---) (labelled with ^{3}H).

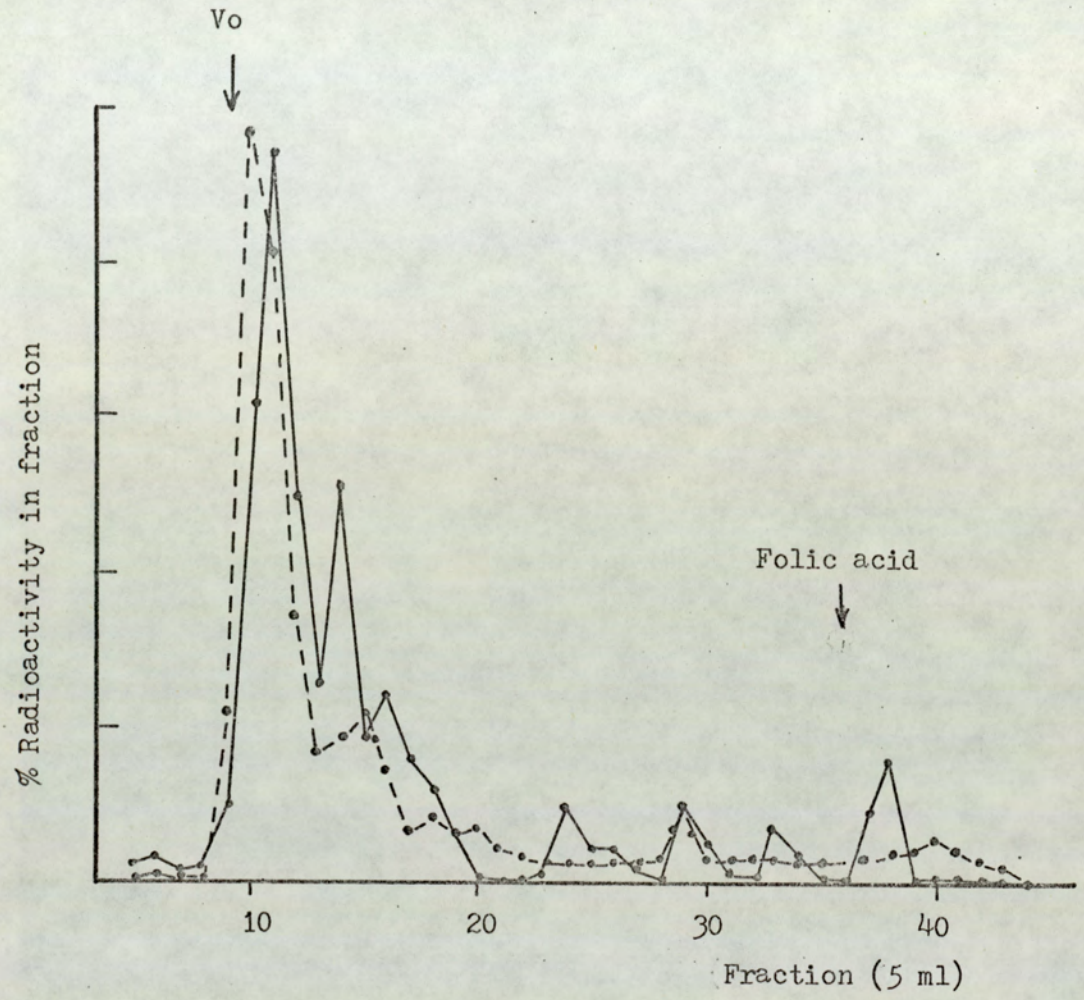


Figure 4.3 Sephadex G15 chromatography of a mixture of hepatic high molecular weight folate (liver extract A) (——) (^{14}C labelled) and pteroylheptaglutamate (-·-·-·-) (^{3}H labelled) incubated for 1 h at pH7 in buffer alone (control incubation).

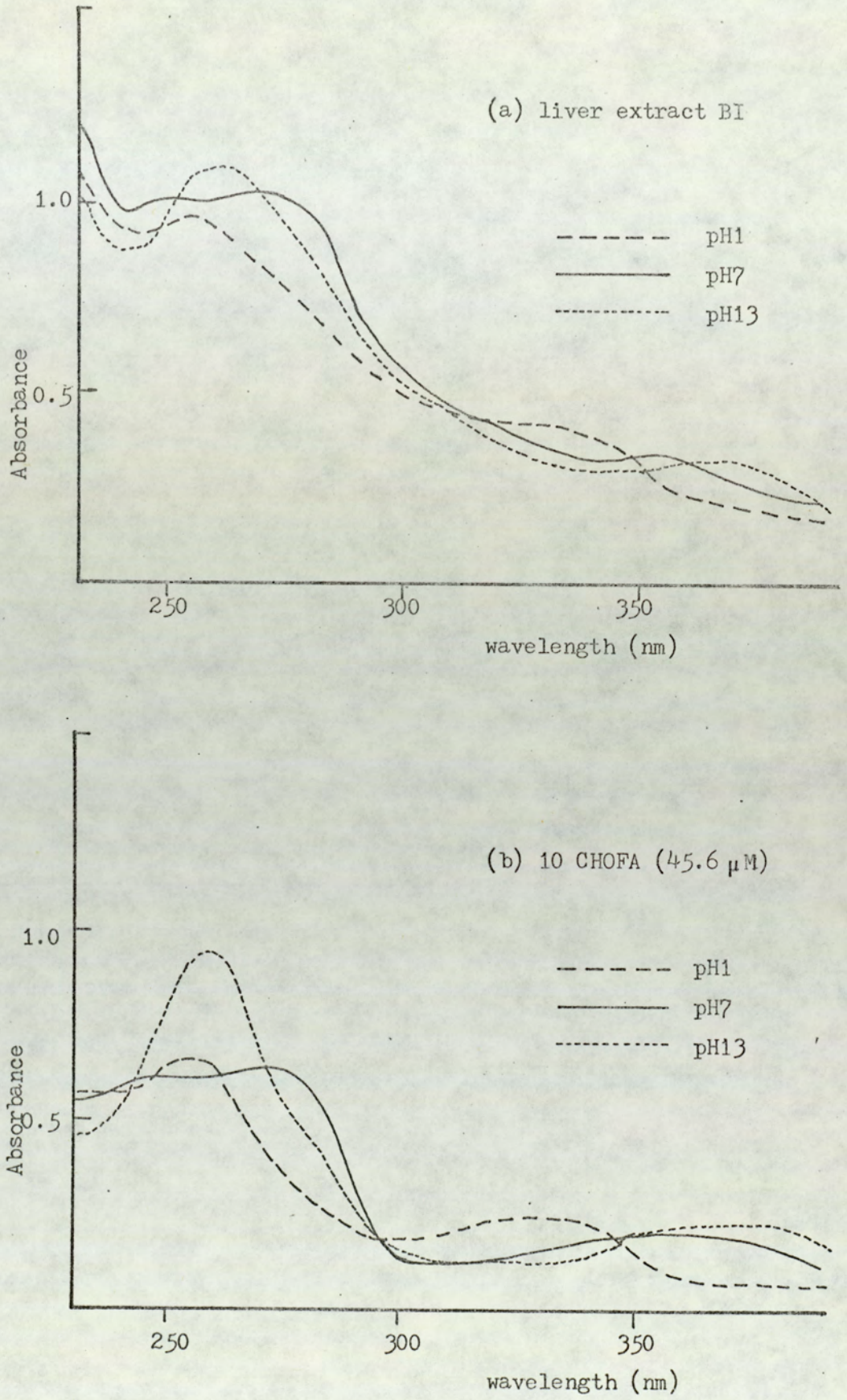


Figure 4.4 The ultraviolet spectra of hepatic high molecular weight folate (liver extract BI) and of 10 CHOFA.

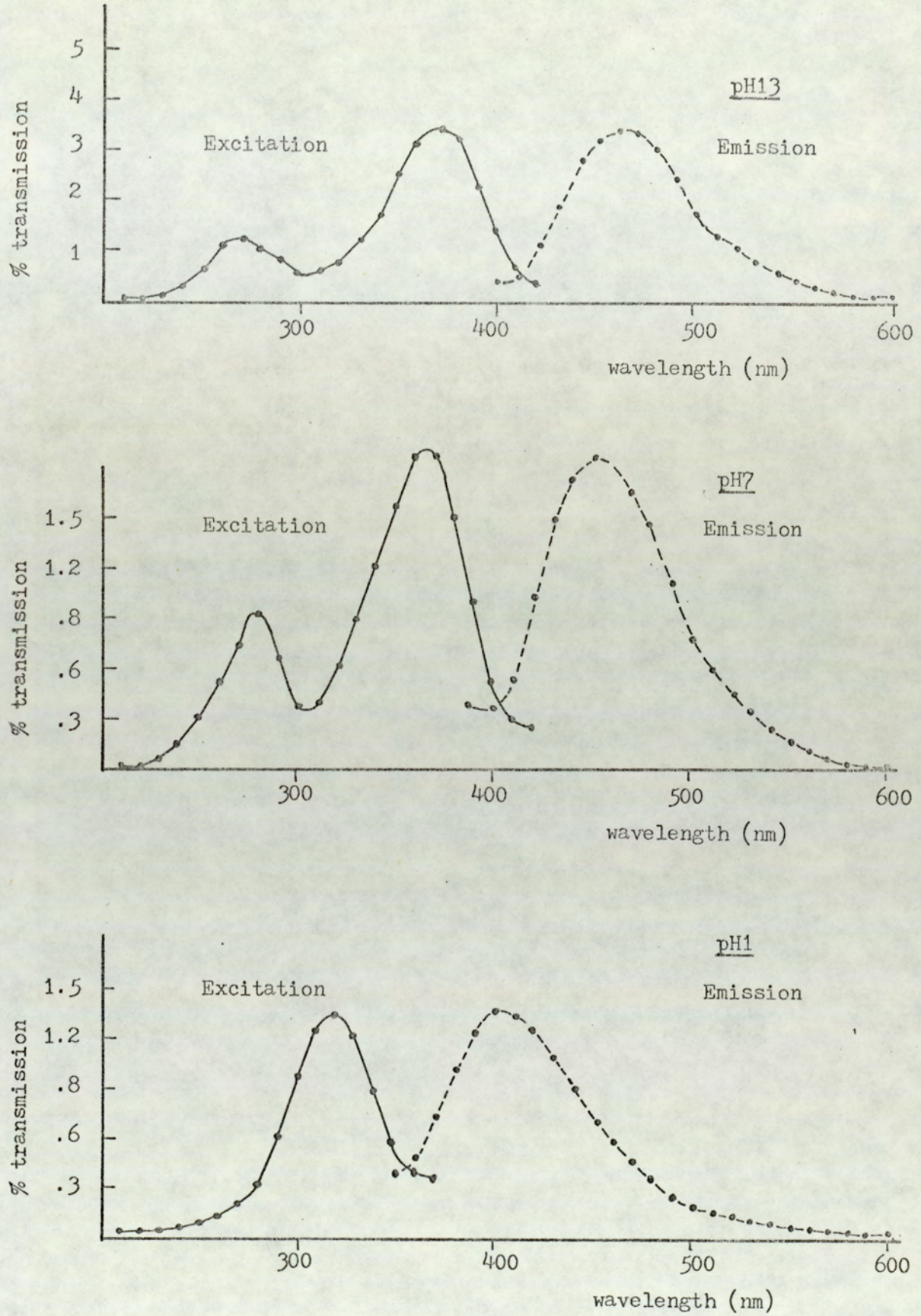


Figure 4.5 The emission and excitation fluorescence spectra of hepatic high molecular weight folate (liver extract BI) at pH1, pH7 and pH13.

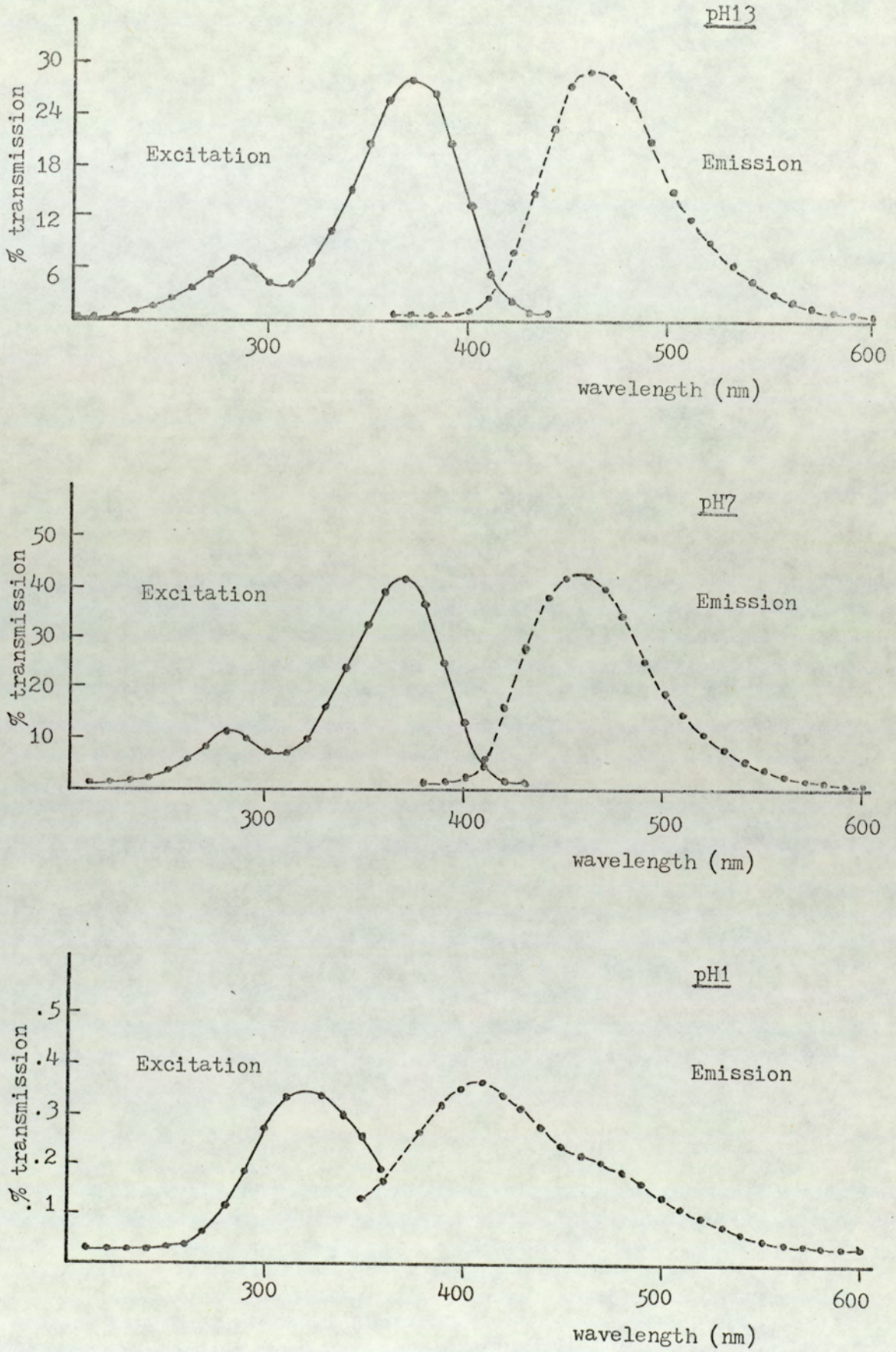
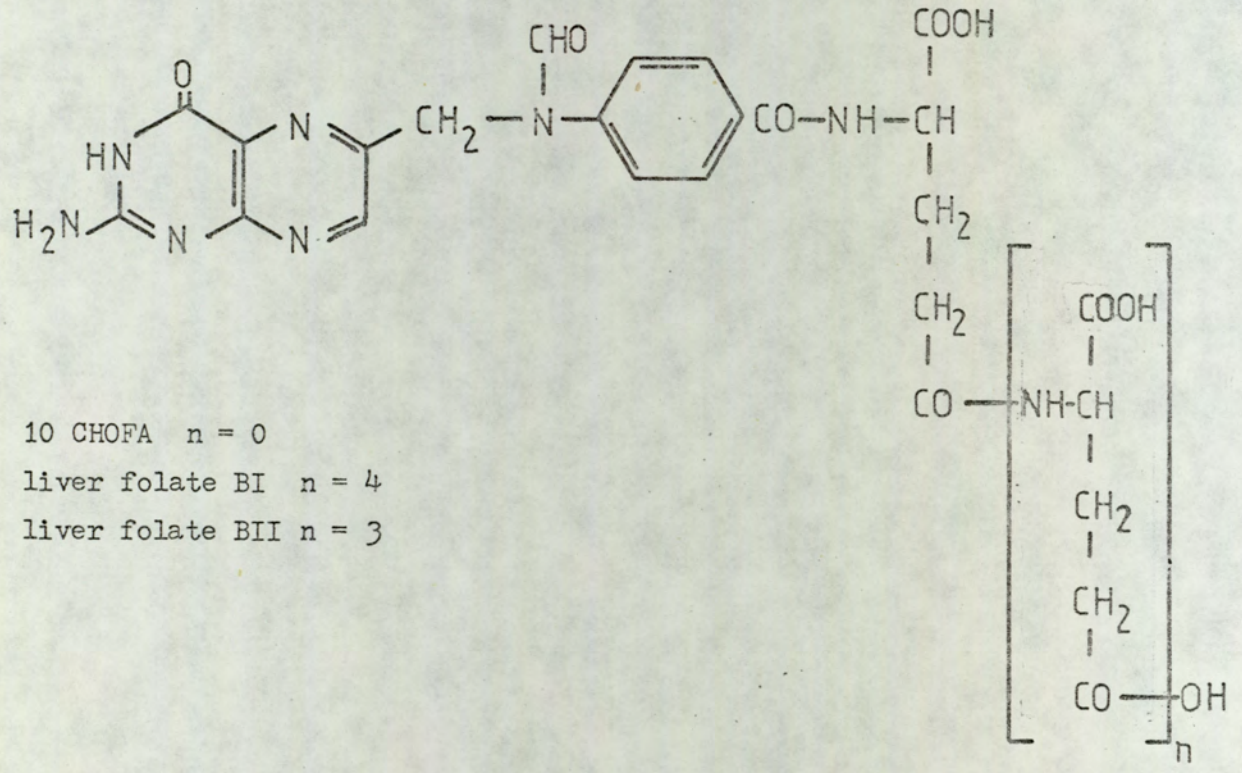


Figure 4.6 The emission and excitation fluorescence spectra of authentic 10 CHOFA at pH1, pH7 and pH13.



10 CHOFA n = 0

liver folate BI n = 4

liver folate BII n = 3

Figure 4.7 The structure of hepatic high molecular weight folate derivatives and of 10 CHOFA

Table 4.1 Recovery of radioactivity during the purification of Extract A.

Material	Volume	Counts (cpm x 5 x 10^{-2})	% ^{14}C Extract
Crude extract	130 ml	32016	100
G15 eluant (1) (9 columns)	290 ml	29163	91
DE52 eluant (2 columns)	95 ml	27688	87
DEAE-Sephadex eluant (2 columns)	15 ml	17850	56
G15 eluant (2) (1 column)	35 ml	12802	40

Table 4.2 Recovery of radioactivity and microbiological activity during the purification of Extract B

Stage of Purification	Recovery of radioactivity		Microbiological assay L. Casei (µg)		+ conjugase / - conjugase	Yield microbiological activity
	% ^{3}H	% ^{14}C	- conjugase	+ conjugase		
Crude extract	100	100	915	3,942	4	100
G15 eluant (1) (12 columns)	73	72	343	2,202	6	56
DE52 eluant* (2 columns)	70	70	29	1,179	41	30
DEAE-Sephadex eluant						
Peak (1) (B I)	58	59	19	533	28	14
Peak (2) (BII)	8.5	8.7	4.4	52	12	1.3
(Total)	(66.5)	(67.5)	(23.4)	(585)	(25)	(15.3)
G15 eluant						
B (I)	55.1	54.1	N.D.	641.5 (1.45 µM)		16.3
B (II)	7.4	6.6	N.D.	26.5 (.06 µM)		.67
(Total)	(62.5)	(60.7)		(668) (1.51 µM)		(17)

* Radioactivity resolved incompletely into 2 components, separable on DEAE-Sephadex;
N.D. = not detected

Table 4.3 Distribution of liver radioactivity between the crude extract and the non-extractable radioactivity

% Radioactivity	% ^{3}H	% ^{14}C
Extract	86.6	83.5
Precipitate 1	10.0	9.3
Precipitate 2	7.6	7.2
Total	104.2	100

Table 4.4 Total extracted and recovered microbiological activity and radioactivity during the purification of rat liver extracts.

Extract	A (500 g liver)	B (800 g liver)
Recovery of radioactivity (^{14}C)	40%	60.7%
Recovery of *L. casei* activity (total after conjugase)	(17%)	17%
% ^{14}C in extract as high molecular weight (H.M.W.) folate	91%	72%
Yield of labelled H.M.W.folate in extract (*L. casei* activity)	228.1 µg .52 µmoles	668 µg 1.51 µmoles
Total labelled H.M.W. folate in extract (*L. casei* activity)	1.18 µmoles i.e. 2.36 nmol g^{-1}	1.8 µmoles (B I = 1.60, BII = 0.20) i.e. 2.25 nmol g^{-1}
Total radioactivity in H.M.W. folate	0.45 µCi ^{14}C	B I 4.13 µCi ^{14}C 8.86 µCi ^{3}H BII 0.50 µCi ^{14}C 1.19 µCi ^{3}H
Total *L. casei* activity of liver extract	13.9 nmol g^{-1}	13.5 nmol g^{-1}

Table 4.5 Specific radioactivities of dosed folic acid and extracted high molecular weight folate

Extract	Derivative	Specific Radioactivity	Total Radioactivity
A	Folic acid dosed	57.8 mCimM ^{14}C	6 μCi (100%)
	High molecular weight folate	0.38 mCimM ^{14}C	0.45 μCi (7.5%)
B	Folic acid dosed	58.2 mCimM ^{14}C	48 μCi (100%)
		500 mCimM ^{3}H	65 μCi (100%)
BI	High molecular weight folate	2.58 mCimM ^{14}C	4.13 μCi (8.6%)
		5.54 mCimM ^{3}H	8.86 μCi (13.6%)
BII	High molecular weight folate	2.50 mCimM ^{14}C	0.50 μCi (1.04%)
		5.95 mCimM ^{3}H	1.19 μCi (1.8%)

Table 4.6 Differential microbiological activity* of the liver extracts and Pte glu_7 before and after conjugase treatment

Extract	Conjugase** treatment	L. Casei	S. Faecalis	P. Cerevisiae
A	-	4.2	N.D.	N.D.
	+	100	88	11.6
	+/-	23.8	∞	∞
BI	-	N.D.	N.D.	N.D.
	+	100	78.1	39.1
BII	-	N.D.	N.D.	N D.
	+	100	100	N.D.
Folic acid hexaglutamate Pte glu_7	-	8.5		
	+	100		
	+/-	11.8		

N.D. = not detected

* Values are expressed for each extract as the growth response relative to the L. casei value after conjugase treatment (100%)

** Chicken pancreas conjugase, for details see text.

Table 4.7 The products of action of chicken pancreas and rat intestine conjugases on pteroyl-heptaglutamate and folate conjugate (BI)

Elution Position Products (Kav)[1]

Additions to assay[2]	^{3}H labelled products i.e. derived from Folic acid $(glu)_6$			^{14}C labelled products i.e. derived from the liver folate conjugate		
Kav[1]	.06	.84	1.57	.18	.48	.78
Compound	Folic acid $(glu)_6$	Folic acid glu	Folic acid	Extract	10 CHOFA glu	10 CHOFA
Chicken pancreas conjugase	6%	64%	4%	13%	58%	4%
Rat intestinal conjugase	4%	6%	86%	14%	11%	65%
None	70%	17%	N.D.	80%	8%	N.D.

1 $Kav = \frac{Ve - Vo}{Vt - Vo}$

N.D. = not detected

2 Flasks 5, 6 and 7 as described in text

Table 4.8 The chromatographic properties of liver extract BI, the products of conjugase action on BI and of 10 CHOFA

System	Method of detection*	Liver extract BI	BI after conjugase	10 CHOFA
DEAE-cellulose ion exchange (molarity NaCl)	A	.55 M	.5M	.5M
Sephadex G15 gel filtration (Kav)	A	0.18	0.72	0.72
t.l.c. (Rf values)				
1) n-propanol/ ammonia/water (200/1/99)	A, B	.08	.31	.31
2) 3% aq.ammonium chloride	A, B	.96	.68	.66
3) 0.05 M-sodium phosphate buffer pH7	A,B	-	.71	.71
Thin layer gel filtration (t.l.g.) (Migration relative to dextran blue)	B, C	.94	.39	.39
Electrophoresis (Migration relative to folic aicd)	B, C	1.56	.72	.72

* refers to method of detection of BI

A by radioactivity measurement

B by fluorescence detection

C by bioautography

Table 4.9 The electrophoretic behaviour of liver extract BI and standard folate monoglutamates (0.1 M-phosphate buffer pH7)

	Paper			Cellulose acetate		
	^{+}d(mm)	$^{*}\mu x10^{4}$	μ/μ_{FA}	^{+}d(mm)	$^{*}\mu x10^{4}$	μ/μ_{FA}
a) t = 30 min., 20 V cm^{-1} (320 V per strip)						
BI	38	1.05	1.91	37	1.02	1.15
Folic acid	20	.55	1.00	32	.89	1.00
b) t = 60 min., 20 V cm^{-1}						
BI	50	.69	1.57			
Folic acid	32	.44	1.00			
5 MeTHF	36	.50	1.14			
10 CHOFA	37	.51	1.16			

$^{+}$ d = migration i.e. distance moved from origin towards the anode

* μ (mobility) = $\frac{v}{E}$ where v = cm sec^{-1}

E = volts cm^{-1}

Table 4.10 U.V. absorbance and fluorescence spectra of 10 CHOFA and the liver extract BI

a) U.V. absorbance spectra

Material	λmax (Abs) nm
10 CHOFA[1]	
pH1	254 (.64), 329 (.22)
pH7	247 (.60), 268 (.61), 352 (.17)
pH13	259 (.92), 372 (.19)
BI[2]	
pH1	255 (.92), 330 (s) (.36)
pH7	249 (.98), 268 (1.01), 350 (s) (.30)
pH13	261 (1.07), 365 (s) (.24)

b) Fluorescence spectra

	Emission			Excitation		
	Excitation wavelength	λmax	% trans	Emission Wavelength	λ max	% trans
10 CHOFA[3]						
pH1	320	408	.36	400	320	.35
pH7	370	458	42	450	370	42
pH13	370	463	29.4	450	370	28
Extract BI[2]						
pH1	320	405	1.35	400	320	1.35
pH7	370	455	3.66	450	370	3.80
pH13	370	465	7.20	450	370	7.0

1 Concentration 29.4 μML^{-1}

2 Concentration 12.6 μML^{-1} (microbiological assay)

3 Concentration 38.4 μML^{-1}

Table 4.11 Glutamic acid content of liver extract BI

	Extract BI	Molar ratio glutamate to folate	Paper purified extract BI	Molar ratio glutamate to folate
^{14}C cpm	8608		29,540	
Glutamic acid (nM)	26.5		67.8	
10 CHOFA Concentration				
(A) Fluorimetric (nM)	1.46	18.1	2.4	28.3
(B) U.V. absorbance (nM)	4.67	5.7	16.03	4.2
(C) Microbiological Assay (nM)	2.23	11.9	12.55	5.4

CHAPTER 5

STUDIES ON THE METABOLISM OF 10 FORMYLFOLIC ACID DERIVATIVES IN NORMAL AND WALKER 256 TUMOUR BEARING RATS

Introduction

Microbiological assay of food stuffs suggest that considerable quantities of food folate exist in conjugated forms, i.e. in the form of folate polyglutamate derivatives (Butterworth et al, 1963; Santini, et al, 1964). Since 10 CHOTHF readily undergoes oxidation in air even at room temperature (Robinson, 1971) any 10 CHOTHF conjugates in food are likely to be oxidised to 10 CHOFA derivatives during storage, preparation and cooking. Studies on the intestinal absorption of synthetic folate polyglutamate derivatives have shown that the folate moiety is readily transported but that this occurs only with the prior loss of the peptide side chain (Rosenberg and Godwin, 1971; Halsted et al, 1976). The level of free folate monoglutamate in the average diet is probably below the required minimal daily intake and thus food folate conjugate is necessarily a source of folate (Blakley, 1969). Although intestinal folate conjugase has an acid pH optimum below the luminal pH and hydrolysis of peptide forms would therefore be expected to be slow it has recently been proposed that the acid microclimate at the gut surface, shown by work in this laboratory to be a major factor in the intestinal absorption of folate monoglutamate (Blair and Matty, 1974), could also be the site of intestinal conjugase activity (Rowe, 1978).

The metabolism of 10 CHOFA derivatives despite their probable nutritional importance has a rather limited coverage in the literature and some controversy exists over their handling and metabolic fate. Several in vitro studies using purified or partially purified DHF reductases from various sources have been published in which the substrate suitability of 10 CHOFA has been assessed. Zakrewski (1960) using a partially purified chicken liver folic acid reductase at pH 5.0 found that 10 CHOFA was not reduced and that it inhibited folic acid reduction. A more detailed study on a 1,000 fold purified chicken

liver reductase (Mathews and Huennekens, 1963) indicated that 10 CHOFA was reduced at the same rate as folic acid at pH 7.5 but that at pH 4.0 although folic acid was reduced at ten times the rate at pH7 10 CHOFA was reduced at only three times the rate. In the same study 10 CHODHF was reduced at 6.5 times the rate of folic acid at pH 7.5 and 1.5 times the rate at pH 4.5. They concluded, surprisingly, that "compared to DHF or folic acid, 10 CHOFA is a poor substrate at either pH whereas 10 CHODHF is reduced slowly at both pH values".

In a study of a mammalian derived (Erlich ascites cells) DHF reductase (Bertino et al, 1965) and an assay at pH 7.5 no measurable substrate activity for either 10 CHOFA or 10 CHODHF was detected. In their system both 10 CHOFA and folic acid acted as competitive inhibitors of DHF (Km DHF = 1.3×10^{-6} M; Ki 10 CHOFA = 6.1×10^{-9} M; Ki folic acid = 1.1×10^{-7} M). The same group reported similar values in a later study (d'Urso-Scott et al, 1974) giving 10 CHOFA an I_{50} = 80 nM. This value is comparable with that of Friedkin et al (1975) for a mouse leukaemia reductase (I_{50} = 100 nM) and they also reported that under similar conditions 10 CHOFA$(glu)_4$ was found to be eight times as inhibitory.

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

Given the substrate properties of 10 CHOFA for DHF reductase it would not be expected to be metabolised into the folate pool unless deformylation could occur. d'Urso-Scott et al (1974) found that

10 CHOFA accumulated in liver slices on incubation with [2-^{14}C] folic acid and since this was not inhibited by methotrexate suggested a specific folic acid formylation system, although no direct evidence was presented. However since to achieve complete dihydrofolate reductase inhibition requires massive doses of methotrexate (Goldman, 1977) their conclusion may well be largely speculative. Whether the proposed deformylation could work in the reverse direction was not discussed.

Detailed *in vivo* studies of 10 CHOFA metabolism are lacking. Beavon and Blair (1975) found that [2-^{14}C] 10 CHOFA was poorly metabolised by the rat in keeping with its dihydrofolate reductase inhibition properties, but in tumour inhibition trials against the R_1 lymphoma in mice no antitumour effects were found although up to a 73.5% increase in survival time was found using methotrexate (Beavon, 1973). Other studies performed in man suggested that 10 CHOFA was slowly metabolised to other folate derivatives (Ratanasthien, 1975).

In the present Chapter results of a detailed study of 10 CHOFA metabolism in the normal and the Walker 256 tumour bearing rat, and the first *in vivo* study of the metabolism of a 10 CHOFA polyglutamate (10 CHOFA$(glu)_4$) is reported.

5.1 The Metabolism and Handling of a Mixture of [2-^{14}C] 10 CHOFA $(glu)_4$ and [3', 5', 9(n) - ^{3}H] 10 CHOFA$(glu)_4$ in the Normal Rat

The isolation of radiolabelled 10 CHOFA$(glu)_4$ from rat liver (Chapter 4) provided the opportunity to investigate the metabolism of a major dietary folate in the intact animal and facilitated comparison with the more readily available folate monoglutamate species normally used in studies of folate metabolism.

General

10 CHOFA$(glu)_4$ (obtained by the column purification of rat liver as described in Chapter 4) was dosed orally to three male Wistar rats

(250 g) at a dose of 280 nM Kg^{-1} (0.18 μCi ^{14}C and 0.39 μCi ^{3}H per rat) equivalent to 126 μg folic acid Kg^{-1} estimated microbiologically. The rats were housed in metabolism cages set up to collect respired CO_2, using traps containing 15 ml of Fisons absorber P. Urine samples were collected over 0-6 h, 6-24 h and 24-48 h periods into 0.05 M-sodium phosphate buffer containing sodium ascorbate (2%w/v) and dithiothreitol (DTT) (5 mg 100 ml^{-1}) and urines from each rat analysed individually by chromatography. Urea in urine samples was assayed as described below. After 48 h the rats were killed and organs removed for the measurement of retained radioactivity. The livers of two rats were removed, washed in ice-cold isotonic saline, divided into two portions and extracted by the following methods:

a) Hot ascorbate : The liver was chopped and dropped into 4 vol. of boiling 0.05 M-sodium phosphate buffer pH7 containing sodium ascorbate (2% w/v) and DTT (5 mg 100 ml^{-1}). After 10 min this was cooled, homogenised and centrifuged. The supernatant was recovered and analysed.

b) Cold extraction : The liver was chopped and homogenised in ice-cold 0.05 M-sodium phosphate buffer pH7 containing sodium ascorbate (2%w/v) and DTT (5 mg 100 ml^{-1}). The homogenate was centrifuged (bench centrifuge) and the supernatant recovered and analysed.

Urea Assay

During chromatographic analysis of urines a ^{14}C only labelled species was found eluting in the void volume on DEAE-cellulose anion exchange and at about fraction 18 on Sephadex-G15, on both occasions cochromatographing with endogenous urea. Since urea was a possible candidate for the ^{14}C species the peak was incubated with urease and the CO_2 produced assayed for radioactivity using the following method. The metabolite containing peak (10 ml) was incubated with urease (5 ml of a solution of Jack Bean urease capable of hydrolysing 67 mg of urea

h^{-1}) for 1 h at 37°C during which a continual stream of air was passed through (using a water vacuum pump) and effluent air bubbled through a CO_2 absorber (15 ml Fisons absorber P). A similar incubation was performed but using bovine serum albumin (50 mg) instead of urease. The reaction was terminated in both cases by the addition of 1 ml 1 M-H_2SO_4. The recovered $^{14}CO_2$ was found by counting the absorber "P".

Results and Discussion

The distribution of radioactivity in the tissues, faeces, urine and expired air over 48 h is given in table 5.1. The ratio of ^{3}H to ^{14}C in the tissues was similar to that of the dosed compound but this was not the case for the urine and faeces where an excess of ^{3}H was present. The finding of labelled carbon dioxide in the expired air although small (6% dose) was unexpected and indicated that considerable degradation of the pteridine ring had occurred.

The liver, kidneys and intestine contained the bulk of the retained radioactivity. The low levels of radioactivity prevented qualitative examination of the latter two tissues but G15 chromatography of hot liver extracts showed that high molecular weight folate, i.e. folate polyglutamate formed the bulk of the retained radioactivity (70%). This behaved on ion exchange cellulose (DE52) analogously to that of the dosed compound eluting just before 5 MeTHF and is probably a formylfolate tetraglutamate. The cold extraction method gave a protein bound peak (35% ^{14}C, 25% ^{3}H) and several breakdown products possibly including pABglu and tritiated water (34% ^{3}H) and pterin (35% ^{14}C). Confirmation of the identity of the latter derivatives by further chromatography was inconclusive due to the low levels of radioactivity.

The excess of tritium over carbon-14 found in the urine demonstrated that degradation of the dosed compound was occurring in vivo. DE52 ion-exchange and Sephadex-G15 column chromatography of 0-6 h and 6-24 h urine samples (the amount of radioactivity in the 24-48 h urine

was too low for meaningful chromatographic analysis), is illustrated in figure 5.1 and figure 5.2. Several intact folate derivatives including 10 CHOTHF and 5 MeTHF as well as 10 CHOFA were present indicating that the polyglutamate derivative dosed had been metabolised into the folate pool. The large amount of 10 CHOFA found in the 0-6 h sample suggests that this was derived from the deconjugation of the 10 CHOFA$(glu)_4$ rather than by oxidation of 10 CHOTHF during collection or in vivo, although a small amount of it may be derived from the latter source.

Several singly labelled derivatives were also present, this being surprising since previous studies in this laboratory had suggested that the excess ^3H found in urine samples after administration of dual-labelled folates was due to ^3H exchange in vivo as the only ^{14}C metabolites identified were intact folate derivatives (Barford and Blair, 1975). However initial studies reported in Chapter 3 of this thesis indicated that a pteridine derivative derived from [2-^{14}C] folic acid was present in the urine and an independent study using [3', 5', 9(n) - ^3H] folic acid reported during the present investigation verified the existence of urinary folate catabolites (Murphy et al, 1976). In the present study several solely tritium labelled metabolites were found in the urine including p-acetamidobenzoate and ^3H_2O, neither of which have been reported previously, and small amounts of a derivative which cochromatographed with p-acetamidobenzoyl-L-glutamate, claimed by Murphy et al (1976) as a long term folate catabolite.

A pteridine derivative which had lost most of its tritium was also found in urine samples after 10 CHOFA$(glu)_4$ indicating that it was a pterin derivative which had lost the p-aminobenzoyl-L-glutamate side chain during metabolism. The characterisation and identification of p-acetamidobenzoate, ^3H_2O and the pterin derivative are dealt with in Chapter 6 of this thesis.

The urinary tritiated catbolites identified and the unidentified pterin can all be accounted for by simple C9-N10 bond cleavage *in vivo* (Blair, 1957) with acetylation and transpeptidation at some stage. However small amounts of labelled CO_2 were found in the expired air and on incubation with urease $^{14}CO_2$ was liberated from urine suggesting that the early eluting urinary derivative was urea. Neither of these compounds had been recorded in previous studies, although subsequently both have been recovered from rats dosed with 10 CHOFA or folic acid (Pheasant, Connor and Blair, unpublished observations), and an explanation of their occurrence is much more problematical.

5.2 The Metabolism and Handling of a Mixture of [2-^{14}C] 10 CHOFA and [3', 5', 9(n) - ^{3}H] 10 CHOFA in the Rat at Three Different Doses

Despite its widespread occurrence and probable nutritional significance only a sparse literature on 10 CHOFA metabolism exists. 10 CHOFA (5 mg) given orally to human volunteers is readily absorbed and peak serum levels (detected microbiologically) were found between 1-1½ h after dosing (Ratanasthien, 1975). Bio-autography revealed that the microbiological activity was entirely due to unmetabolised 10 CHOFA, indicating little or no intestinal metabolism occurred at this dose. Analysis of urine in the same study revealed that 5% of the dose was excreted as 10 CHOFA, 1% as 5 MeTHF and 0.2% as 10 CHOTHF within 3 h indicating that 10 CHOFA was slowly entering the body folate pool. In contrast a study utilizing [2-^{14}C] 10 CHOFA suggested that 10 CHOFA did not contribute to the folate pool in the rat or at least did so extremely slowly (Beavon, 1973). The latter study employed a single radio-label and since it relied on t.l.c. for metabolite identification extensive purification and treatment of urine prior to chromatography was required, a process which could conceivably have resulted in the loss or degradation of some metabolites. The collection of urine into acid (1 ml c.HCl) may have also facilitated chemical degradation.

Since 10 CHOFA$(glu)_4$ was shown to contribute to the folate pool in the rat (section 5.1), giving a variety of intact folates and degradation products in urine a more detailed investigation of 10 CHOFA metabolism using a dual label to facilitate indentification of metabolites was undertaken.

Materials and Methods

Three groups of normal male Wistar rats (200 g) were dosed orally with a mixture of [2-^{14}C] 10 CHOFA and [3', 5', 9(n) - ^{3}H] 10 CHOFA (synthesised as described in Chapter 2) at 10.5, 52.4 or 104.7 μg kg^{-1} body weight. After dosing they were housed as described in section 5.1 with the expired air being passed in some cases sequentially through a water trap (U-tubes immersed in a methanol/dry ice bath) and CO_2 traps (containing 15 ml of 5M-KOH).

After 48 h the animals were killed and the tissues removed for the estimation of retained radioactivity. Freshly excised livers were washed in ice-cold 0.15 M-NaCl and extracted by one of the following methods:

a) Hot ascorbate extraction (see method (a) in section 5.1).

b) Liver was chopped and homogenised in 30 ml 0.25 M-sucrose in 0.05 M-sodium phosphate buffer, pH7. Protein was precipitated out by the addition of 10 ml TCA (10% w/v). After centrifuging the supernatant was adjusted to pH7 and retained for analysis.

c) Liver was chopped and homogenised in 4 vol. 0.05 M-sodium phosphate buffer pH7 containing sodium ascorbate (2% w/v). In some cases 10 ml of TCA (10% w/v) was added to precipitate protein. The homogenates were centrifuged and the supernatant retained for analysis.

d) Liver was chopped and homogenised in 0.05 M-sodium phosphate buffer pH7 containing sodium ascorbate (2% w/v) and incubated in a shaking water bath at 37°C to allow autolysis to proceed. After 1 hour the reaction was either terminated by the addition of 10 ml TCA (10%

w/v) or after centrifuging to remove insoluble material the supernatant was recovered and frozen for analysis.

e) Liver was homogenised and subjected to subcellular fractionation by differential centrifugation according to the method of Cook and Blair (1979). The high speed supernatant (i.e. cytoplasmic fraction) was harvested and examined chromatographically.

f) Liver was chopped and homogenised in 3 vols of 10% TCA. After centrifuging the supernatant was harvested and stored frozen for analysis. The pH of the supernatant was about pH4.2.

Results

Quantitative Distribution of Radioactivity

Table 5.3 shows the distribution of radioactivities retained in the tissues 48 h after each dose of 10 CHOFA. The bulk of the retained radioactivity was in the liver, gut and kidneys with much smaller amounts in the other tissues examined. The ratio of isotopes in each tissue was similar to that of the dosed compound in all cases except the liver from rats dosed at 10.5 $\mu g\ kg^{-1}$ where more ^{3}H (10.82%) than ^{14}C (9.55%) was found ($p < 0.02$). Overall tissue retention decreased with increasing dose (14.93% ^{14}C & 16.20% ^{3}H at 10.5 $\mu g\ kg^{-1}$ to 9.41% ^{14}C & 9.63% ^{3}H at 104.7 $\mu g\ kg^{-1}$) and a reciprocal trend was evident in the urinary recoveries (table 5.4). The decrease in tissue retention with increasing dose was most marked in the liver and intestine (figure 5.3) with significantly more of the ^{14}C ($p < 0.05$ and $p < 0.01$ respectively) and ^{3}H ($p < 0.02$ and $p < 0.05$ respectively) being retained at the low dose compared to the high dose.

There was a marked imbalance in the ratio of ^{14}C to ^{3}H found in the urine and faeces compared to the dose over 48 h. The comparative excess of ^{14}C over ^{3}H in the faeces was highly significant at each dose as was the reciprocal excess of ^{3}H over ^{14}C in the urine. Faecal ^{14}C was reasonably constant at each dose (37.56%, 41.87% and 36.94% at

the low, middle and high dose respectively) and no statistically significant differences were found. Faecal ^{3}H showed a slight tendency to increase with dose and faecal ^{3}H at the high dose (29.35%) was significantly higher than at the low dose (23.93%) by a Student's "t" test ($0.02 < p < 0.05$).

The relative distribution of ^{14}C and ^{3}H in urine samples changed with time. At all doses significantly more ^{3}H than ^{14}C was present in the 0-6 h and 6-24 h urine ($p < 0.001$ in all cases) but the ^{3}H excess over ^{14}C in the 24-48 h samples was statistically significant only at the middle and high dose ($p < 0.05$ and $p < 0.001$ respectively by a paired "t" test). At the low dose although more ^{3}H than ^{14}C was found (4.3% ^{3}H and 3.70% ^{14}C) this was not significant (by a paired "t" test).

More radioactivity was excreted in the 0-6 h urine as the dose increased and highly significant differences ($p < 0.001$) between urinary recovery of radioactivity at the three doses were found (table 5.4 and table 5.5). Similar percentages of the dosed radioactivity were found in the 6-24 h urine samples at each dose and no statistically significant differences were found although a slight decrease in urinary ^{3}H with increasing dose was apparent (table 5.4). The % ^{14}C and % ^{3}H of the 24-48 h urines decreased as the dose increased, a significantly higher % ^{14}C being excreted at the low dose compared to the middle and high doses ($p < 0.05$ in both cases) and a higher % ^{3}H at the low dose compared to the high dose ($p < 0.05$).

Total recoveries of radioactivity in the tissues, faeces, urine and expired air are given in table 5.6. Both $^{14}CO_2$ and $^{3}H_2O$ (the latter quantified only at the middle dose) were found in the expired air and there was a trend towards decreased $^{14}CO_2$ production as the dose increased. The total overall recoveries of radioactivity were between 70-80%. Since the tissues examined accounted for only 10% of

the body weight the missing radioactivity (20-30%) can be readily accounted for by dilution over the whole body.

Qualitative Analysis of Radioactivity

a) Urinary Radioactivity

DEAE-cellulose chromatography resolved urine samples into several components (see figures 5.4; 5.5; 5.6) and on rechromatography of the peaks on Sephadex G15 further resolution of radioactivity into at least nine derivatives was obtained. Several intact folates including 10 CHOFA, 10 CHOTHF, 5 MeTHF and an unidentified folate(S), and five cleavage products $^{3}H_2O$, p-acetamidobenzoic acid, p-acetamidobenzoyl-L-glutamate, the unidentified reduced pterin (P) and small amounts of a metabolite with the properties of urea were present. Typical examples of DEAE-cellulose chromatography of the 0-6 h, 6-24 h and 24-48 h urine are illustrated in figures 5.4; 5.5; 5.6 respectively (middle dose). Although intact folates (10 CHOFA, 10 CHOTHF and 5 MeTHF) dominate the 0-6 h urines relatively more of the cleavage products are found in the later urines. The distributions of radioactivity amongst the metabolites with time for each dose are given in tables 5.7; 5.8; and 5.9 and the quantities (% dose) of metabolites excreted at each dose are given in table 5.10 and illustrated in figure 5.7. Considerable amounts of unmetabolized 10 CHOFA were found at the high dose level and this fell off dramatically at the middle and low dose levels. Since at the high dose level 10 CHOFA was excreted in the 0-6 h, 6-24 h and 24-48 h urines in steadily decreasing amounts some of the 10 CHOFA apparently persisted unmetabolized within the animal. Faecal radioactivity was relatively constant suggesting that absorption was similar at each dose, although the trend towards raised faecal tritium at the higher dose may suggest that a small amount of 10 CHOFA remained unabsorbed.

Surprisingly it was found that a considerable proportion of the

excess tritium in the urine was not associated with folate catabolites but with intact folates. Since the excess of ^{3}H over ^{14}C was evident in all the urinary folates including 10 CHOFA this is unlikely to be the result of a simple tritium exchange process and is suggestive of an isotopic effect at some stage of handling and metabolism *in vivo*. It may also be deduced that some of the excess ^{14}C over ^{3}H in the faeces is due to larger amounts of ^{14}C labelled intact folates compared to ^{3}H labelled folates being lost by this route. A consequence of this observation is that the extent of cleavage of folate *in vivo* cannot be deduced from simple quantitative analysis of urinary recoveries following the administration of [$2^{14}C$] and [3', 5', 9(n) - ^{3}H] labelled folate.

b) Hepatic Radioactivity

The results obtained for each method of extraction are described below. Extracts were analysed by Sephadex G15 gel filtration followed by further analysis on ion-exchange columns where required. The chromatographic profiles varied extensively with the extraction procedures used. No significant qualitative or quantitative differences were found between livers extracted under similar conditions at the 3 dose levels indicating that in each case 10 CHOFA was handled and metabolised by the liver in a similar manner.

Method (a) : The bulk of the radioactivity extracted by "hot ascorbate" was dual-labelled, eluted close to the void volume on Sephadex G15 (table 5.11) and also had the same elution characteristics on DEAE-cellulose as 10 CHOFA$(glu)_4$. It was therefore assigned the latter structure. The residual acitivity included 10 CHOFA and singly labelled components including pterin, tritiated water and a species related to p-aminobenzoyl-L-glutamate. The occurrence of the latter 3 compounds in the extracts is indicative of the existence of a labile derivative in liver which has undergone decomposition during extraction.

A slight excess of ^{3}H over ^{14}C in the 10 CHOFA(glu)$_4$ peak may be due to the isotope effect referred to in the urine analysis. It was found that 84% ^{14}C and 84% ^{3}H were extracted from liver by this method.

Method (b) : Gel filtration of sucrose extracts after TCA precipitation to remove protein gave a major peak close to the void volume (folate polyglutamate derivatives) and variable amounts of 10 CHOFA, 10 CHOTHF (and 5 CHOTHF ?), pterin, $^{3}H_2O$ and p-aminobenzoyl-L-glutamate (table 5.11). When the folate polyglutamate peak was incubated with a rat intestinal conjugase preparation a variety of products were found including folate polyglutamate derivatives (16% ^{14}C; 24% ^{3}H), 10 CHOTHF (7% ^{14}C; 12% ^{3}H), 10 CHOFA (7% ^{14}C; 16% ^{3}H), 5 CHOTHF (24% ^{14}C; 19% ^{3}H) and decomposition fragments (41% ^{14}C; 21% ^{3}H). Quantitative determination of radioactivity showed that 66% ^{14}C and 69% ^{3}H were extracted from liver by this method.

Method (c) : Gel filtration of extracts prepared by this method gave similar elution profiles to those of extracts obtained by method (b). The distribution of radioactivity (table 5.12) amongst the folate polyglutamate, formylfolate and degradation products (pterin, p-aminobenzoyl-L-glutamate and in one sample p-aminobenzoic acid) was more variable and a lower yield of high molecular weight folate was obtained. When the extract was treated with TCA following homogenisation a higher yield of folate polyglutamate was obtained (46% ^{14}C with TCA; 17% ^{14}C without TCA). Using method (c) 68% ^{14}C and 74% ^{3}H were extracted from liver.

Method (d) : Gel filtration of extracts prepared by this method revealed that signficant hydrolysis of folate polyglutamates occurred during autolysis (table 5.13). The major products of autolysis were 10 CHOTHF, 10 CHOFA and 5 CHOTHF although considerable variations in the relative distributions were found. Termination of autolysis by the addition of TCA to remove protein resulted in the appearance of

considerably larger amounts of degradation products compared to termination by freezing, suggesting that the autolysis products are stablized by the presence of protein. Using autolysis 78% ^{14}C and 77% ^{3}H were extracted from liver.

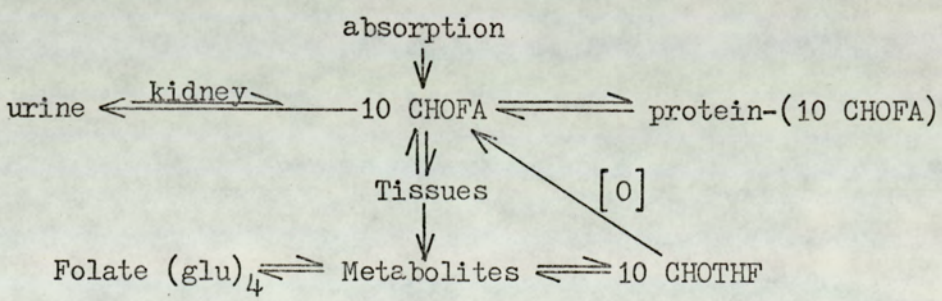
Method (e) : The subcellular distribution of radioactivity is given in table 5.14. The bulk of the radioactivity was divided between the high speed supernatant (cytoplasm) and the mitochondrial (I) fractions. Chromatography of the supernatant (after precipitation of the protein with TCA) gave a folate polyglutamate peak (9% ^{14}C; 10% ^{3}H), 10 CHOFA (46% ^{14}C; 63% ^{3}H) and pterin (36% ^{14}C; 20% ^{3}H). The major folate species found was 10 CHOFA. Since ascorbate interferes with the assay of marker enzymes (e.g. protein assays) it is necessarily omitted from the subcellular fractionation procedure and this may explain the finding of 10 CHOFA rather than 10 CHOTHF.

Method (f) : Gel filtration of the products obtained when liver was homogenised and stored in TCA gave a mixture of overlapping peaks comprising largely degradation fragments including small amounts of xanthopterin and a compound which may be dihydropterin (discussed at the end of the Chapter) and larger amounts of pterin (60% ^{14}C) and $^{3}H_2O$ (20% ^{3}H). A small amount of folate polyglutamate (7% ^{14}C; 11% ^{3}H) and possibly 10 CHOFA (4% ^{14}C; 13% ^{3}H) were found indicating that extensive cleavage of both the C9-N10 bond and the gamma-glutamyl side chain had occurred. Quantities (about 30% ^{3}H) of a tritiated low molecular weight fragment were also present (p-aminobenzoyl-L-glutamate derivative).

Discussion

At the three doses studied administration of 10 CHOFA gave rise to a variety of metabolites in the urine including intact folates and folate catabolites. Since these derivatives are similar to those derived from folic acid (Pheasant, Connor and Blair, unpublished observations) 10 CHOFA does enter the folate pool in the rat.

The metabolites excreted were dose dependent, this being most marked with 10 CHOFA and the higher urinary recoveries of radioactivity at the higher doses is largely due to increased amounts of 10 CHOFA in the urine. Since 10 CHOFA apparently persisted even into the 24-48 h urines (at the high dose) the factors controlling its excretion are evidently complex. Similar faecal radioactivities found at each dose indicate that absorption of the dose across the intestine was unsaturated and since 10 CHOFA absorption is probably rapid the higher urinary recoveries in initial urine samples represents excretion of free plasma 10 CHOFA above the renal threshold. Since 10 CHOFA was found in 6-24 h and 24-48 h samples but not at the low dose, some form of "storage" of unmetabolised 10 CHOFA occurs. This may be due to protein-binding in the plasma (and possibly the tissues). The interaction of the various processes is shown below.



Serum is known to contain considerable quantities of folate binding proteinsand in some studies these have been shown to have a high affinity for oxidized folate derivatives (Waxman and Schreiber, 1973; Fischer et al, 1975), although in the latter case information on 10 CHOFA binding is poorly covered in the literature. Using the model above low doses of 10 CHOFA will be almost fully bound in the plasma and tissues and the amount of free 10 CHOFA passing through the kidney will be small. Metabolism in the tissues to other folates will effectively reduce the free plasma [10 CHOFA] and 10 CHOFA (in equilibrium with the protein bound form) will be released and become available to the tissues. Thus after an initial period (a few hours) during which the plasma [10 CHOFA] is above the renal threshold the equilibrium between protein binding and tissue metabolism will ensure no further loss of 10 CHOFA into the urine. The folate binding proteins thus fulfill the role of a temporary store. At the higher doses the system will still operate although relatively more 10 CHOFA will be lost into the urine initially. Since the protein bound 10 CHOFA is in equilibrium with the free 10 CHOFA and assuming that the process of tissue metabolism is at a maximum (this is quite likely in view of the low rate of reduction expected) 10 CHOFA will continue to be lost into the urine until the free [10 CHOFA] released from the bound "store" falls below the level at which the tissue metabolism is no longer saturated and the renal threshold no longer exceeded.

The model proposed explains the tissue dependent uptake and the similar metabolites found in the tissues and urine. Although relatively more of the dose was retained in the liver at the low dose compared to the high dose the difference is less than a factor of 2 despite a ten-fold difference in dose. Urinary folates (figure 5.7) show similar trends. If the determining factor in maintaining 10 CHOFA within the body was simply the renal threshold we would have expected much more

marked differences in tissue retention and urinary folates between the extreme doses. Larger amounts of 10 CHOFA would have been found in the urine at the middle and high doses than found since the occurrence of 10 CHOFA in the urine even at the low dose suggests that the renal threshold and tissue uptake were both exceeded at a dose of 10 $\mu g\ kg^{-1}$.

Since relatively more of the dosed radioactivity was found in the 24-48 h urine at the low dose compared to the middle and high doses, although the 6-24 h urines contained similar amounts of the dose in all three cases, it is tentatively suggested that a proportion of the 24-48 h urinary metabolites is derived from metabolites produced in the tissues from 10 CHOFA taken up in the intial stages. The model proposed predicts that at the low dose relatively more of the radioactivity would enter the tissues initially and be metabolised. A proportion of this will be retained for use by the tissue and the surplus excreted. Since the 24-48 h urine is dominated by catabolites it is suggested that these are derived from the retained tissue folate, and that the observed dose dependent occurrence of catabolites in the urine is due to the relatively slower entry of 10 CHOFA into the tissues at the higher doses. The mechanism and production of the individual catabolites will be discussed in more detail later.

By various methods of extraction the retained derivatives were shown to be largely folate polyglutamates. Hot ascorbate extraction, which gave the most efficient recovery of tissue radioactivity, gave the same derivative as after folic acid administration i.e. 10 CHOFA $(glu)_4$. The milder "cold" extraction methods gave less reproducibility and more variable amounts of unconjugated folates and degradation products, probably due to slower inactivation of endogenous folate metabolising enzymes. Conjugase incubation of the polyglutamate peak obtained by sucrose extraction gave several folates including 10 CHOFA, 10 CHOTHF and 5 CHOTHF as well as degradation products,

although 10 CHOFA was the product derived from 10 $CHO(glu)_4$ (Chapter 4). This suggests that sucrose extraction gave 10 $CHOTHF(glu)_4$, this more labile derivative giving a less clean product with conjugase. Autolysis of liver produced 10 CHOTHF again supporting 10 $CHOTHF(glu)_4$ as the initial derivative. The lability of the liver folate was confirmed in the TCA extracts where largely degradation products were produced, although 10 CHOFA is relatively stable under these conditions. TCA extraction will be discussed in more detail in the next section, (5.3).

The results from the present study using a mixture of [2-^{14}C] 10 CHOFA and [3', 5', 9(n) - ^{3}H] 10 CHOFA suggest that an isotope effect may be occurring during handling and metabolism leading to a ^{3}H excess over ^{14}C in the urinary folates and a reciprocal excess in the faecal derivatives. The cause of this process is unknown but has also been found in studies using mixtures of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] folic acid (Pheasant, Connor and Blair, unpublished observations). The most attractive explanation would be a differential rate of transport across the intestine although current opinion suggests that folate transport relies on passive diffusion through an acid microclimate at the gut surface (Blair and Matty, 1974) and it is difficult to envisage how the incorporation of ^{14}C at the C2 position would affect this. Leslie and Rowe (1972) have identified a binding protein (although specific for folic acid) on the brush boder of the intestine which binds almost irreversibly and it may be possible that if binding occurs in the benzene ring region of the molecule the ^{3}H atoms in the 3', 5' positions may reduce this with the effect of enabling [3', 5', 9(n) - ^{3}H] 10 CHOFA to enter the intestine whilst the [2-^{14}C] 10 CHOFA is slightly retarded and more of it lost in the faeces. Different chromatographic properties may be observed between [3', 5', 9(n) - ^{3}H] folic acid and [2-^{14}C] folic acid, the two being slightly resolved on DEAE-cellulose

and Sephadex G15 chromatography (M.J. Connor, A.E. Pheasant and J.A. Blair, unpublished observations). The slight difference in molecular weight cannot account for this anomaly and may indicate a more serious chemical difference between the two forms, a real possibility since they are synthesised by distinct chemical routes.

5.3 The Metabolism and Handling of a Mixture of [2-^{14}C] 10 CHOFA and [3', 5', 9(n) - ^{3}H] 10 CHOFA in the Rat Bearing Implanted Walker 256 Tumours

In view of the many published reports of differences in the handling of folate in rapidly dividing compared to slow growing tissues and the reported *in vitro* properties of 10 CHOFA described earlier a study of the effect of an implanted rapidly dividing tumour on the whole animal metabolism of 10 CHOFA seemed particularly appropriate. The tumour of choice was the Walker 256 carco-sarcinoma, this tumour being readily available, undergoing a steady and uniform growth on implantation, has been the subject of several studies on *in vivo* folate metabolism in our laboratory previously (Chapter 2 of this thesis; Beavon, 1975; Barford and Blair, 1978) and the levels of folate metabolising enzymes are fairly well documented (Lepage *et al*, 1972; Poirier, 1973).

Materials and Methods

Five male Wistar rats (200-250 g) bearing implanted Walker 256 carco-sarcinoma were dosed orally with a mixture [2-^{14}C] 10 CHOFA and [3', 5', 9(n) - ^{3}H] 10 CHOFA (52.4 μg kg^{-1} body weight) and treated as described in the previous section. Tumour tissue (2 x 10^{6} cells) had been subcutaneously implanted into the flank of the rats 7 days prior to dosing. After 48 h the animals were killed and the livers and tumours rapidly excised, washed in ice-cold saline and extracted by methods (a) (hot ascorbate), (d) (autolysis), (e) (subcellular fractionation) and (f) (10% TCA) described in the previous section.

Cursory examination of the excised tumour tissue revealed no evidence of necrosis. Four of the tumours were of similar weight (about 9.5 g) with the fifth being much smaller (2.4 g). Tumour growth was restricted to the subcutaneous lump at the site of implantation and there was no apparent incursion into the body cavity although it had to be cut from the skin with a scalpel blade.

Results

The distribution of radioactivity in the tissues of tumour bearing rats and the appropriate normal controls are given in table 5.15. Significantly more radioactivity was retained in the livers of tumour bearing rats (host livers) compared to the controls ($p < 0.05$) and a similar trend was found in the kidneys and intestine (on a weight for weight basis). The tumours (8.19 g) were of similar size to the livers (7.72 g host livers; 8.3 g control livers) but contained only one third of the radioactivity of host livers. Host livers contained relatively more ^{3}H than ^{14}C compared to the dose although this was not statistically significant ($0.1 > p > 0.05$ by a paired "t" test).

The total urinary radioactivity in both groups of rats over 48 h (table 5.16) and over the 0-6 h, 6-24 h and 24-48 h periods was very similar. Faecal radioactivity was lower in the tumour bearing rats (32.6% ^{14}C; 20.7% ^{3}H) compared to controls (41.9% ^{14}C; 27.3% ^{3}H) but this was not statistically significant ($0.1 > p > 0.05$). The radioactivity of the tumour (4.7% ^{14}C; 5.2% ^{3}H) and the increase in radioactivity of host livers compared to controls (4.8% ^{14}C; 7.5% ^{3}H) were sufficient to account for the lower faecal radioactivities of the tumour bearing rats.

Qualitative Analysis of Urine

DEAE-cellulose chromatography of urine gave chromatograms similar to those portrayed in figures 5.4, 5.5 and 5.6. However when the peak

containing 5 MeTHF in the 0-6 h and 6-24 h sample was rechromatographed on Sephadex G15 a novel metabolite (metabolite W) was found, absent from the 5 MeTHF peak of control urines. Sephadex G15 chromatography of the 5 MeTHF peak obtained by DEAE-cellulose chromatography of 0-6 h and 6-24 h urines from tumour bearing and control rats are illustrated in figures 5.8 and 5.9. The 5 MeTHF peak of control urine gave two peaks on sephadex G15 (i.e. 5 MeTHF and its oxidation product), whilst that of the tumour bearing rats has an additional peak (metabolite W) between these two.

The relative amounts of urinary metabolites changed with time (see table 5.17) and closely followed the pattern found in control urines (see figures 5.10 and 5.11). Total excretion of metabolites over 48 h is given in table 5.18. The 5 MeTHF level in tumour bearing rat urine was reduced to 68% control and folate "S" to 60% control, although in the latter case this figure is likely to be inaccurate due to the difficulties in obtaining homogenous peaks free from overlap by the comparatively large amounts of 5 MeTHF in urine.

Qualitative Analysis of Liver and Tumour Radioativity

Hot ascorbate extracts of livers and tumours (method (a)) on gel filtration gave essentially similar profiles (figure 5.12). The distribution of radioactivities between the various components present is given in table 5.19. Slightly less folate polyglutamate was found in the tumours compared to the host liver and slightly more degradation products. The latter included largely pterin, $^{3}H_2O$ and a p-aminobenzoyl-L-glutamate derivative. This suggests that the tumours contained relatively more of the heat-labile derivatives although the significance of this observation is complicated by several factors which will be discussed later.

Autolysis (method (d)) resulted in the liberation of 10 CHOTHF from liver (see figure 5.13 and table 5.19), and a small amount of

5 CHOTHF. When the 10 CHOTHF peak was rechromatographed on DEAE-cellulose the major peak cochromatographed with 10 CHOTHF but small amounts of 10 CHOFA and 5 CHOTHF were also found. When rechromatographed back on to Sephadex G15 the peak gave largely 10 CHOFA with small amounts of 10 CHOTHF and 5 CHOTHF. Thus under the conditions of extraction and chromatography (at pH7 in phosphate buffer) small amounts of 5 CHOTHF could be generated from 10 CHOTHF by an isomerization process and this presumably gave rise to the small amounts of 5 CHOTHF found in the original chromatograms. The liver autolysate also contained a small amount of folate $(glu)_n$(10 CHOTHF$(glu)_4$) and pterin and tritium labelled degradation products. Chromatography suggested that a small amount of 5 MeTHF was also present, chromatographing with the pterin peak, since rechromatography of the latter gave a small peak chromatographing in the region of the 5 MeTHF oxidation products. Although the latter could not be quantified accurately it amounted to no more than 5% of the liver radioactivity. Sephadex G15 chromatography of the tumour autolysate (figure 5.13) gave a very different pattern. A smaller amount of polyglutamate was present and the amount of 10 CHOTHF was one third that of the liver. The major portion of the radioactivity was composed of pterin (67% ^{14}C) and tritiated fragments (66% ^{3}H). The latter radioactivity was associated with two derivatives, $^{3}H_2O$ and a compound which chromatographed with p-aminobenzoic acid, and is tentatively identified as such. The marked difference in autolysis products despite the similarity in hot ascorbate extracts suggests that whilst the *in vivo* handling of folate is similar, very different enzymic activities prevail *in vitro* between the two tissues.

Chromatography of TCA extracts (method (f)) (figure 5.14; table 5.19) gave a complex mixture of degradation products and no intact folate monoglutamates were found. The similarity of the degradation products of both liver and tumour supports the suggestion that the

folate derivatives present in the two tissues are identical. Small amounts of high molecular weight species were present although a 100% excess of tritium over ^{14}C suggests that this is a mixture of folate polyglutamate and p-aminobenzoyl-L-glutamate (glutamate)n. Three ^{14}C only labelled derivatives were present the two minor ones eluting in the same place as xanthopterin (compound I) and pterin (III) and these also cochromatographed on DEAE-cellulose. The major metabolite (II) on rechromatography decomposed to give pterin, suggesting that it was a reduced pteridine compound. Since the aerobic oxidation of THF is known to ultimately produce pterin and xanthopterin, the former compound via 7,8 dihydropterin (Pearson, 1974) the identity of compound II as dihydropterin seemed likely. Dihydropterin is difficult to obtain in a pure form and readily oxidizes at neutral pH. A solution of crude dihydropterin, simply prepared by dithionite reduction of pterin in mercaptoethanol was applied to a Sephadex G15 column and the U.V. absorbance of the peaks obtained determined. Two peaks, both with the U.V. spectrum of pterin, were found chromatographing in the position of compound II and of authentic pterin. It was concluded therefore that compound II was probably dihydropterin, its persistence in the TCA extract presumably being due to stabilization at the low pH. The two major tritiated compounds were not identified conclusively although the early eluting one (compound IV) was volatile and therefore probably $^{3}H_2O$ or formaldehyde. The identity of the other derivative is unknown but it is unlikely to be a degradation product of p-aminobenzoyl-L-glutamate since this derivative is stable under the extraction conditions used (Connor and Vince, unpublished observations). A possible identity may be p-N-methylaminobenzoyl-L-glutamate, this derivative being a known product of the chemical reduction of 5,10 CH=THF under acid conditions (Baugh et al, 1979). By postulating the formation of 10 CH_3THF (after Baugh et al, 1979) a derivative

which has the strong electron withdrawing group (-CHO) at N10 replaced by an electron donating group and lacks the steric hindrance conferred by substitution at the N5 position (as in 5,10 CH=THF) spontaneous cleavage reactions, characteristic of THF, can occur but with the generation of the methyl derivative of p-aminobenzoyl-L-glutamate. The stage at which loss of the peptide side chain occurs is unknown since this would be expected to be very slow at pH4 (the extract pH) although the long contact time at the low pH and the existence of apparent p-aminobenzoyl-L-glutamate peptides in the extract would suggest that this occurred after cleavage.

Subcellular fractionation of host liver gave a similar distribution of radioactivity to normal liver although slightly more radioactivity was found in the high speed supernatant. The subcellular distribution of radioactivity in the tumours differed markedly from that of the host liver, larger amounts being recovered in the supernatant (table 5.20). Direct comparison between the tumour and liver is complicated however since the Walker 256-carcosarcinoma is not derived from liver but originally arose in mammary tissue of a female rat bearing a transplantable carcinoma (Earle, 1935). Recovery of marker enzymes also differed between the two tissues particularly the acid phosphatase (lysosomal) marker which was distributed much less cleanly in the tumour subcellular fractions. The latter observation may be indicative of tumour necropsy although no gross visual indication of this was observed.

Discussion

Walker-256 tumour bearing rats metabolised 10 CHOFA in a similar manner to normal controls with the following noticeable differences.

i) The presence of a novel urinary metabolite (W). This compound which has a lower ratio of ^{3}H to ^{14}C than the dose (80% of the ^{3}H of the dosed compound) is unidentified. As the compound has lost

20% of the ^{3}H present in the dosed 10 CHOFA and since the 10 CHOFA had 41% of the ^{3}H in the benzene ring it would appear that metabolite W is a folate which has lost tritium during metabolism since a cleavage product even if it retained all the C9 ^{3}H intact (which is unlikely) would have lost twice as much ^{3}H as metabolite W. Even allowing for the isotope effect found in these studies which resulted in the excretion of 5 MeTHF and other metabolites apparently containing excess ^{3}H (1.14 times more ^{3}H for the 5 MeTHF recovered in the urine from these rats) the observed ^{3}H to ^{14}C ratio of metabolite W would not account for a 6-alkyl pterin derivative. Although chemical models of ^{3}H exchange from folate exist (Hachey et al, 1978) the only known example from folate biochemistry is the loss of protons during thymidylate biosynthesis where 5,10 CH_2THF is oxidised to 7,8 dihydrofolate (Friedkin, 1973) and this reaction involves none of the labelled sites. A detoxification mechanism by which methotrexate is hydroxylated at the C7 position is known (Jacob et al, 1976) and several pterins are substrates for xanthine oxidase e.g. isoxanthopterin (7-hydroxypterin) being produced from pterin (Lowry et al, 1949). It may be possible for hydroxylation of the C7 position to account for loss of ^{3}H although evidence for such hydroxylation reactions with intact folate derivatives is lacking. Since some necrosis of the tumours may have occurred (cf. the lysosomal distribution) metabolite W may reflect chemical or enzymic degradation of folate under extreme physiological conditions.

ii) Although hot extraction procedures demonstrated that similar metabolites were present in the tumour and liver and this was supported by the finding of comparable degradation products in TCA extracts the products of autolysis of the two tissues were markedly different. Autolysis of liver produced mainly 10 CHOTHF and a small amount of 5 MeTHF whereas autolysis of tumour produced small amounts of 10 CHOTHF but larger amounts of the degradation products pterin,

^3H_2O and p-aminobenzoic acid. The Walker-256 carco-sarcinoma is known to contain much lower levels of the enzymes FIGLU transferase, CH_2= THF dehydrogenase, dihydrofolate reductase and 10 CHOTHF synthetase and comparable or greater amounts of serine hydroxymethyltransferase (Lepage et al, 1972) compared to host liver and reduced amounts of folate binding proteins (Wagner, unpublished observations). During autolysis of the tumour any THF generated from the 10 CHOTHF$(glu)_4$ initially present (e.g. by 10 CHOTHF dehydrogenase) would be subsequently metabolised only slowly and thus available for oxidative degradation. In the liver where protein binding is available to stabilize folate derivatives no such degradation would occur. Since p-aminobenzoic acid may have been formed during autolysis of tumour (but not liver) the existence of a mammalian p-aminobenzoyl-L-glutamate carboxypeptidase is suggested.

iii) The hepatic retention of radioactivity by the host liver was significantly higher than that of normal liver and a smaller amount of 5 MeTHF was excreted in the urine of tumour bearing rats. Both these factors are suggestive of the tumour induced folate deficiency previously recorded (Poirier, 1973); 5 MeTHF, the major serum transport folate, being more extensively taken up by the relatively folate deficient tissues.

5.4 A Study of the Metabolism of [3', 5', 9(n) - ^3H] [10-^{14}CHO] Folate in the Rat

10 CHOFA, despite its reported dihydrofolate reductase inhibition properties, enters the folate pool in the rat (section 5.2) where it is handled in a similar manner to folic acid (Pheasant, Connor and Blair, unpublished observations). In order to elucidate the mechanism of entry into the folate pool in vivo a pilot experiment was performed on the metabolism of 10 CHOFA labelled in the folate structure with ^3H as before but with the ^{14}C label in the formyl group.

Materials and Methods

Two normal male Wistar rats (250 g) were dosed orally with [3', 5', 9(n) - ^{3}H] [10-^{14}CHO] folate (250 μg kg^{-1}; 70 μCi ^{3}H and 0.1 μCi ^{14}C per rat). They were housed in sealed metabolism cages and treated as described in Section (1), with expired CO_2 being collected into 5M-KOH. After 48 h the animals were killed and the livers, intestine and kidneys removed for the determination of retained radioactivity. Livers were extracted by methods (a) (hot ascorbate) and by method (b) (isotonic sucrose) with protein removed by TCA precipitation).

Results

The distribution of radioactivity in the tissues, faeces, urine and expired CO_2 is given in table 5.21. The distribution of ^{3}H was comparable with the results obtained from studies with [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA metabolism. The low hepatic radioactivity (3.72% ^{3}H) is consistent with the dose related tissue uptake observed in Section 2 of this Chapter since the dosed 10 CHOFA for this experiment was 250 μg kg^{-1}, necessitated by the low specific radioactivity (^{14}C) of the compound. The distribution of retained ^{14}C is very different from the ^{3}H and much less of the dosed radioactivity was retained in the animals the majority being excreted in the urine, faeces and expired air.

Both methods used to extract radioactivity from liver failed to extract substantial amounts of ^{14}C although the ^{3}H was recovered with the usual efficiency. Less than 20% of the ^{14}C was extracted by hot ascorbate and none was detected in the TCA precipitated sucrose extracts. These observations suggest that the ^{14}C was lost with the liver protein. Gel filtration of extracts by methods (a) and (b) gave three ^{3}H peaks corresponding to 10 CHOFA$(glu)_4$ (82% and 72% respectively) and degradation products (5% and 12% respectively) showing

that the folate molecule was metabolised as in previous experiments. The only ^{14}C peak detected was found eluting at fractions 29/30 and accounted for 9% of the liver ^{14}C. The compound was not identified but it was not associated with ^{3}H and was detected only in the hot ascorbate extracts.

Chromatography of the 0-6 h urine sample revealed that most of the tritium was associated with 10 CHOFA and with small amounts of 10 CHOTHF and 5 MeTHF. Significant amounts of ^{14}C were associated with the 10 CHOFA peak only. The 6-24 h sample revealed several tritium peaks ($^{3}H_2O$ 7%; p-acetamidobenzoic acid 36%; 10 CHOFA 27%; 10 CHOTHF 7%; 5 MeTHF 21%) and ^{14}C was found associated with the 10 CHOFA peak (20% ^{14}C sample). Several ^{14}C derivatives were present all unassociated with ^{3}H but they were not identified. The ^{14}C peaks eluted from DEAE-cellulose in the following positions:

a) at the void volume, 15% ^{14}C;

b) 0.26 M-NaCl, 13% ^{14}C;

c) 0.7 M-NaCl, 8% ^{14}C;

d) 0.8 M-NaCl, 11% ^{14}C

Discussion

The absence of folate derivatives other than 10 CHOFA labelled with ^{14}C from the urine fails to provide definitive evidence on the route of entry of 10 CHOFA into the folate pool. Since the rate of flux of one carbon units is known to be rapid (Nixon et al, 1973) the presence of 10 CHOTHF and 5 MeTHF lacking the ^{14}C label in urine precludes neither direct reduction of 10 CHOFA via dihydrofolate reductase nor the prior loss of the formyl group before reduction. The persistence of a ^{14}C label in the 10 CHOFA of the 6-24 h urine demonstrates that 10 CHOFA can persist within the animal for some time without metabolism and thus the 10 CHOFA found in the urine of rats dosed [2-^{14}C] & [3', 5', 9(n) - ^{3}H] 10 CHOFA and 10 CHOFA$(glu)_4$ which

persisted even in the 24-48 h urine was almost certainly derived from the unmetabolized dosed compound.

When [2-^{14}C] folic acid was administered orally to rats at low doses (3 μg kg^{-1} + 30 μg kg^{-1}) labelled folic acid was not detected in the urine (Dransfield and Blair, 1972); however after low doses given intramuscularly (about 2 μg kg^{-1}) Murphy et al (1976) found labelled folic acid in urine samples although they did not quantify this. The difference in the two observations indicates that at low doses intestinal metabolism of folic acid is significant. After administration of larger oral doses of labelled folic acid (100 μg kg^{-1}) unmetabolized folic acid is found in urine in small amounts up to 48 h after the dose (Pheasant et al, 1979). In the latter study examination of bile folate revealed that labelled folic acid was absent. Thus once absorbed across the intestine folic acid can persist within the animal and subsequently be excreted in the urine even when the concentration is comparatively low (presumably by the same processes described for 10 CHOFA described in Section 5.2). If 10 CHOFA entered the folate pool via prior loss of the formyl group followed by reduction of folic acid then from the properties of folic acid described above we should expect to find at least a small amount of folic acid in the urine Interestingly, if 10 CHOFA exhibits in vivo the dihydrofolate reductase inhibition properties ascribed to it in vitro, then at higher doses more folic acid could be expected to be produced (in the urine) since once generated the folic acid could not be reduced. The absence of folic acid from the urine of rats dosed with up to 250 μg kg^{-1} of 10 CHOFA thus suggests that this is not an intermediate in the metabolism of 10 CHOFA.

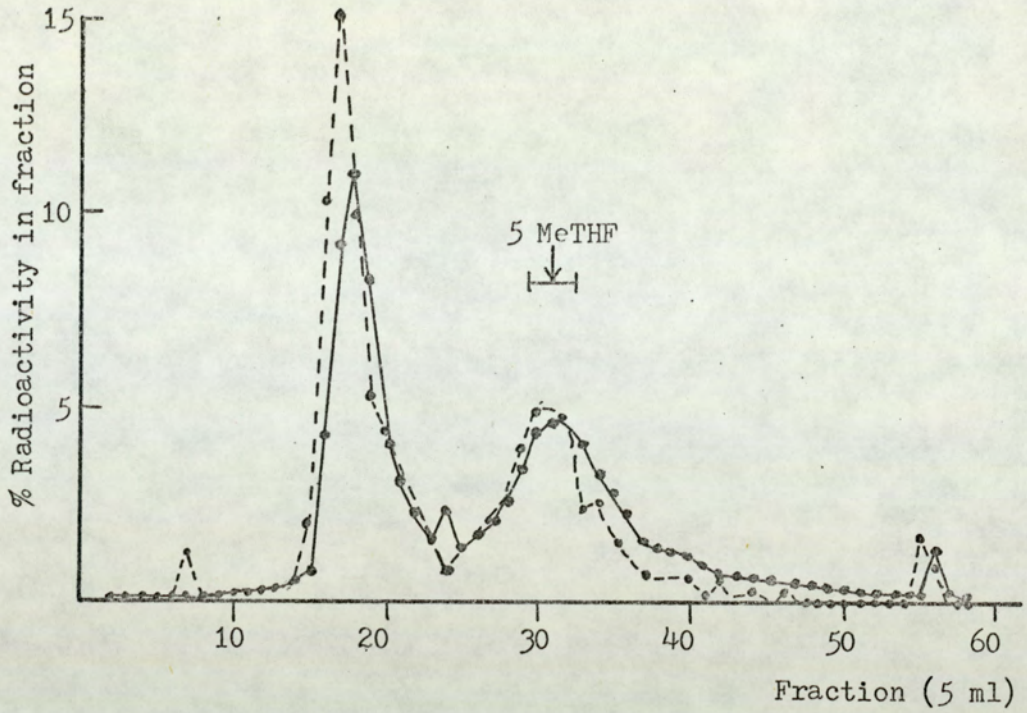
Direct reduction of 10 CHOFA to 10 CHOTHF via a dihydrofolate reductase mediated reaction would account for the release of the comparatively large amount of $^{14}CO_2$ produced (15%) and the number of

^{14}C labelled metabolites derived from the 10 CHO group found in urine samples. The main pathway of formate oxidation *in vivo* involves tetrahydrofolate (Krebs *et al*, 1976) and folate deficiency results in the appearance of formate in the urine (Stokstad *et al*, 1966). CO_2 can be liberated from 10 CHOTHF directly via 10 CHOTHF dehydrogenase or the one carbon group lost in any of the folate mediated reactions producing purines, pyrimidines, serine or methionine. Incorporation of label into the latter two aminoacids could thus account for the protein associated ^{14}C observed in liver.

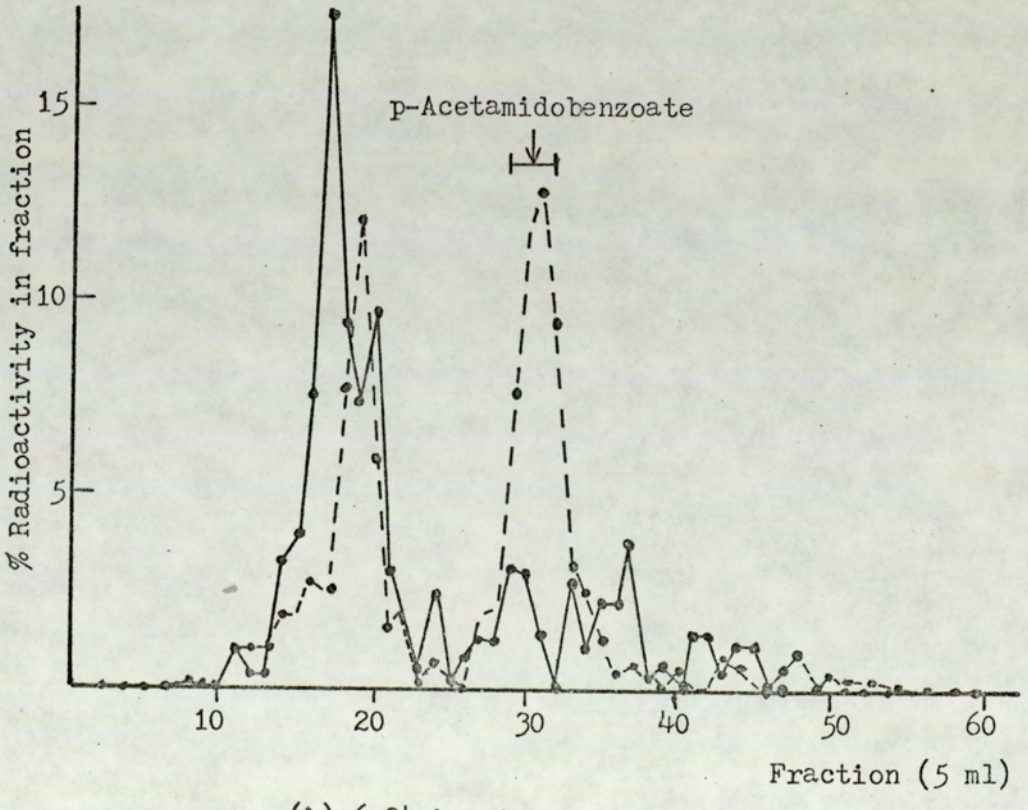
Conclusion

10 CHOFA is metabolised *in vivo* by the normal rat in a similar manner to folic acid. Tissue retention and metabolite excretion were dose dependent but considerable metabolism of 10 CHOFA was found even at very high doses (250 $\mu g\ kg^{-1}$). Entry of 10 CHOFA into the body pool is probably via dihydrofolate reductase reduction this being made possible by a slow release of free 10 CHOFA into the tissues from a protein bound store. The cellular concentration of DHF reductase is very high (Goldman, 1977) and thus the *in vitro* kinetic studies discussed in the introduction to this Chapter are probably invalid in the *in vivo* situation. Studies with Walker 256 implanted rats indicate that tumour induced changes of the folate pool can occur, sufficient to produce a novel urinary metabolite and thus confirming that the folate pool is a potential source of tumour markers.

10 CHOFA$(glu)_4$ was hydrolysed to 10 CHOFA, presumably at the absorption stage, and metabolised to 10 CHOFA metabolites. This important food folate is thus of nutritional importance.

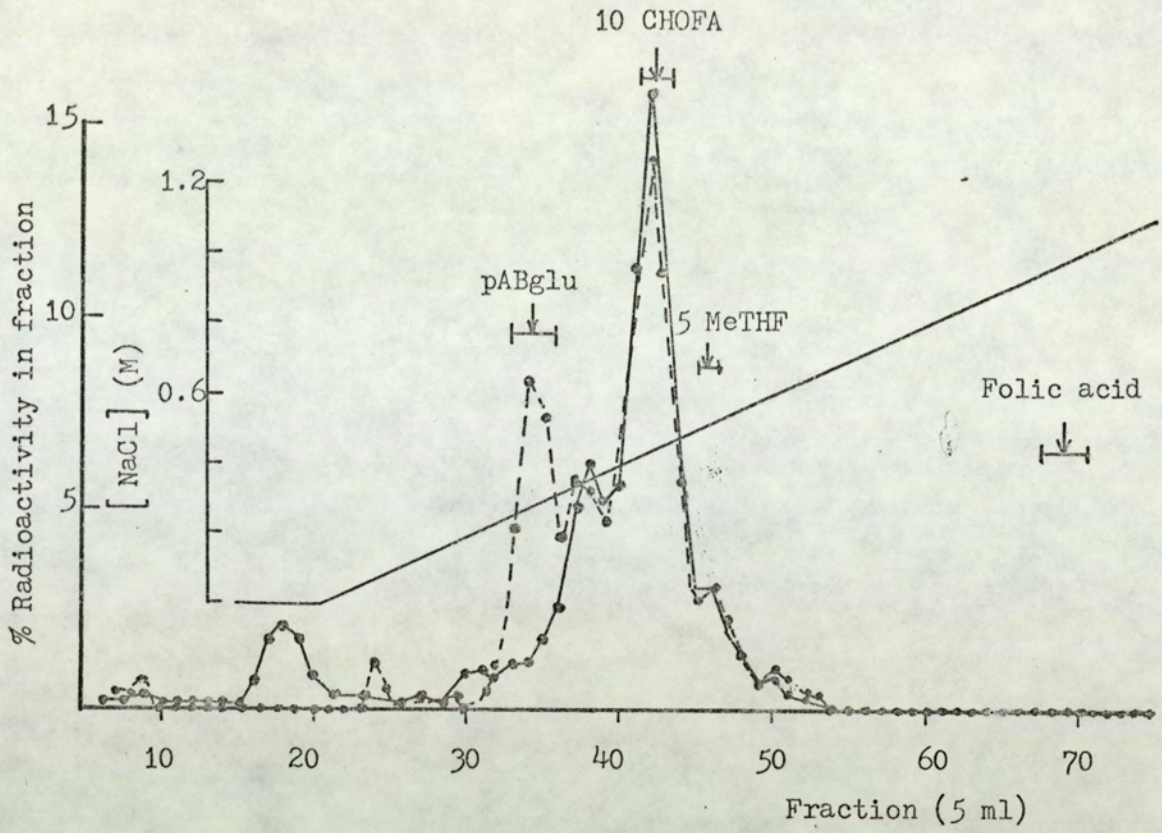


(a) 0-6 h urine

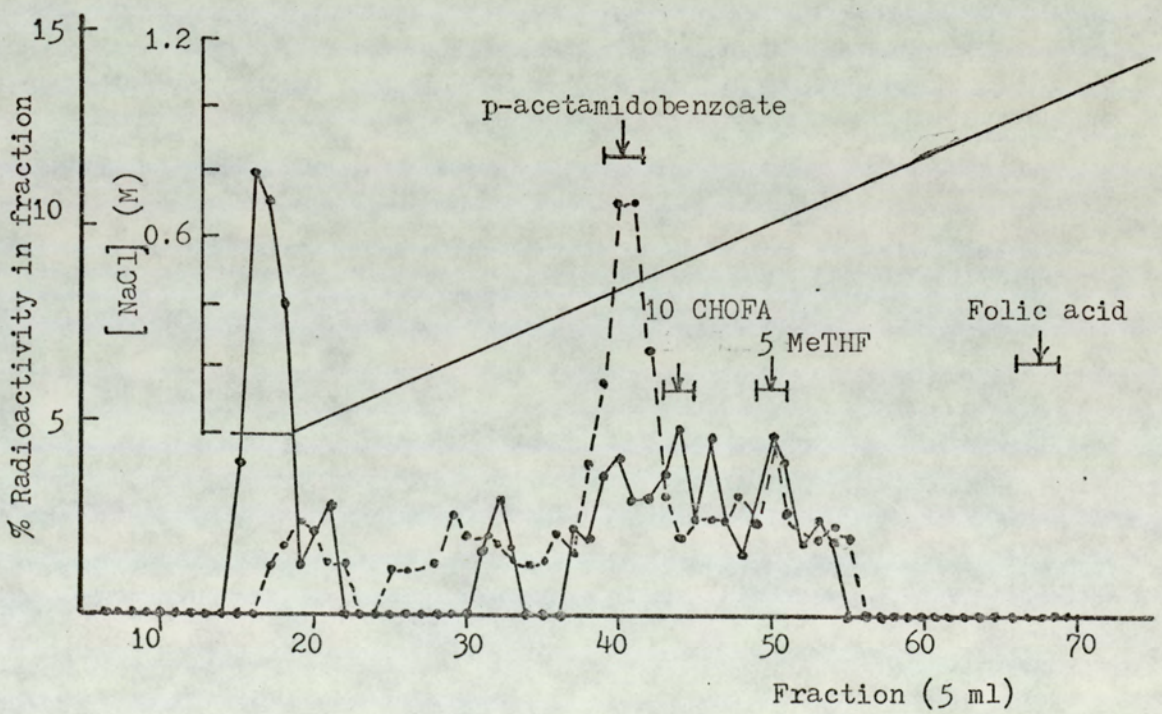


(b) 6-24 h urine

Figure 5.1 Sephadex G15 chromatography of rat urine samples collected 0-6 h and 6-24 h after the administration of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA(glu)$_4$.
(•——•——•) ^{14}C radioactivity; (•–•–•–•) ^{3}H radioactivity.



(a) 0-6 h urine



(b) 6-24 h urine

Figure 5.2 DEAE-cellulose chromatography of rat urine samples collected 0-6 h and 6-24 h after the administration of a mixture of $[2\text{-}^{14}C]$ and $[3', 5', 9(n) - {}^{3}H]$ 10 CHOFA$(glu)_4$.

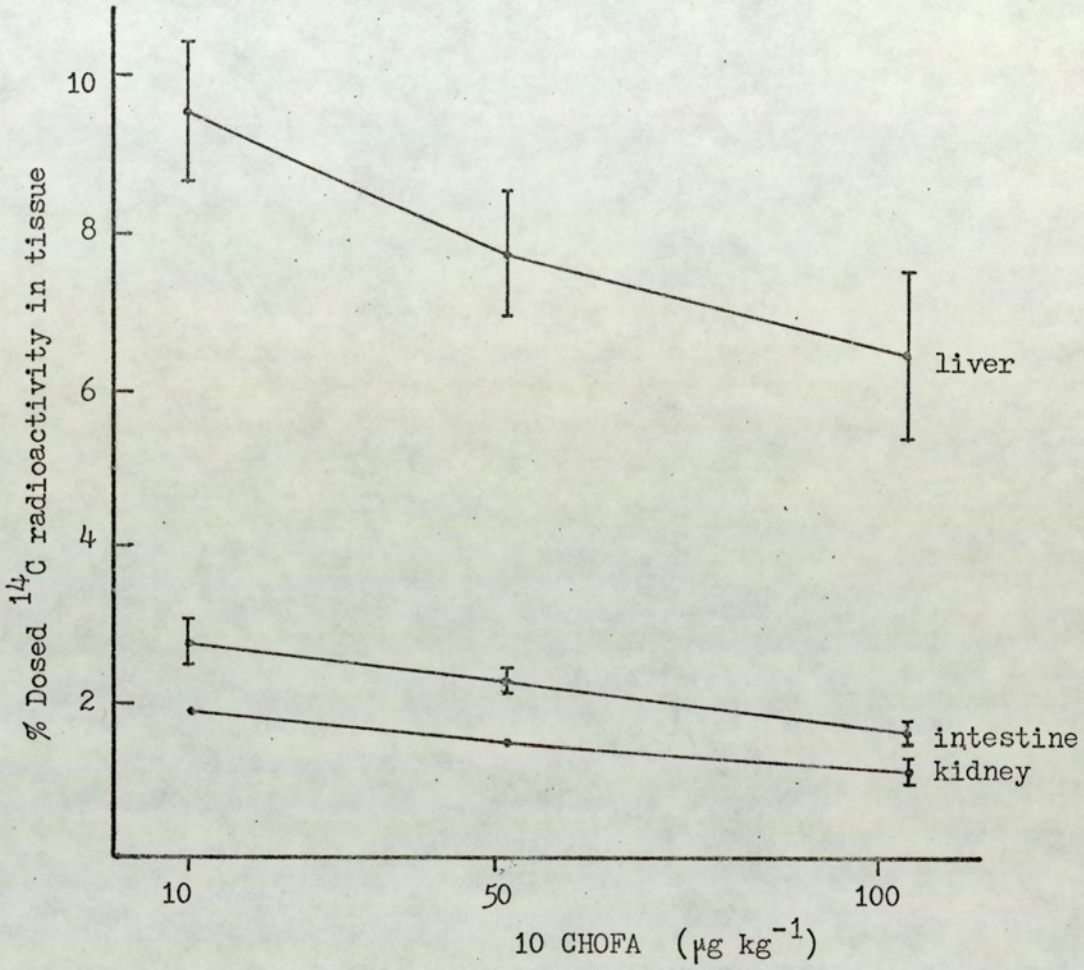


Figure 5.3 The dose dependent retention of radioactivity in rat liver, intestine and kidney 48 h after the administration of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA to normal rats

Points represent the mean and bars the SEM (n=6) of the % dosed ^{14}C radioactivity; unbarred points were determined from samples of pooled tissue (from 6 rats).

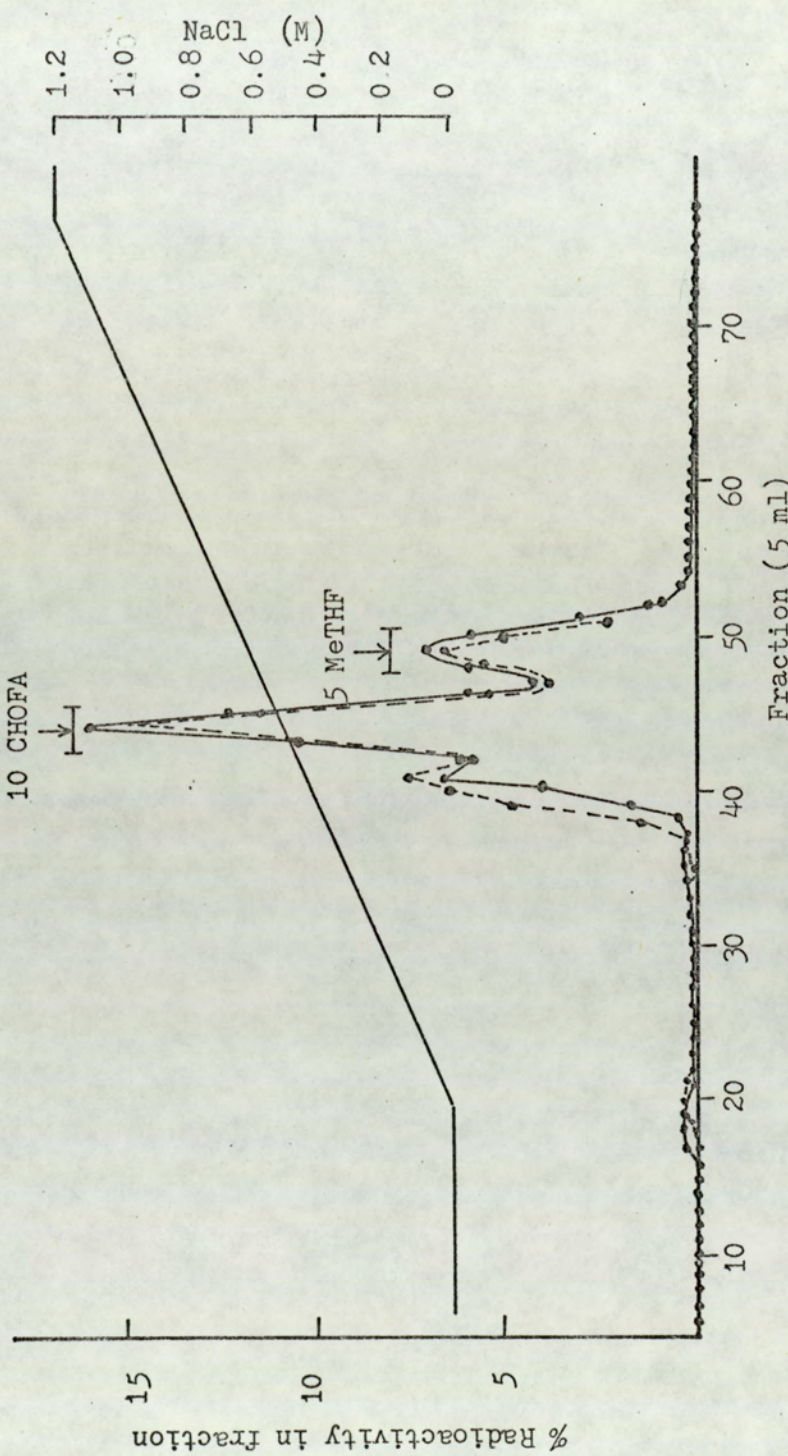


Figure 5.4 DEAE-cellulose chromatography of rat urine collected 0-6 h after the administration of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA (52.4 μg kg^{-1}) (——) ^{14}C radioactivity; (-·-·-·-) ^{3}H radioactivity.

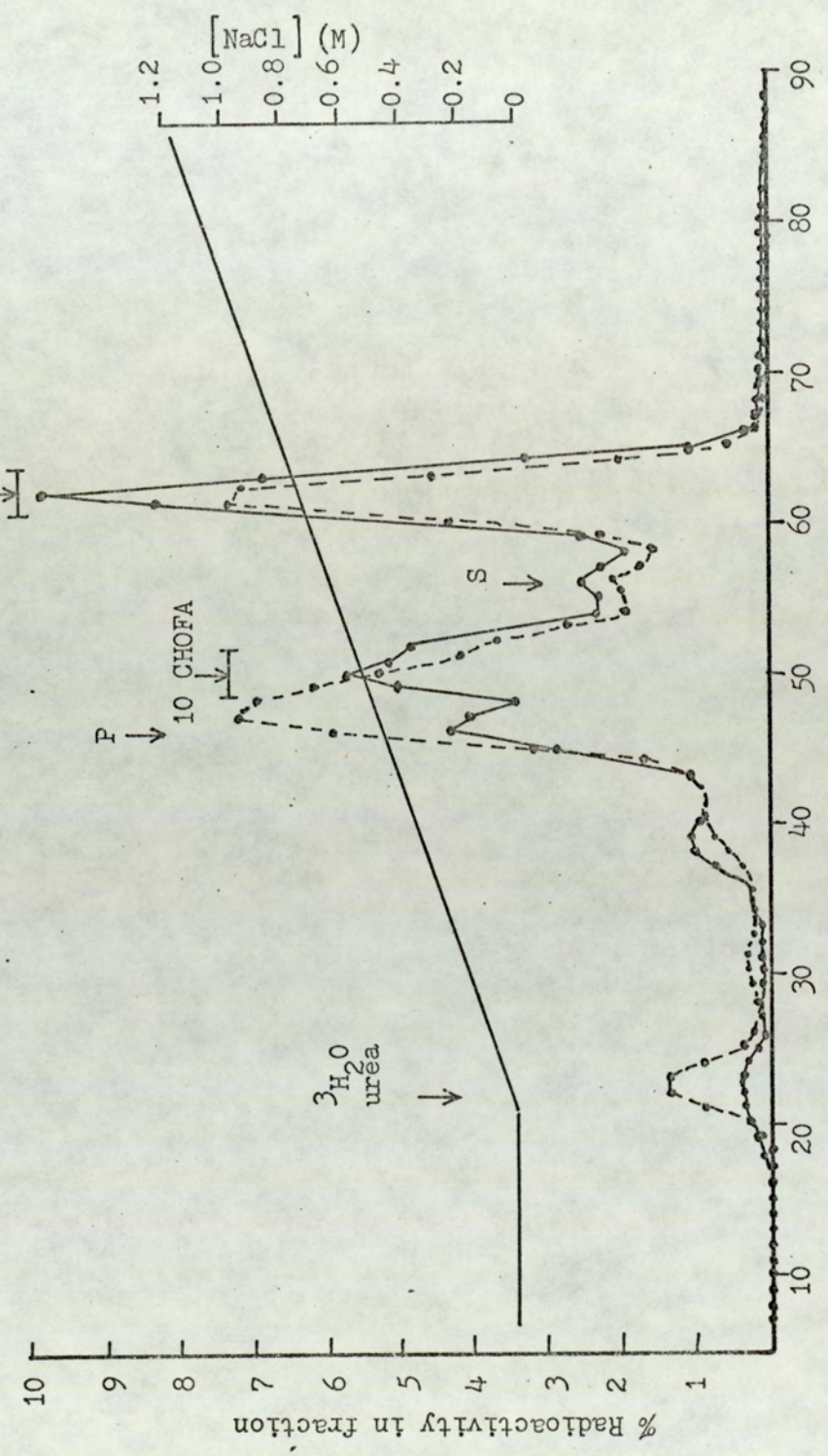


Figure 5.5 DEAE-cellulose chromatography of rat urine collected 6-24 h after the administration of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA (52.4 μg kg^{-1}) (——) ^{14}C radioactivity; (- - - -) ^{3}H radioactivity.

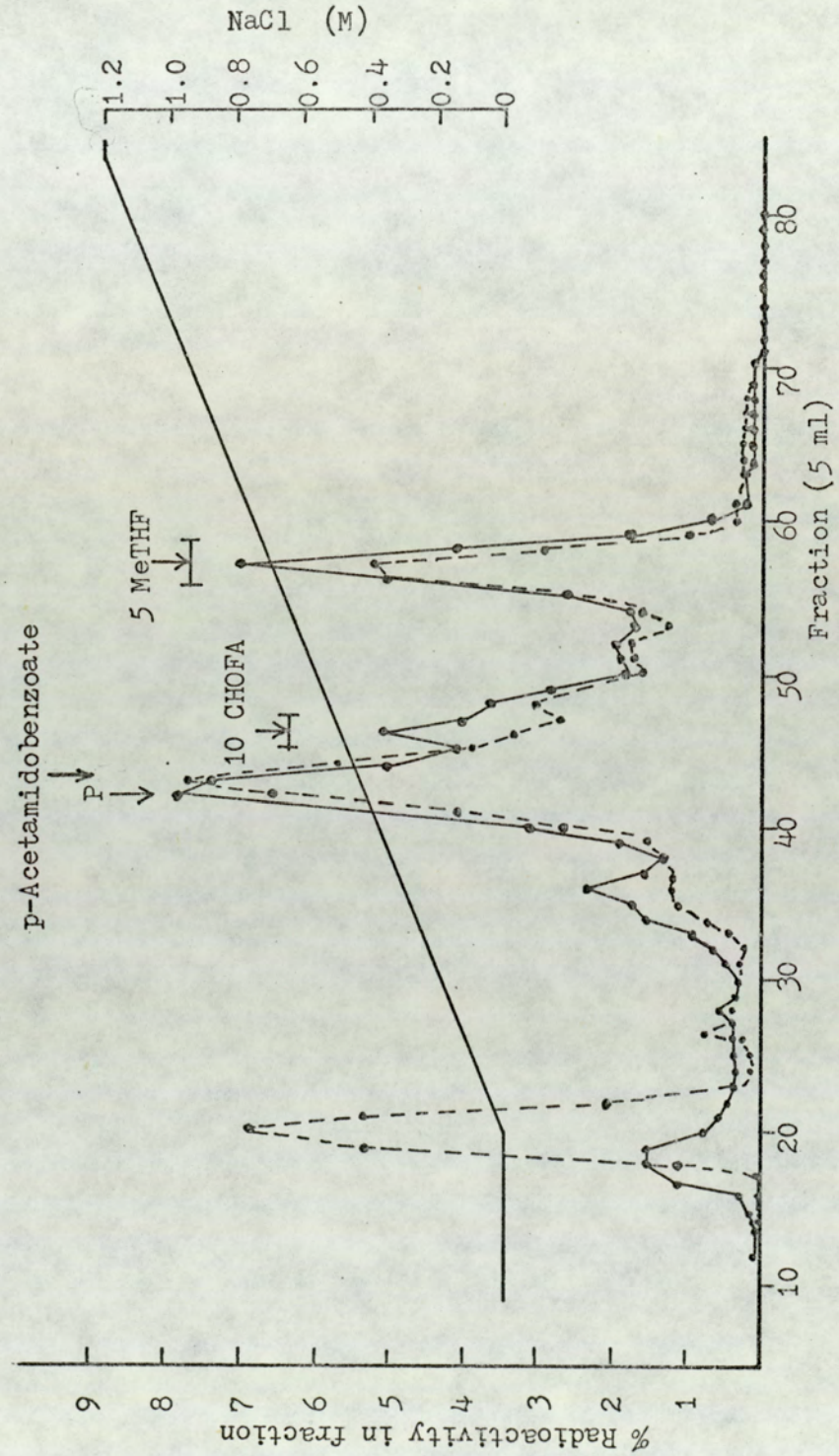


Figure 5.6 DEAE-cellulose chromatography of rat urine collected 24-48 h after the administration of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA (52.4 μg kg^{-1}) (——•——) ^{14}C radioactivity; (- - - - -) ^{3}H radioactivity.

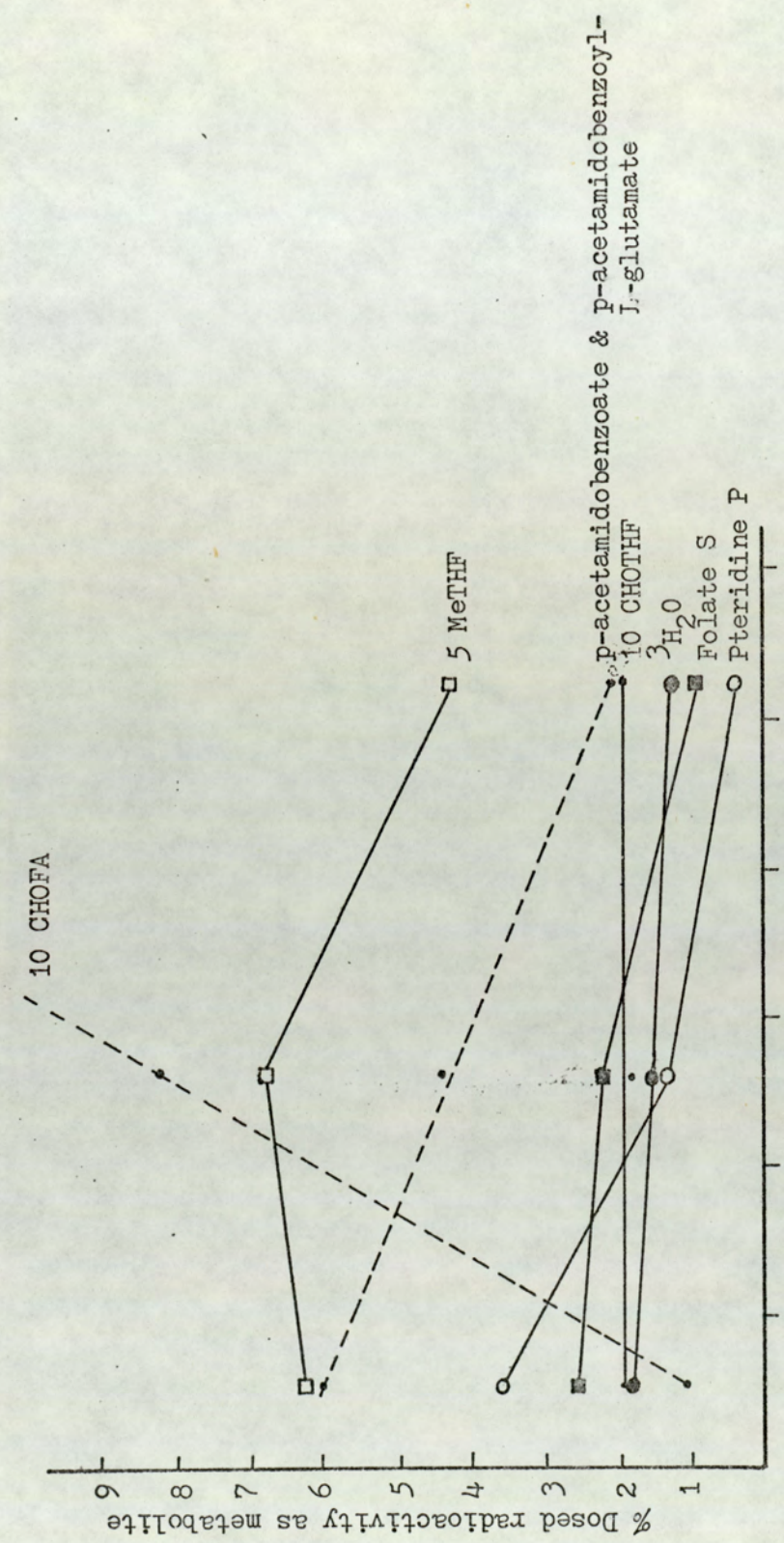


Figure 5.7 Dose dependent excretion of metabolites in the urine of rats 0-48 h after the administration of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA

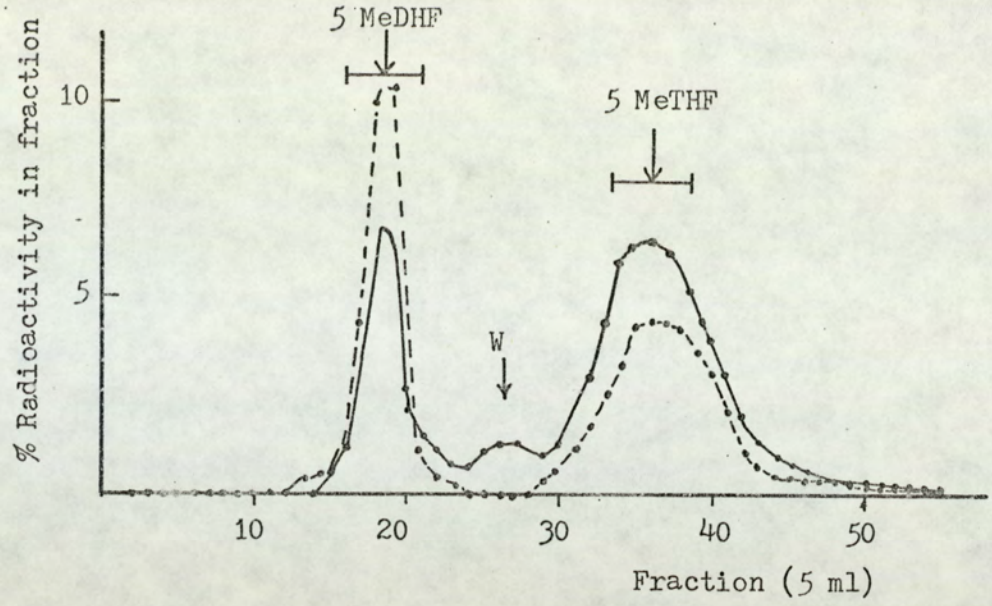


Figure 5.8 Sephadex G15 chromatography of the 5 MeTHF peak (^{14}C radioactivity) obtained on DEAE-cellulose chromatography of urine samples from normal (-•-•-•-) and tumour bearing (——•——) rats collected 6-24 h after 10 CHOFA administration. (52.4 μg kg^{-1}).

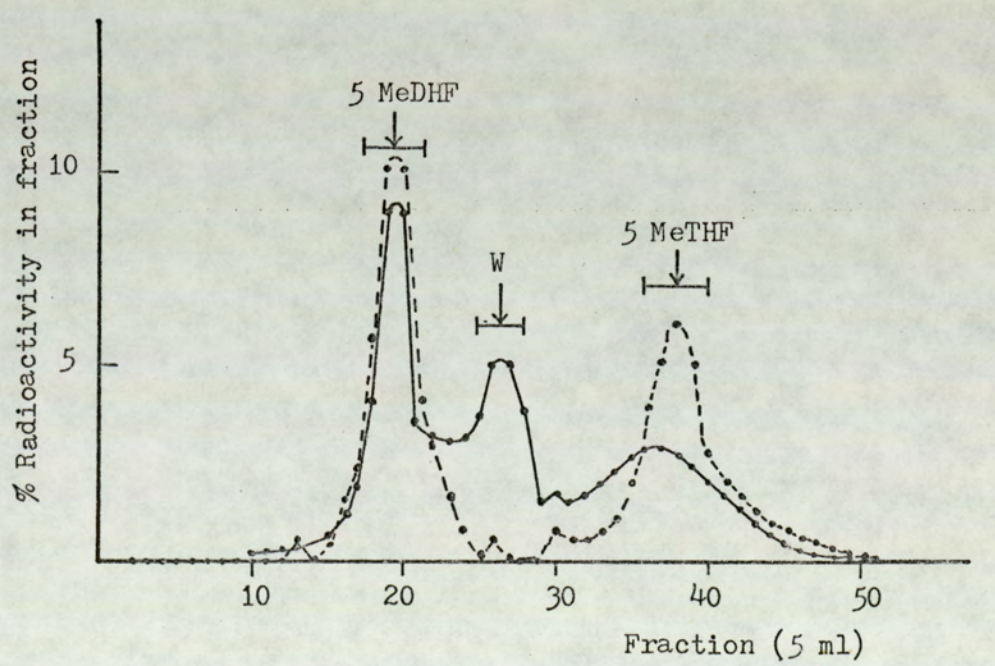


Figure 5.9 Sephadex G15 chromatography of the 5 MeTHF peak (^{14}C radioactivity) obtained on DEAE-cellulose chromatography of urine samples from normal (-•-•-•-) and tumour bearing (——•——) rats collected 0-6 h after 10 CHOFA administration. (52.4 μg kg^{-1})

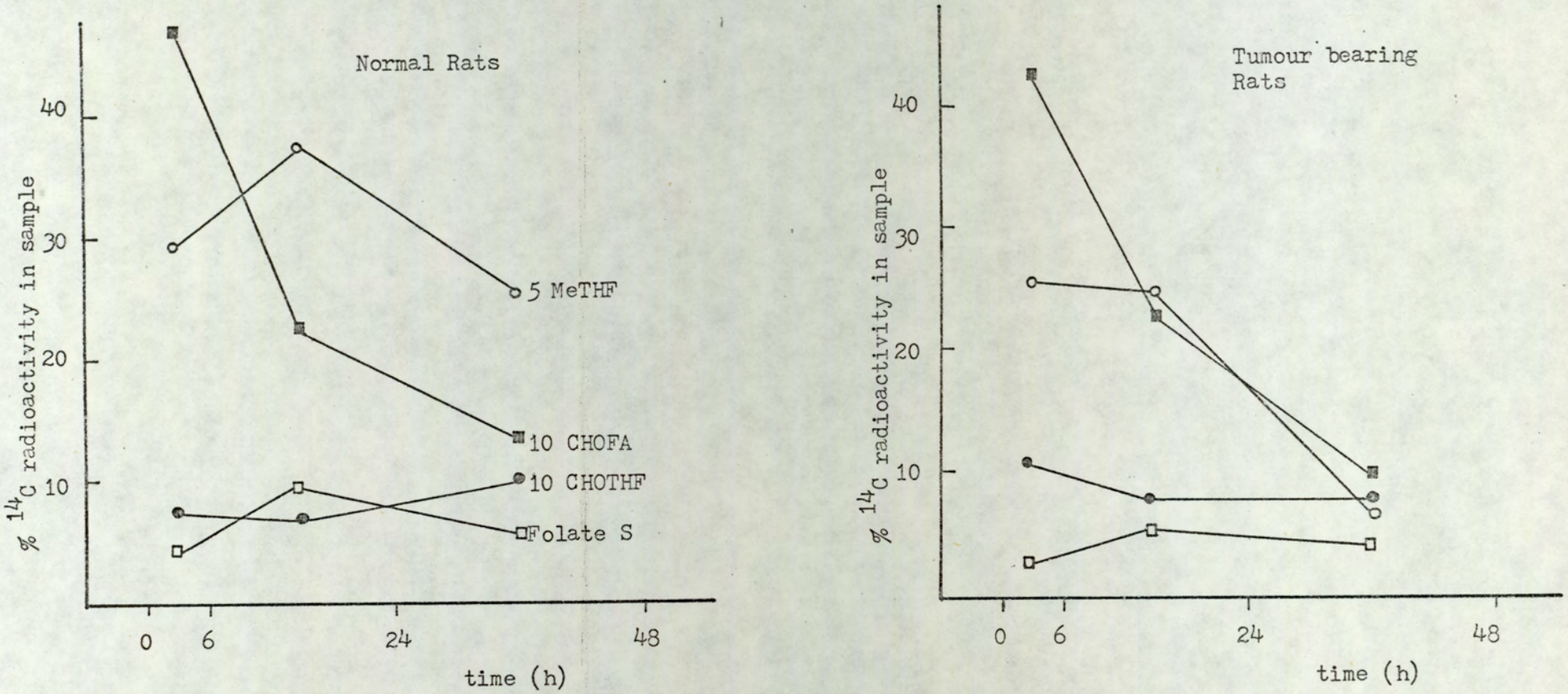


Figure 5.10 Time related changes in the distribution of radioactivity amongst the intact folate derivatives in urine samples 0-48 h after the administration of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA (52.4 μg kg^{-1}) to normal and Walker 256 tumour bearing rats.

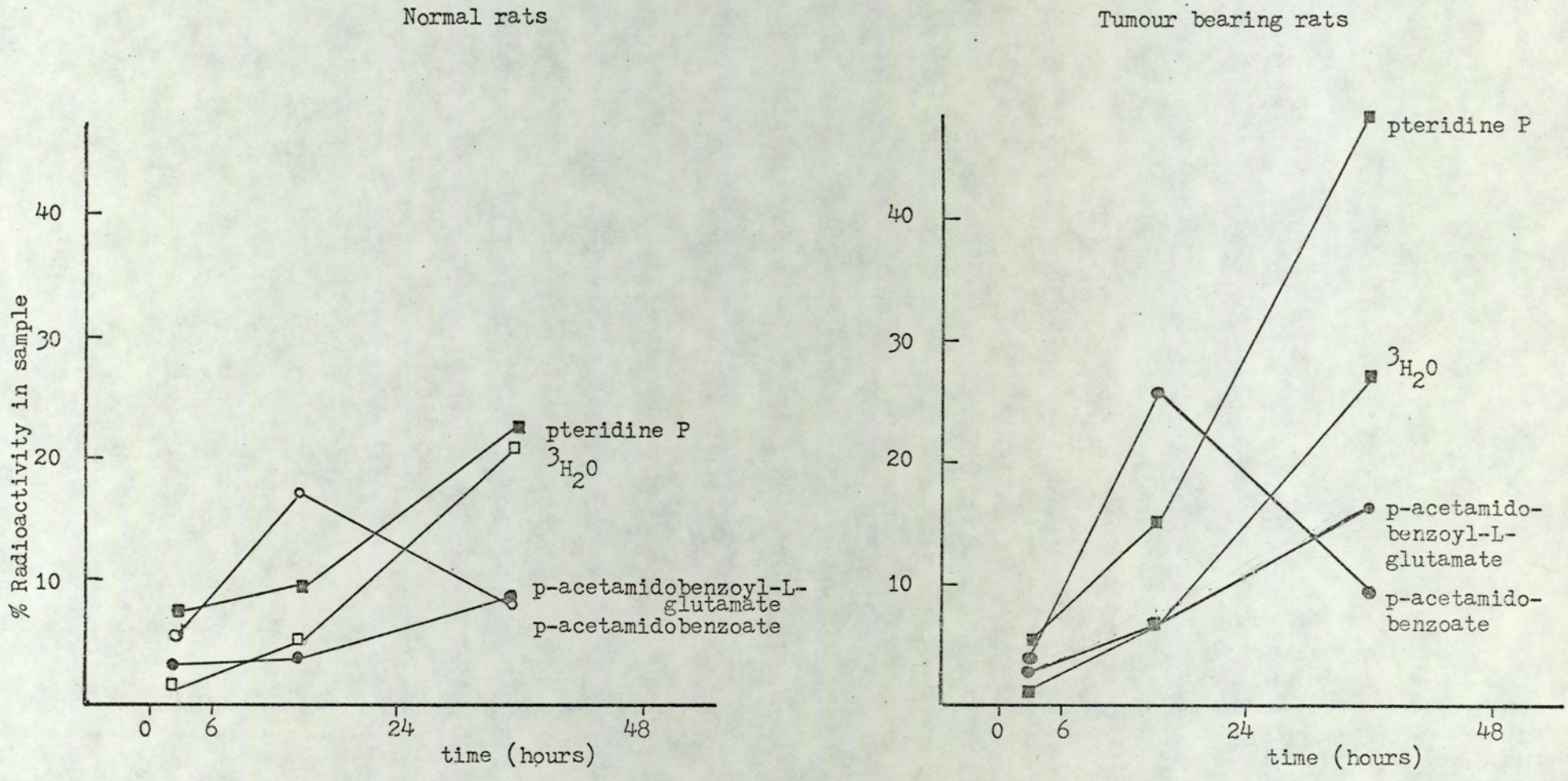
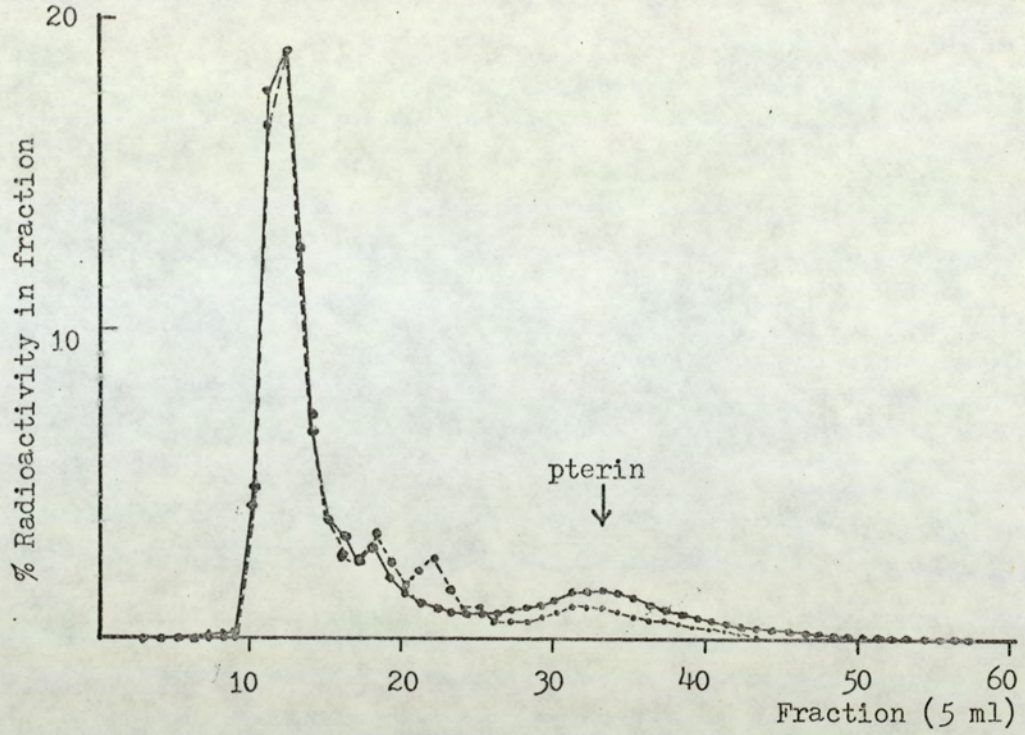
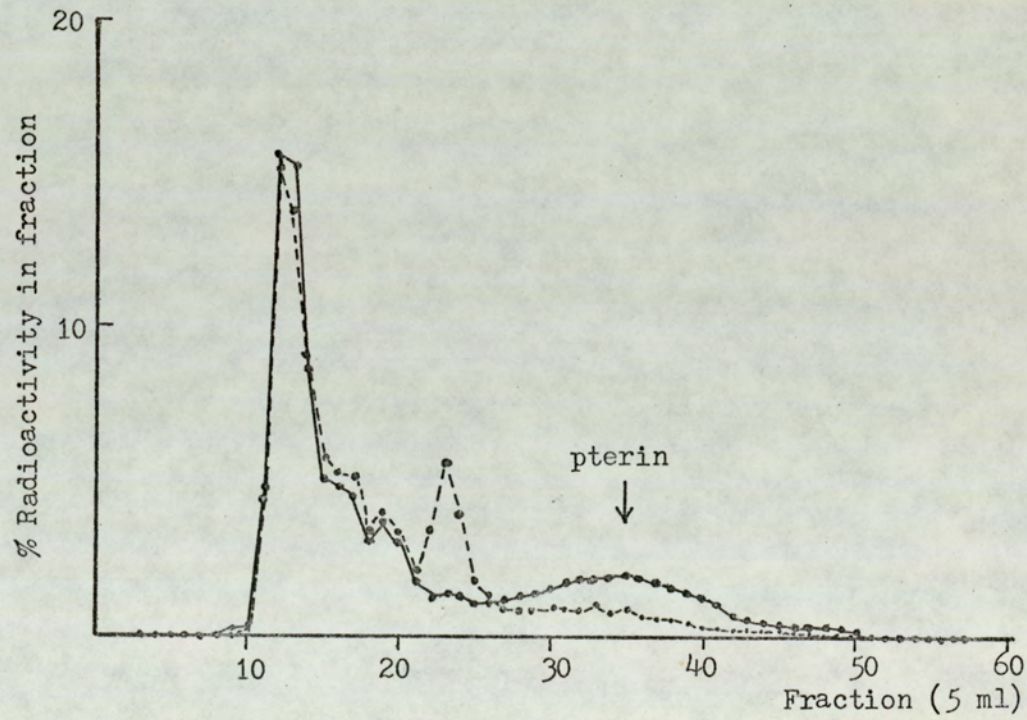


Figure 5.11 Time related changes in the distribution of radioactivity amongst the folate catabolites found in urine samples 0-48 h after the administration of 10 CHOFA (52.4 $\mu g\ kg^{-1}$) to normal and tumour bearing rats.

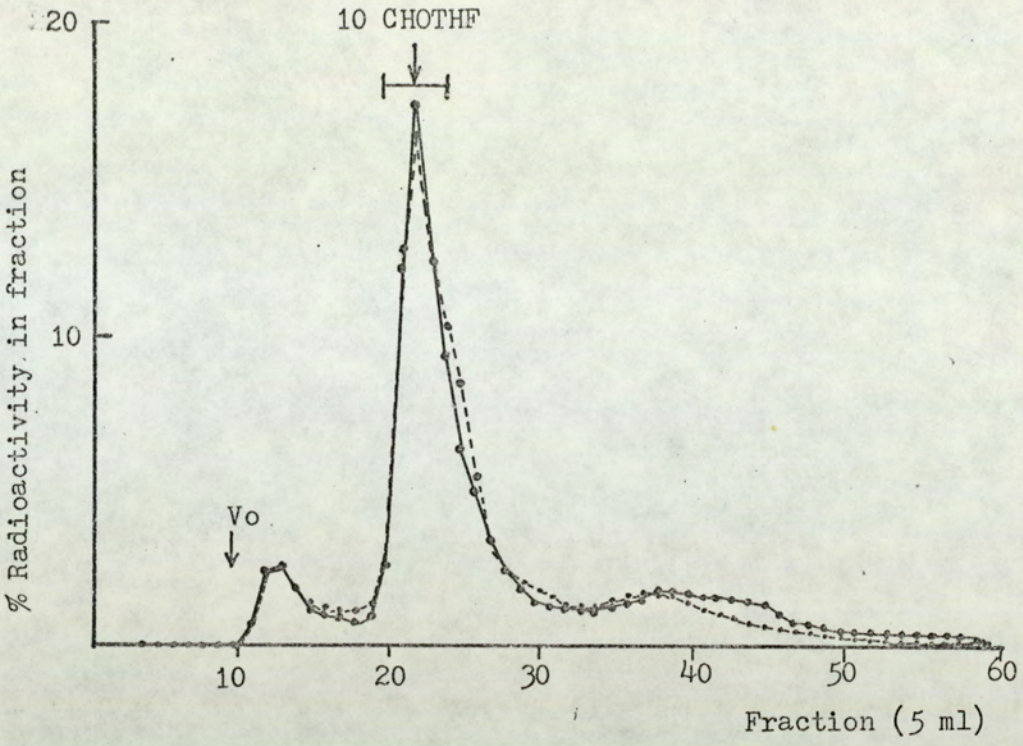


(a) Liver

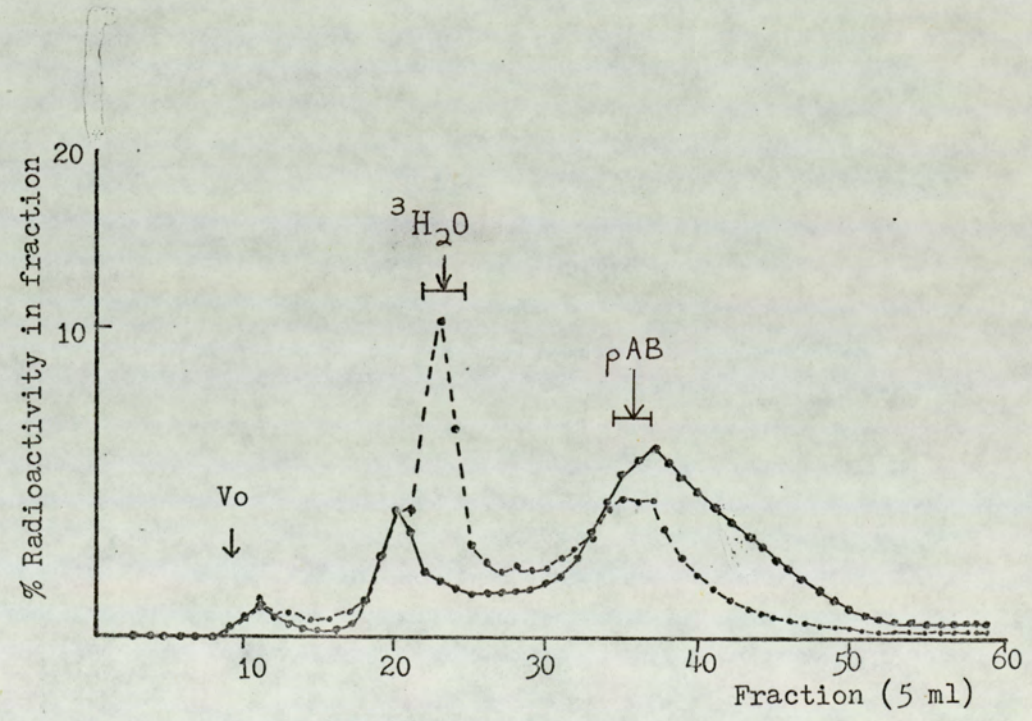


(b) Tumour

Figure 5.12 Sephadex G15 chromatography of hot ascorbate extracts (method (a)) of liver and tumour excised 48 h after the administration of 10 CHOFA to Walker 256 tumour bearing rats.
(·····) ^{14}C radioactivity; (-·-·-·-) ^{3}H radioactivity.

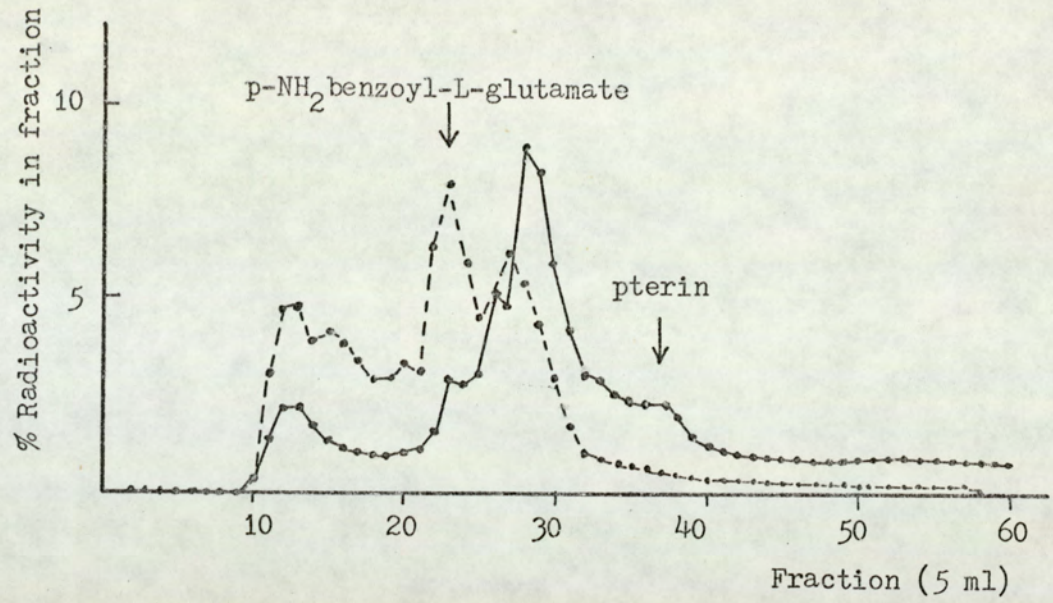


(a) Liver

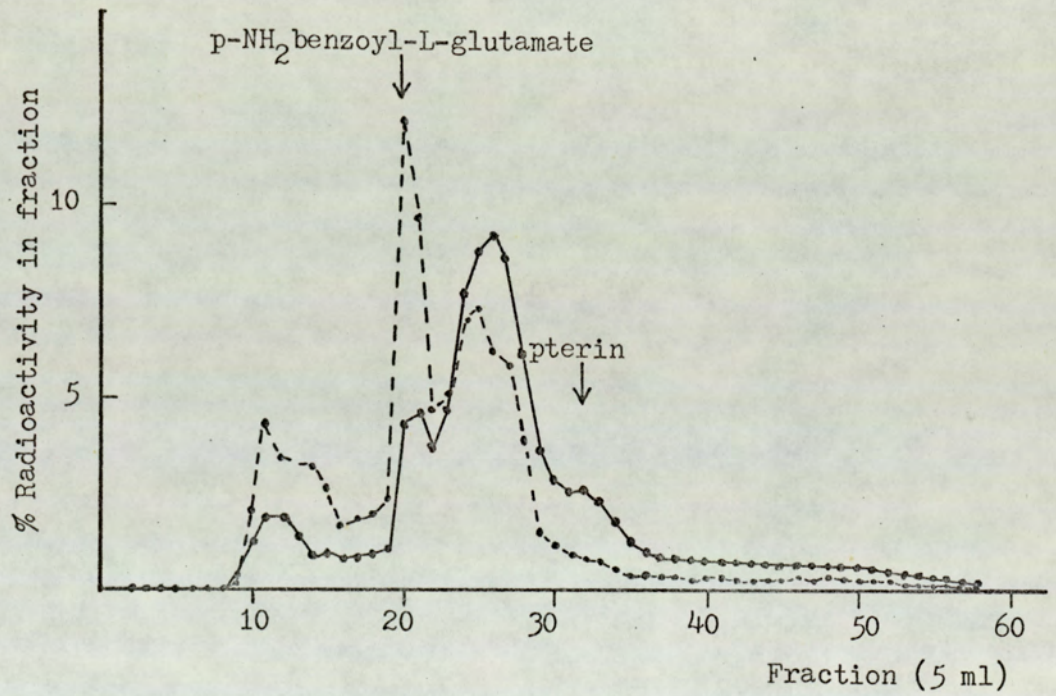


(b) Tumour

Figure 5.13 Sephadex G15 chromatography of autolysates (extraction method (d)) of liver and tumour excised 48 h after the administration of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA to Walker 256 tumour bearing rats.
(——) ^{14}C radioactivity; (-·-·-) ^{3}H radioactivity



(a) Liver



(b) Tumour

Figure 5.14 Sephadex G15 chromatography of TCA extracts (method (f)) of liver and tumour excised 48 h after the administration of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] 10 CHOFA to Walker 256 tumour bearing rats.
(——) ^{14}C radioactivity; (– – –) ^{3}H radioactivity

Table 5.1 The distribution of radioactivity in the faeces, tissues urine and expired air 48 h after an oral dose of [3', 5', 9(n) -^{3}H] and [2-^{14}C] 10-formylfolatetetraglutamate.
Values are expressed as percentages of the dose.

Sample	Wet Weight g	% ^{14}C	% ^{3}H	No. rats
Faeces	7.32	26.3	36.9	2
Liver	7.50	4.89	4.89	2
Intestine	4.50	0.88	1.07	2
Contents Intestine	.54	1.01	0.80	1
Testes	2.48	0.13	0.07	2
Heart	1.00	0.11	0.14	2
Spleen	0.63	0.05	0.08	2
Brain	1.81	< 0.04	< 0.04	2
Kidneys	1.83	0.59	0.81	2
Urine	0- 6 h	22.1	21.9	3
	6-24 h	7.74	13.9	3
	24-48 h	1.75	3.29	1
	Total	31.59	39.09	
CO_2	0- 6 h	.22		2
	6-24 h	5.68		2
	24-48 h	.33		2
	Total	6.23		2
	TOTAL	71.82	83.89	

Table 5.2 The distribution of radioactivity amongst the labelled metabolites found in urine 0-6 h and 6-24 h after an oral dose of [2-^{14}C]& [3', 5', 9(n) - ^{3}H] 10 CHOFA(glu)$_4$

0-6 h urine	% Urinary Radioactivity		% Dosed compound	
	^{3}H	^{14}C	^{3}H	^{14}C
10 CHOFA	30	31	6.6	6 8
10 CHOTHF	22	19	4.8	4.2
5 MeTHF	12	16	2.6	3.5
p-AcBglu*	11	-	2.4	-
p-AcBenzoate*	7	-	1.5	-
Pteridine P	-	7	-	1.5
Urea	-	9	-	2.0
$^{3}H_2O$	2	-	0.4	-
Total	84	82	18.3	18 0
6-24 h urine				
10 CHOFA 10 CHOTHF	9	16	1.3	1.2
5 MeTHF	11	15	1.5	1.2
p-AcBglu*	9	-	1.3	-
p-AcBenzoate*	51	-	7.1	-
Pteridine P	-	16	-	1.2
Urea	-	37	-	2.9
$^{3}H_2O$	6	-	0.8	-
Total	86	84	12	6.5

* p-AcBglu; p-acetamidobenzoyl-L-glutamate

* p-AcBenzoate; p-acetamidobenzoate

Table 5.3 Distribution of radioactivity in the tissues of rats 48 h after the administration of 3 different doses of 10 CHOFA

Dose kg^{-1}	22.18 nM, 10.5 µg			110.9 nM, 52.35 µg			221.82 nM, 104.7 µg		
Tissue	wet weight (g)	% ^{14}C	% ^{3}H	wet weight (g)	% ^{14}C	% ^{3}H	wet weight (g)	% ^{14}C	% ^{3}H
Liver	7.5	9.55	10.82	8.3	7.62	7.59	8.61	6.32	6.13
± SEM	±0.56	±0.95	1.22	±0.52	±0.87	±0.74	±0.48	±1.12	±1.02
Intestine	5.73	2.74	2.70	8.68	2.15	2.05	6.93	1.54	1.74
± SEM	±0.35	±0.32	±0.31	±0.81	±0.23	±0.22	±0.32	±0.01	±0.07
Kidney	1.72	1.86	1.74	1.68	1.42	1.40	1.98	1.07	1.20
Lungs	2.41	0.18	0.22	2.16	0.13	0.12	2.43	0.08	0.09
Testes	2.58	0.17	0.19	2.48	0.16	0.20	2.24	0.08	0.10
Spleen	0.70	0.33	0.40	0.57	0.16	0.16	0.71	0.13	0.13
Heart	0.87	0.09	0.12	1.05	0.16	0.14	1.05	0.06	0.07
Brain	- Not determined -			1.35	0.05	0.06	1.39	0.03	0.04
Sternum	0.13	0.01	0.01	0.13	0.01	0.01	0.22	0.10	0.13
Total		14.93	16.20		11.86	11.73		9.41	9.63

Table 5.4 Distribution of radioactivity in the urine and faeces collected 48 h after the administration of three different doses of 10 CHOFA to rats

Dose per kg	22.18 nM 10.5 µg		110.9 nM 52.35 µg		221.82 nM 104.7 µg	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Urine						
0- 6 h	6.85 ±1.14	8.92 ±1.43	13.01 ±1.19	17.38 ±1.75	22.38 ±1.74	27.40 ±2.14
	$p < 0.001$		$p < 0.001$		$p < 0.001$	
6-24 h	8.96 ±0.99	12.46 ±1.15	7.63 ±0.45	11.58 ±0.47	7.98 ±1.24	10.81 ±1.81
	$p < 0.001$		$p < 0.001$		$p < 0.001$	
24-48 h	3.70 ±0.60	4.30 ±0.68	2.15 ±0.17	2.98 ±0.14	2.12 ±0.19	2.68 ±0.24
	$0.5 > p > 0.3$		$p < 0.05$		$p < 0.001$	
TOTAL	19.53 ±1.37	25.69 ±1.37	22.79 ±1.48	31.94 ±1.64	32.48 ±1.44	40.89 ±1.83
Faeces						
0-48 h	37.56 ±2.10	23.93 ±1.62	41.87 ±3.36	27.29 ±1.87	36.94 ±6.41	29.35 ±3.00
	$p < 0.001$		$p < 0.001$		$0.001 < p < 0.01$	

Values are expressed as the percentage of the dose ± SEM.
The values of P quoted refer to the statistical significance of the difference between the $\%^{14}C$ and $\%^{3}H$ found determined by a paired t test
n = 6 in all cases.

Table 5.5 Summary of the statistical significance of the differences in recovery of radioactivity in the urine at each dose.

Sample	low dose/middle dose	low dose/high dose	middle dose/high dose
0-6 h ^{14}C	$0.001 < p < 0.01$	$p < 0.001$	$p < 0.001$
^{3}H	$0.001 < p < 0.01$	$0.001 < p < 0.01$	$0.001 < p < 0.01$
6-24 h ^{14}C	$0.2 < p < 0.3$	$0.5 < p < 0.6$	$0.7 < p < 0.8$
^{3}H	$0.4 < p < 0.5$	$0.4 < p < 0.5$	$0.6 < p < 0.7$
24-48 h ^{14}C	$0.02 < p < 0.05$	$0.02 < p < 0.05$	$p > 0.9$
^{3}H	$0.05 < p < 0.10$	$0.02 < p < 0.05$	$0.2 < p < 0.3$

Table 5.6 Total recovery of radioactivity 48 h after the administration of 10 CHOFA at three different doses

	Low dose		Middle dose		High dose	
	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
Tissues	14.93	16.20	11.86	11.73	9.41	9.63
Faeces	37.56	23.93	41.87	27.29	36.94	29.35
Urine	19.53	25.69	22.79	31.94	32.48	40.89
CO_2 (0- 6)	1.70	-	2.50	0.05	2.04	-
& H_2O (6-24)	2.65	-	1.01	0.31	0.53	-
(24-48)	-	-	0.06	0.61	0.10	-
Total	4.35	-	3.57	0.96	2.77	-
Total	76.37	65.82	80.09	71.92	81.06	79.87

Table 5.7 Distribution of metabolites in urine samples after a dose of 10.5 μg kg^{-1} 10 CHOFA to rats (low dose)

	0-6 h		6-24 h		24-48 h	
Metabolite	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
10 CHOFA	12.2	13.1	ND	ND	5.6	4.8
	(0.8)	(1.2)			(0.2)	(0.2)
10 CHOTHF	15.3	13.5	5.2	4.3	6.9	5.1
	(1.0)	(1.2)	(0.5)	(0.5)	(0.3)	(0.2)
5 MeTHF	29.6	26.2	39.8	33.0	16.8	12.6
	(2.0)	(2.3)	(3.6)	(4.1)	(0.6)	(0.5)
Folate S	15.8	16.3	20.9	14.6	13.0	10.5
	(1.1)	(1.4)	(1.9)	(1.8)	(0.5)	(0.4)
p-acetamidobenzoate	-	9.1	-	19.8	-	24.0
		(0.8)		(2.5)		(1.0)
p-acetamidobenzoyl-L-glutamate	-	5.0	-	5.3	-	9.0
		(0.4)		(0.7)		(0.4)
$^{3}H_2O$	-	2.7	-	5.4	-	19.1
		(0.2)		(0.7)		(0.8)
Pterin "P"	4.3	2.0	11.2	2.9	32.0	5.2
	(0.3)	(0.2)	(1.0)	(0.4)	(1.2)	(0.2)
Urea	1.5	-	1.4	-	7.3	-
	(0.1)		(0.1)		(0.3)	
Total	78.7	87.9	78.5	85.3	81.6	90.3

Values in parenthesis refer to the% of dosed radioactivity

Table 5.8 Distribution of metabolites in urine samples after a dose of 52.35 $\mu g kg^{-1}$ 10 CHOFA (middle dose) to rats

	0-6 h		6.24 h		24.48 h	
Metabolite	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
10 CHOFA	46.5	42.5	22.0	17.5	13.0	9.9
	(6.1)	(7.4)	(1.7)	(2.0)	(0.3)	(0.3)
10 CHOTHF	7.1	6.2	7.0	5.8	9.8	6.3
	(0.9)	(1.1)	(0.5)	(0.7)	(0.2)	(0.2)
5 MeTHF	29.0	25.3	37.3	30.6	25.2	20.8
	(3.8)	(4.4)	(2.9)	(3.5)	(0.5)	(0.6)
Folate S	4.2	4.0	9.0	7.7	5.6	5.1
	(0.5)	(0.7)	(0.7)	(0.9)	(0.1)	(0.1)
p-acetamidobenzoate	-	5.6	-	17.1	-	7.8
		(1.0)		(2.0)		(0.2)
p-acetamidobenzoyl-L-glutamate	-	3.1	-	3.8	-	7.8
		(0.5)		(0.4)		(0.2)
$^{3}H_2O$	-	1.3	-	5.2	-	21.2
		(0.2)		(0.6)		(0.6)
Pterin P	7.3	4.0	9.4	1.2	22.4	5.4
	(0.9)	(0.6)	(0.7)	(0.1)	(0.5)	(0.2)
Urea	1.2	-	3.8	-	6.7	-
	(0.2)		(0.3)		(0.1)	
Total	95.3	88.7	88.5	88.9	82.7	84.3

Table 5.9 Distribution of metabolites in urine samples after a dose of 104.7 $\mu g kg^{-1}$ 10 CHOFA (high dose) to rats

	0-6 h		6-24 h		24-48 h	
Metabolite	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
10 CHOFA	79.0	75.7	50.6	54.2	31.6	38.2
	(17.7)	(20.7)	(4.0)	(5.8)	(0.7)	(1.0)
10 CHOTHF	7.2	6.2	4.1	3.8	3.5	2.4
	(1.6)	(1.7)	(0.3)	(0.4)	(0.1)	(0.1)
5 MeTHF	8.8	7.6	23.2	21.3	18.0	15.5
	(2.0)	(2.1)	(1.8)	(2.3)	(0.4)	(0.4)
Folate S	Not resolved		4.2	3.8	5.2	4.7
			(0.3)	(0.4)	(0.1)	(0.1)
p-acetamidobenzoate	Not resolved		-	12.9	-	9.5
				(1.4)		(0.3)
p-acetamidobenzoyl-L-glutamate	Not resolved			trace		8.6
						(0.2)
$^{3}H_2O$	-	.6	-	4.3	-	17.4
		(0.2)		(0.5)		(0.5)
pterin "P"	Not resolved		5.7	1.7	17.9	3.4
			(0.5)	(0.2)	(0.4)	(0.1)
Urea	0.5	-	2.9	-	4.9	-
	(0.1)		(0.2)		(0.1)	
Total	95.5	90.1	90.7	102	81.1	99.7

Table 5.10 Radiolabelled metabolites excreted over 48 h as a percentage of the dosed radioactivity after three doses of 10 CHOFA to normal rats

Total Radioactivity (% of dose)

	10.5 g kg^{-1}		52.4 g kg^{-1}		104.7 g kg^{-1}	
Metabolite	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
10 CHOFA	1.0	1.4	8.1	9.7	22.4	27.5
10 CHOTHF	1.8	1.9	1.6	2.0	2.0	2.2
5 MeTHF	6.2	6.9	7.2	8.5	4.2	4.8
Folate S	3.5	3.6	1.3	1.7	0.4	0.5
p-acetamidobenzoate	-	4.3	-	3.2	-	1.7
p-acetamidobenzoyl-L-glutamate	-	1.5	-	1.1	-	0.2
$^{3}H_2O$	-	1.7	-	1.4	-	1.2
Pterin P	2.5	0.8	2.1	0.9	0.9	0.3
Urea	0.5	-	0.6	-	0.4	-
Total	15.5	22.1	20.9	28.5	30.3	38.4

Table 5.11 Derivatives present in liver after administration of 10 CHOFA at three different doses to rats

Method (a)

	Dose (10 CHOFA)					
	10.5 μg kg^{-1}		52.4 μg kg^{-1}		104.7 μg kg^{-1}	
Derivative	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
Folate $(glu)_n$	64	69	60	69	70	80
Formyl folates + tritiated fragments		23		13		19		9		15
pterin	31	-	8	-	15	-

Method (b)

	Dose (10 CHOFA)			
	52.4 μg kg^{-1}		104.7 μg kg^{-1}	
Derivative	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
Folate $(glu)_n$	68	66	51	59
Formyl folates	8	9	24	27
tritium fragments	-	17	-	12
pterin	8	-	17	-

Table 5.12 Derivatives found in liver after 10 CHOFA administration to rats

Method (c)

Without TCA treatment

Dose level	10.5 μg kg^{-1}	
	% ^{14}C	% ^{3}H
Folate $(glu)_n$	17	29
Formyl folates	27	35
tritium fragments	-	32
pterin	52	-

With TCA treatment

Dose level	52.4 μg kg^{-1}	
	% ^{14}C	% ^{3}H
Folate $(glu)_n$	46	54
Formyl folates	22	25
tritium fragments	-	21
pterin	26	-

Table 5.13 Derivatives found in liver after 10 CHOFA administration to rats

Method (d)

Without TCA treatment

Dose level	52.4 μg kg^{-1}	
	% ^{14}C	% ^{3}H
Folate $(glu)_n$ & protein bound folate	42	40
Formyl folates	41	43
tritiated fragments	-	10
pterin	15	7

With TCA treatment

Dose level	104.7 μg kg^{-1}	
	% ^{14}C	% ^{3}H
Folate $(glu)_n$	22	26
Formyl folates tritiated fragments	50	62
pterin	25	9

Table 5.14 Subcellular distribution of radioactivity in rat liver cells 48 h after administration of radiolabelled 10 CHOFA (104.7 μg kg^{-1})

		% of total fractions				
	% Recovery from homogenate	Mitochondria (1)	Mitochondria (2)	Nuclear	Microsomal	Supernatant
^{3}H	85	44.9	10.6	0.7	1.8	42.1
^{14}C	81	47.0	11.2	0.5	2.4	38.8
Protein	69	24.6	11.1	4.7	15.7	43.9
Acid phosphatase	80	13.1	56.1	0.9	14.3	15.6
Succinate-cytochrome c reductase	76	93.4	6.5	0.2	-	-
RI-NADH-cytochrome c reductase	83	22.3	20.3	2.1	49.1	7.6

Each value represents the mean of two separate fractionations

Table 5.15 Distribution of radioactivity in the tissues 48 h after the administration of 10 CHOFA (52.4 $\mu g\ kg^{-1}$) to normal rats and rats with implanted Walker 256 tumours

	Normal rats (n = 6)			Tumour bearing rats (n = 5)		
Tissue	Wet wt.	% ^{14}C	% ^{3}H	Wet wt.	% ^{14}C	% ^{3}H
Liver	8.3	7.62	7.59	7.72	12.43*	15.13
(± SEM)	(±0.52)	(±0.87)	(±0.74)		(±1.22)	(±1.50)
Tumour				8.19	4.73	5.18
(± SEM)					(±1.21)	(±1.33)
Intestine	8.68	2.15	2.05	3.94	1.74	1.98
(± SEM)	(±0.81)	(±0.23)	(±0.22)		(±0.21)	(±0.27)
Kidney	1.68	1.42	1.40	1.93	2.38	2.66
Lungs	2.16	0.13	0.12	1.68	0.19	0.20
Testes	2.48	0.16	0.20	2.32	0.22	0.22
Spleen	0.57	0.16	0.16	0.66	0.29	0.32
Heart	1.05	0.16	0.14	0.96	0.06	0.06
Sternum	0.13	0.01	0.01	0.08	< 0.01	< 0.01
TOTAL		11.86	11.73			

* Significantly more radioactivity was found in the livers of tumour bearing rats compared to normal controls ($p < 0.05$)

Table 5.16 Distribution of radioactivity in the urine, faeces and expired air collected 48 h after the administration of 10 CHOFA (52.4 $\mu g\ kg^{-1}$) to normal rats and rats with implanted Walker 256 sarco-carcinoma

	Normal rats (n = 6)		Rats with tumours (n = 5)	
	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
Urine				
0- 6 h	13.01±1.19	17.38±1.75	11.91±1.03	14.30±1.24
	$p < 0.001$		$p < 0.001$	
6-24 h	7.63±0.45	11.58±0.47	7.18±1.22	11.20±1.60
	$p < 0.001$		$p < 0.01$	
24-48 h	2.15±0.17	2.98±0.14	1.78±0.27	2.33±0.35
	$p < 0.05$		$p < 0.01$	
Total	22.79±1.48	31.94±1.64	20.87±1.35	27.82±1.64
	$p < 0.001$		$p < 0.001$	
Faeces				
0-48 h	41.87±3.36	27.29±1.87	32.60±2.77	20.68±2.46
	$p < 0.001$		$p < 0.001$	
Expired CO_2				
0- 6 h	2.50		0.95	
6-24 h	1.01		1.58	
24-48 h	0.06		0.05	
Total	3.58		2.58	

Values are expressed as the percentage of the dose ± SEM. The values of P quoted refer to the statistical significance of the difference between the % ^{14}C and the % ^{3}H found, determined by a paired "t" test.

No significant differences were found between recoveries of radioactivity in the urine and faeces of the two groups of rats.

Table 5.17 Distribution of metabolites in urine samples after a dose of 52.4 μg kg^{-1} of 10 CHOFA to rats bearing implanted Walker 256 tumours

	0-6 h		6-24 h		24-48 h	
Metabolite	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
10 CHOFA	43.2	38.7	23.2	18.9	10.3	9.1
	(5.1)	(5.5)	(1.7)	(2.1)	(0.2)	(0.2)
10 CHOTHF	11.2	10.4	8.2	5.8	7.3	5.3
	(1.3)	(1.5)	(0.6)	(0.6)	(0.1)	(0.1)
5 MeTHF	26.1	24.2	25.1	17.7	6.5	4.7
	(3.1)	(3.6)	(1.8)	(2.0)	(0.1)	(0.1)
Folate S	2.9	3.1	5.4	4.4	4.4	3.2
	(0.3)	(0.4)	(0.4)	(0.5)	(0.1)	(0.1)
p-acetamidobenzoate	-	3.4	-	25.3	-	9.2
		(0.5)		(2.8)		(0.2)
p-acetamidobenzoyl-L-glutamate	-	2.4	-	6.9	-	16.0
		(0.3)		(0.8)		(0.4)
$^{3}H_2O$	-	0.8	-	6.7	-	26.9
		(0.1)		(0.7)		(0.6)
Pterin P	5.2	0.9	15.1	4.4	48.1	6.0
	(0.6)	(0.1)	(1.1)	(0.5)	(0.9)	(0.1)
Urea	1.0	-	5.7	-	10.3	-
	(0.1)		(0.5)		(0.2)	
Pteridine W	1.8	1.4	4.1	3.3	ND	ND
	(0.2)	(0.2)	(0.3)	(0.3)		
Total	91.4	85.3	86.8	93.4	86.9	80.4

Table 5.18 Radiolabelled metabolites excreted in the urine of normal rats and rats with implanted Walker 256 sarco-carcinoma 0-48 h after the administration of 10 CHOFA (52.4 $\mu g\ kg^{-1}$)

	Normal rats % Dose		Rats with tumours % Dose		% Normal rat metabolite level	
	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
10 CHOFA	8.1	9.7	7.0	7.8	86	80
10 CHOTHF	1.6	2.0	2.0	2.2	125	110
5 MeTHF	7.2	8.5	5.0	5.7	69	67
Folate S	1.3	1.7	0.8	1.0	62	59
p-acetamidobenzoate	-	3.2	-	3.5	-	109
p-acetamidobenzoyl-L-glutamate	-	1.1	-	1.5	-	136
$^{3}H_2O$	-	1.4	-	1.4	-	100
Pterin P	2.1	0.9	2.6	0.7	124	78
Urea	0.6	-	0.8	-	133	-
Compound W	ND	ND	0.5	0.5		
Total	20.9	28.5	18.7	24.3		

Table 5.19 Summary of the derivatives found in extracts of host livers and tumours prepared by different methods (for details see text)

Extraction Method	Host liver		Tumour	
	% ^{14}C	% ^{3}H	% ^{14}C	% ^{3}H
Hot ascorbate (a)				
Folate $(glu)_n$	70	69	58	60
10 CHOTHF	9	10	11	12
Fragments	21	20	30	27
Autolysis (d)				
Folate $(glu)_n$	9	10	3	5
Formyl folates	70	73	24	26
Fragments*	20*	16*	67	66
* Possibly includes a small amount of 5 MeTHF (about 5%)				
TCA (f)				
High mol.wt.derivs.	12	28	11	23
^{14}C I (Xanthopterin)	10	-	14	-
II (Dihydropterin)	58	-	51	-
III (pterin)	17	-	14	-
^{3}H I	-	32	-	31
II	-	36	-	43

Table 5.20 Subcellular distribution of radioactivity in tumour and host liver cells 48 h after the administration of radiolabelled 10 CHOFA (52.35 $\mu g\ kg^{-1}$) to rats bearing implanted Walker 256 tumours

	% Recovery from homogenate		% of total of fractions									
			Mitochondria (1)		Mitochondria (2)		Nuclear		Microsomal		Supernatant	
	Liver	Tumour	Liver	Tumour	Liver	Tumour	Liver	Tumour	Liver	Tumour	Liver	Tumour
^{3}H	86.5	74	32.1	9.1	7.6	7.8	1.7	9.6	1.3	1.2	57.3	72.3
^{14}C	89.5	115	34.7	8.8	7.3	5.9	2.2	9.6	3.9	5.8	51.5	69.9
Protein	75	80	25.8	12.8	14.4	8.2	6.1	16.4	11.5	9.4	42.2	53.3
Acid phosphatase	74	137	17.4	17.5	55.5	15.9	3.9	15.5	8.7	8.1	14.5	43.1
Succinate - cytochrome c reductase	52	47	82.3	62.4	17.1	37.6	0.6	0	0	0	0	0
RI-NADH-cytochrome c reductase	69	48.5	26.8	9.4	24.6	5.0	1.9	0	44.2	81.9	2.5	3.7

Each value represents the mean of two separate fractionations

Table 5.21 The distribution of radioactivity in the tissues, faeces, urine and expired air of normal rats (n = 2) 48 h after the administration of [3', 5', 9(n) - ^{3}H] [10-^{14}CHO] folate

Tissue		Wet weight	% Dose % ^{14}C	% Dose % ^{3}H
Liver		7.3 g	0.81	3.72
Intestine		6.1 g	0.80	1.01
Kidney		1.73 g	0.25	0.78
Faeces		6.1 g	27.62	17.75
Urine	0- 6 h		19.85	30.01
	6-24 h		8.58	9.59
	24-48 h		8.02	2.80
	Total		36.45	42.40
Expired CO_2	0- 6 h		2.49	-
	6-24 h		4.80	-
	24-48 h		7.27	-
	Total		14.56	-
TOTAL			80.49	65.66

CHAPTER 6

THE IDENTIFICATION AND CHARACTERIZATION OF THE PRODUCTS OF FOLATE CATABOLISM IN THE RAT

Studies reported in this thesis have shown that following the administration of folic acid, 10 CHOFA and 10 CHOFA$(glu)_4$ to rats fragmentation products are found in the urine and that folate derived hydrogen and carbon are found in the breath as H_2O and CO_2. To elucidate the mechanism of catabolism *in vivo* the identification of the folate catabolites is essential. Blair (1958 a) and more recently Murphy *et al* (1978) suggested that a possible metabolic route of folate catabolism would be via cleavage of the C9-N10 bond in the tissues and subsequent excretion of the resultant fragments and their metabolites. Other workers have shown that the structurally related antifolate drug methotrexate undergoes catabolism by loss of the glutamic acid residue yielding a pteroate analogue, probably arising from metabolism by the gut microflora (Valerino *et al*, 1972; Valerino, 1972). Although the enzyme has not been reported in the mammal both the bacterial enzyme known as carboxypeptidase G1 (Albrecht *et al*, 1978) and the related protozoal (Crithidia) enzyme (Iwai *et al*, 1979) will cleave folic acid and other folate derivatives. Since the biliary excretion of folate is extensive (Lavoie and Cooper, 1974; Hillman *et al*, 1977; Pheasant, Connor and Blair, unpublished observations) catabolism by the gut bacteria could occur during enterohepatic circulation.

Work in this laboratory using dual labelled folates has demonstrated the presence of three tritium catabolites and at least two ^{14}C labelled catabolites (Connor *et al*, 1977; Connor *et al*, 1979) in rat urine. Experiments on the determination and characterisation of these catabolites are described in the present Chapter and their possible origin is discussed.

6.1 Solely Tritium Labelled Catabolites

Materials and Methods

DEAE-cellulose chromatography of the urine of animals dosed with mixtures of [2-^{14}C] and [3', 5', 9-^{3}H] folate and sequential Sephadex

G15 gel filtration resolves the urine into three solely tritiated peaks. These were identified as ^3H_2O, p-acetamidobenzoate, and p-acetamidobenzoyl-L-glutamate by the methods described below.

1. ^3H_2O

One of the tritium metabolites eluted at the void volume on DEAE-cellulose ion exchange chromatography indicating that it was a neutral or positively charged molecule at pH7. The metabolite eluted on Sephadex G15 at the salt resolution limit (about fraction 20) in the precalibrated position of authentic tritiated water. On freeze drying of the chromatographed metabolite or of raw urine sample the radioactivity attributable to it was lost demonstrating the volatility of the compound. On crude distillation of the peak containing the metabolite (after passage down an ion exchange column) the radioactivity distilled over with water (at 100°C) and continued to do so on three subsequent distillations. During these distillations the specific radioactivity (counts per ml) remained constant. The only other volatile derivative likely to be produced from folate catabolism or degradation is formaldehyde derived from the C9 carbon atom released during the chemical oxidation of THF and DHF (Chippel and Scrimgeour, 1970) and this differed in chromatographic properties; any formaldehyde generated *in vivo* would probably be oxidised to formate and water. Since ^3H_2O was also found in the expired air it was concluded that the volatile metabolite was ^3H_2O.

2. p-acetamidobenzoate

Gel filtration on Sephadex G15 of the major tritium containing peak obtained by DEAE-cellulose chromatography of urine samples gave a major peak with K_{av}=1.73 (Metabolite Q). The material was not volatile and was soluble in both ethylacetate and methanol. Repeated extraction of the acidified metabolite in water with ethylacetate gave steadily diminishing recoveries in the organic phase and the partition coefficient

(conc. in acidified water ÷ conc. in ethylacetate) was not constant (5.2; 55.3; 490 for three successive partitions) and thus the simple partition law was disobeyed. This preliminary data was consistent with the behaviour of a dissociable organic acid and in view of its origin the probable retention of the p-aminobenzoate moiety. On chromatographic analysis using column and paper systems with several authentic p-aminobenzoate derivatives (table 6.1) the catabolite cochromatographed with p-acetamidobenzoic acid. A mixture of p-acetamidobenzoic acid (50 mg) and the catabolite was hydrolysed in 2 M-HCl for 1 hour at 100°C and the products extracted with ethylacetate (5 vols). On chromatography the radioactivity and u.v. absorbance continued to cochromatograph, but now coeluting with p-aminobenzoic acid (the expected hydrolysis product of p-acetamidobenzoic acid) (table 6.1).

Further confirmation of the identity of the metabolite was achieved by reverse isotope dilution analysis. The catabolite and p-acetamidobenzoate (100 mg) were dissolved in the minimum amount of boiling water. After cooling (slowly) the crystals were recovered and recrystallized a further five times from boiling water. At each stage a small quantity of crystals were removed and the specific radioactivity determined by counting samples in a scintillation counter and estimating the concentration spectrophotometrically (λ max = 262 nm at pH7.0). The graphical plot of radioactivity vs. concentration (absorbance) obtained is illustrated in figure 6.1. The specific radioactivity was constant throughout the recrystallizations with a correlation coefficient between counts and concentration of unity ($r = 1$).

The variety of chemical and physical properties described above identifies p-acetamidobenzoate as a major catabolite of the folate pool (Connor et al, 1979).

3. p-acetamido-benzoyl-L-glutamate

Sephadex G15 chromatography of the catabolite containing peak obtained on DEAE-cellulose chromatography of urine samples contained variable amounts of an early eluting metabolite (metabolite R) (Kav = 0.66) as well as the p-acetamidobenzoate peak. Metabolite R was soluble in methanol, slightly soluble in ethylacetate and eluted in a similar position to p-aminobenzoyl-L-glutamate on column chromatography although on cochromatography the radioactive peak (ie metabolite R) and the absorbance of the p-aminobenzoyl-L-glutamate marker did not match exactly, metabolite R eluting slightly later on both DEAE-cellulose and Sephadex G15. Independent studies (Murphy et al, 1976) suggested that p-acetamidobenzoyl-L-glutamate was a long-term folate catabolite and this was therefore compared to metabolite R. On Sephadex G15, DEAE-cellulose and paper chromatography (Propanol : ammonia : water; 200 : 1 : 99) p-acetamidobenzoyl-L-glutamate and R were inseparable. In view of the precedent of Murphy et al and the preliminary observations reported above it was concluded that metabolite R was p-acetamidobenzoyl-L-glutamate.

6.2 Catabolites Containing Carbon-14

Urine from rats dosed with [2-^{14}C] folate contained at least two ^{14}C labelled catabolites in appreciable quantities and in some urine samples traces of a third compound. The latter derivative when present appeared in small amounts (less than 2% ^{14}C of urine samples) and was evident as a slowly migrating peak on Sephadex G15 chromatography of raw urine samples. It eluted in a similar position to dihydroxanthopterin (fraction 50-55) although further confirmation of its identity was not obtained due to the low concentrations found. The unpredictable occurrence and possible identity of the compound (i.e. dihydroxanthopterin) suggest that it is probably a degradation product formed from a labile precursor (see below) during urine collection. Of the two

significant ^{14}C containing catabolites the minor one has been characterised as urea (Chapter 5). Although the major derivative (pteridine P) has not been identified it has been extensively characterised and this is described below.

Characterization of Pteridine P

1. Isotope Content

Pteridine P from the urine of rats dosed with mixtures of [2-^{14}C] and [3', 5', 7, 9 - ^{3}H] folate contained both isotopes. Compared to the dosed compound (10 CHOFA expts) pteridine P had a lower ^{3}H content (1.94 μCi ^{3}H per μCi ^{14}C for pteridine P; 5.0 μCi ^{3}H per μCi ^{14}C for the dosed compound) retaining 38% of the original ^{3}H present. The commercially obtained tritiated folic acid used contained 59% of the tritium in the C7 and C9 positions determined by degradation studies. Recent ^{3}H n.m.r. studies (Radiochemical Centre, Amersham) indicate that about 20% of the ^{3}H is located at C7.

2. Chromatography on Sephadex G15 : the Elimination of Simple Pteridines

On Sephadex G15 pteridine P eluted at fraction 41 (kav = 2.05). By cochromatography its non-identity with the following pteridines was established (for a summary of their chromatographic properties see Chapter 2): xanthopterin, isoxanthopterin, pterin-6-COOH, pterin-6-CHO, 6OH-methylpterin, 6-methylpterin, pterin, dihydropterin, 6OH-methyldihydropterin, lumazine, 6-oxolumazine, 6,7-dihydroxylumazine, 7-oxolumazine, leucopterin, dihydroxanthopterin, neopterin, biopterin, dihydrobiopterin, tetrahydrobiopterin, 7,8-dihydropterin-6-aldehyde, pterin-6-sulphonic acid, lumazine-6-COOH. Solutions of dihydrofolate, 5 MeTHF, 10 CHOFA or folic acid at pH7 (0.05 M-sodium phosphate buffer) stored at room temperature for 48 hours failed to decompose to products similar to pteridine P.

3. Microbiological Assay

Aliquots of pteridine P were assayed for microbiological

activity with the folate requiring bacteria L. casei, S. faecalis and P. cerevisiae and with the biopterin dependent protozoan Crithidia fasciculata. No activity was observed with any of the microorganisms tested. Since the folates occur in microgram quantities in rat urine and considerable dilutions of samples are normally required for efficient assay, it was concluded that pteridine P is microbiologically inactive.

4. Enzyme Assay for Pteridine Phosphates and Glucuronides

Naturally occurring pteridine phosphates are intermediates in the biosynthesis of both folate (Shiota et al, 1964) and the 6-alkyl-pterin derivatives such as tetrahydrobiopterin (Brown, 1971). Glucuronide conjugation of drugs is a well known detoxification mechanism (Parke, 1968). Both purified pteridine P and raw urine were treated with β-glucuronidase and alkaline phosphatase and the products examined chromatographically.

a) Alkaline phosphatase (After the method of Bessey et al, 1946) Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) (100 μl containing sufficient enzyme to hydrolyse 20 μM-p-nitro-phenol-phosphate per min) and pteridine P (1,000 dpm) were incubated together for 90 minutes at 37°C in the dark in 0.1 M-glycine buffer pH10.4 containing 0.2 mmol L^{-1} $ZnCl_2$, 0.2 mmol L^{-1} $MgCl_2$ and sodium ascorbate (2% w/v) in a total volume of 10 ml. A blank incubation was performed substituting albumin (100 μl of a solution of 1 mg ml^{-1} Bovine serum albumin) for the enzyme.

G15 chromatography of the products gave identical chromatograms for both the blank and the enzyme incubations each containing three peaks: Pteridine P (58% ^{14}C), unknown (possibly xanthopterin) (28% ^{14}C) and a minor unknown (Kav = 1.09) (14% ^{14}C). The latter two derivatives were presumably derived by chemical degradation under the conditions of assay.

b) β- glucuronidase (After the method of Stahl and Touster, 1972) β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3. 2.1.3 from bovine liver) (100 µl containing sufficient enzyme to liberate 10 mg of phenolphthalein from phenolphthalein glucuronide per hour under the assay conditions) and pteridine P (1,000 dpm) were incubated for 90 min in 0.1 M-sodium acetate buffer pH5 containing sodium ascorbate (2% w/v) at 37°C in the dark. A blank incubation substituting 100 µl of albumin (1 mg ml^{-1}) for the enzyme and a similar incubation with 5 ml of raw urine (24-48 h urine sample from normal rats dosed with 52.4 µg kg^{-1} 10 CHOFA) were also performed.

Sephadex G15 chromatograms of the pteridine P incubations were identical for both blank and enzyme incubations each again containing three peaks: Pteridine P (63% ^{14}C); unknown (xanthopterin) (26% ^{14}C) and a minor unknown (Kav = 1.54, different from the minor unknown found in the phosphatase assays) (11% ^{14}C). The blank and enzyme treated urine samples chromatographed identically.

It was concluded that pteridine P was not a phosphate or a glucuronide derivative.

5. Large Scale Purification

In an attempt to obtain sufficient of pteridine P for spectral analysis a large scale purification from urine was attempted. Urine (630 ml collected from rats dosed with 10 CHOFA) was applied to a DEAE-cellulose column, washed with 0.05 M-sodium phosphate buffer pH7 containing DTT (5 mg 100 ml^{-1}) and eluted with a 0-1.2 M-NaCl gradient. Fractions containing P (and 10 CHOTHF + p-acetamidobenzoate) were pooled and freeze dried. The freeze dried residue was taken up in 10 ml of water and applied toa Sephadex G15 column and eluted with 0.05 M-sodium phosphate buffer pH7. The expected pteridine P peak was absent being replaced by two ^{14}C containing peaks eluting at fractions 49 (pteridine I, Kav = 2.56) and fraction 55 (pteridine II, Kav

= 2.95) both retaining ^{3}H in a similar $^{14}C/^{3}H$ ratio as pteridine P. On paper chromatography in butanol : acetic acid : water (80 : 5 : 25) pteridine II gave a single spot (Rf = 0.03) but pteridine I gave two spots (Rf = 0.03 containing 64% ^{14}C and Rf 0.23 containing 35% ^{14}C) suggesting that pteridine II was derived from pteridine I. On paper electrophoresis pteridine I migrated towards the anode (25 mm) whereas xanthopterin (-6 mm) and dihydroxanthopterin (-7 mm) migrated towards the cathode (electrophoresis performed as described in Chapter 2 using a pH4.7 0.1 M-sodium acetate buffer as the electrolyte and a constant voltage of 20 Vcm^{-1}) and may therefore contain an acidic function. On further chromatography in propanol : ammonia : water (200 : 1 : 99) pteridine I gave a spot at Rf = 0.12 and pteridine II at Rf = 0.28. On repeated chromatography however pteridine I decomposed to a derivative which cochromatographed with xanthopterin in this solvent (Rf = 0.14) and also on a Sephadex G15 column.

When the dried paper chromatograms were viewed under u.v. light faint blue and green fluorescent bands were seen at the same place as pteridine I and pteridine II respectively. The spots were eluted (using 1% ammonia) and the u.v. spectra determined (pteridine I : pH1 λ max = 269 nm; pH7 = 265 nm; pH13= 266 nm; 350 nm : pteridine II : pH1 λ max = 274 nm; pH7 λ max = 274 nm; pH13 λ max = 292 nm, 350 nm). Since both pteridines underwent degradation during chromatography and as the degree of purity was unknown exactly which compounds the u.v. absorbance corresponds to is unclear.

The information obtained from this study revealed that pteridine P was labile decomposing on freeze drying to several compounds including probably xanthopterin. Since xanthopterin was apparently produced by degradation of pteridine P then pteridine P is identified as a pterin derivative (i.e. a derivative of 2-amino-4OH-pteridine) and is not a substituted lumazine (i.e. a derivative of 2,4 dihydroxy pteridine)

as have been reported as degradation products of tetrahydrobiopterin metabolism in the rat (Rembold et al, 1971).

6. The Production of Pteridine P after Large Doses of Folic Acid to the Rat

A single male Wistar rat (200 g) housed as described in Chapter 2 was dosed with [2-^{14}C] folic acid (2 μCi; 77 μg kg^{-1}) and 6 hours later with unlabelled folic acid (50 mg). The same dose schedule was repeated for a further 3 days. Urine and faeces were recovered for analysis up to 24 h after the administration of the last dose of unlabelled folic acid. Total recovery of radioactivity in the urine was determined daily (table 6.2) and the excretion of Crithidia factor (i.e.biopterin like compounds) measured by microbiological assay with C. fasciculata. The animal failed to produce faeces after day 2 and on autopsy at the end of the experiment was found to have a considerable amount of dry faecal pellets retained in the lower intestine indicating severe intestinal malfunction. Urinary volume was high on day 1 and day 2 (51 and 43 ml respectively) returning to normal levels on days 3 and 4 (20 ml and 18 ml respectively). Excretion of Crithidia activity remained constant during the experiment (50 μg in the 24 h before the experiment; 76.5 μg on day 1, 55.4 on day 2, 52.6 μg on day 3, 56.3 μg on day 4).

Urine samples were chromatographed on DEAE-cellulose and the radioactive peaks rechromatographed on Sephadex G15. The metabolites identified are given in table 6.3 for each daily urine. The peaks containing pteridine P were pooled and reapplied to a DEAE-cellulose column to concentrate the material, and the u.v. spectrum determined (pH1 λ max = 260 nm, 269 nm; pH7 λ max = 258 nm; pH13 λ max = 240 nm , 266 nm, 296 nm). No fluorescence activity associated with the radioactivity was detected. The purified material was then subjected to the chemical degradations described below.

Chemical Degradation Studies

1. Permanganate Oxidation (Results summarised in table 6.4)

acid : Fractions containing pteridine P from chromatography of urine samples were acidified to pH1 using a few drops of c.HCl and 50 µl KMO_4 (4% in water) was added. A slight excess persisted since the solution remained pale pink. Three peaks were found on column chromatography of the product a minor (fraction 19, kav = 0.70, 5% ^{14}C) major (fraction 25, kav = 1.03, 65% ^{14}C), minor (fraction 32, kav = 1.47, 24% ^{14}C). The major peak had a $^{14}C/^{3}H$ ratio similar to pteridine P but tritium was not detected in peak 3 (kav = 1.47).

alkali : Fractions containing pteridine P were adjusted to pH12 with 200 µl c.NaOH.$KMnO_4$ (100 µl 4% solution) was added and the reaction mixture stirred for 5 min after which time H_2O_2 (200 µl) was added to remove excess permanganate. The pH was readjusted to pH7 and decanted to remove the small particles of MnO_2 present. Sephadex G15 chromatography gave 2 overlapping peaks; a major kav = 0.70 (xanthopterin) (61% ^{14}C) and a minor at fraction 25 kav = 1.03 (39% ^{14}C). Tritium had been lost from both peaks and a volatile ^{3}H peak produced, although 50% of the ^{3}H of pteridine P was retained in the xanthopterin peak. When an excess of xanthopterin (50 mg) was added to peak 1 and stirred for 1 hour at room temperature over 50% of the radioactivity was lost from solution.

2. Iodine Oxidation (Results summarised in table 6.4)

These were performed on pteridine P (labelled with ^{14}C only) obtained from the high dose folic acid experiment.

Mild oxidation : To pteridine P in 0.1 M-sodium acetate buffer pH5 was added iodine (1 ml of a solution of 30 mg iodine in 100 ml 0.1 M-sodium iodide) and the mixture heated to 70°C for 30 min. A similar reaction was performed in which ascorbate (0.2 g) was added after heating. Three peaks were found on Sephadex G15 chromatography

at kav = 0.70 (xanthopterin ?) (12% ^{14}C and 24% ^{14}C respectively) at kav = 1.47 (19% ^{14}C and 36% ^{14}C respectively) and at kav = 2.05 (unchanged pteridine P) (68% ^{14}C and 40% ^{14}C respectively).

Vigorous oxidation : Since degradation of pteridine P appeared to be incomplete in the "mild" iodine oxidation reactions described above pteridine P was oxidised with a large excess of iodine (10 ml of iodine solution) at pH7 for 30 min at 70°C. Sephadex G15 chromatography gave a single peak at kav = 1.03 (fraction 25) although a shoulder was evident at about fraction 32 (kav = 1.47). The peak and shoulder were freeze dried and subjected to paper chromatography (propanol : ammonia : water 200 : 1 : 99). The peak gave 3 spots at Rf = 0.13 (possibly xanthopterin) (68% ^{14}C) Rf = 0.29 (17% ^{14}C) and Rf = 0.45 (possibly pterin) (15%) whilst the shoulder gave a single identifiable peak at Rf = 0.45 (possibly pterin).

3. Dithionite Reduction

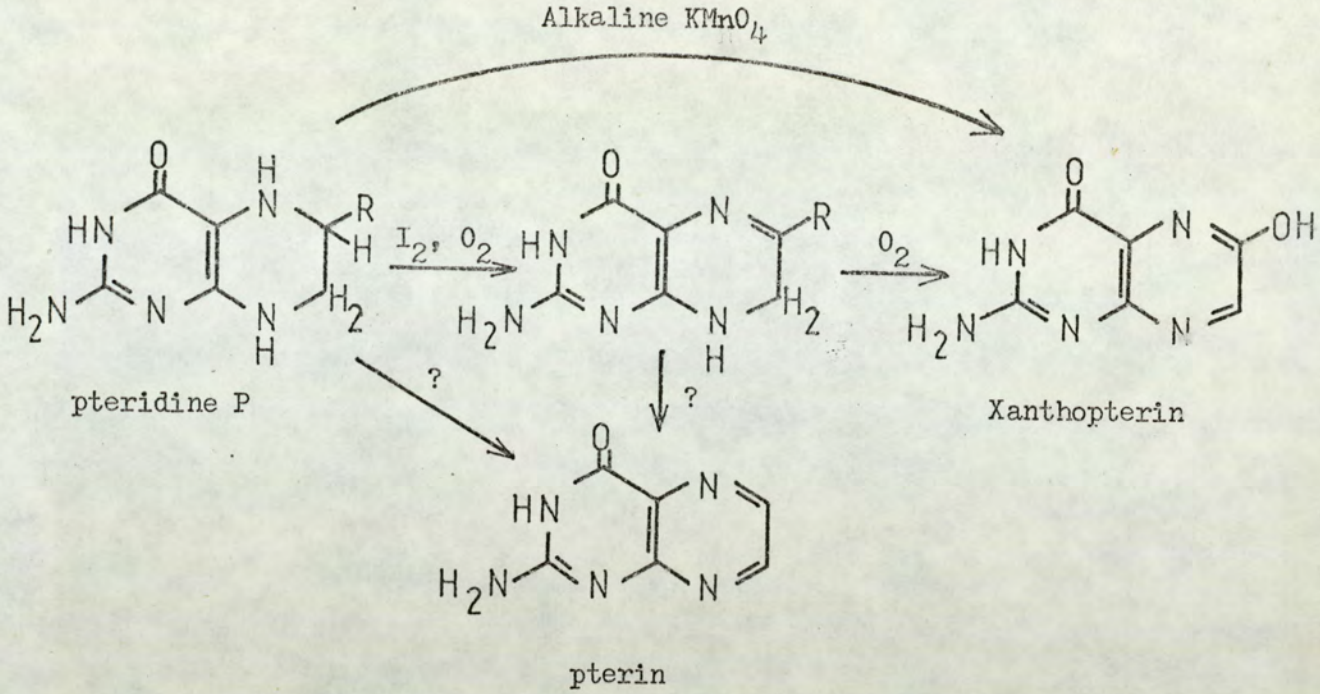
To pteridine P in 0.1 M-sodium acetate buffer, pH5 (containing sodium ascorbate 2% w/v) was added sodium dithionite (100 mg). The reaction vessel was left in the dark at room temperature for 1 hour and then frozen. On Sephadex G15 chromatography a single peak of unchanged pteridine P was observed.

The variable products obtained with the different oxidation procedures employed (table 6.4) and the failure to effect reduction of pteridine P with dithionite are incompatible with a fully oxidised ring system. The ultimate products of oxidation included largely xanthopterin but small amounts of pterin-like material were produced. The appearance of the latter indicates a fully reduced ring system i.e. to the tetrahydro level rather than the dihydro level of reduction since pterin is a product of the oxidation of THF but not DHF (Chippel and Scrimgeour, 1970). Both iodine (Bailey and Ayling, 1977) and acidic permanganate treatment can partially oxidise substituted

reduced pterins leaving the side-chain group (at C6) intact. Alkaline permanganate oxidation of substituted pterins is more severe and the side chain is either oxidised to pterin 6 COOH (Maclean et al, 1966) or lost. In the present study pteridine P was oxidisable to xanthopterin apparently via an air labile derivative (kav = 1.03). This may indicate that under certain conditions (I_2 at pH7 and acidic permanganate) pteridine P is either rearranging to a more stable reduced form (under the conditions of oxidation) or that a partially reduced derivative is being formed. In view of the known behaviour of pteridine derivatives under the latter conditions production of the partially reduced derivative would seem more likely. Chemical models exist for the production of partially reduced intermediates, for example oxidation of THF produces pterin (via dihydropterin) and dihydroxanthopterin (Chippel and Scrimgeour, 1970; Pearson, 1974) and oxidation of DHF and dihydrobiopterin to dihydropterin-6-CHO (Whiteley et al, 1968; Mengel et al, 1977).

Confirmation of the reduced state of pteridine P can also be deduced from the albeit limited spectral properties observed. Reduced pterins tend to have both simpler u.v. absorbance spectra and λ max at lower wavelengths than the corresponding oxidised forms (Blakley, 1969) and this may explain the absence of absorbance above 300 nm in the spectrum obtained from the column purified pteridine P. Several reduced pterin derivatives fail to exhibit the often marked fluorescence of their oxidised forms (e.g. tetrahydropterin and dihydropterin compared to pterin) and thus the absence of fluorescence associated with pteridine P is not entirely unexpected.

The observed chemical properties of P may be summarised as below:



The structure of pteridine P illustrated is of a C6 substituted tetrahydropterin. Naturally occurring substituted pteridines are usually substituted at the N5 or C6 position. Since the N5 substituted compounds such as 5 MeTHF and 5 CHOTHF resist oxidation, probably due to steric hindrance at N5 and the inability to form the transitory quinonoid structures believed to be required prior to loss of the side chain, pteridine P is unlikely to be N5 substituted. Although substitution at the C7 position is possible the formation of xanthopterin from pteridine P and the retention of some of the C7 tritium (observed in the oxidation of the dual labelled material) are incompatible with a structure of this type.

The nature of the side chain remains unknown. Since aerial oxidation of pteridine P apparently leads to the formation of an acidic intermediate (as observed in 6.2.5) it may contain an acidic function. The failure to detect interference with Crithidia factor excretion by large doses of folic acid would suggest that the side chain of pteridine P bears little resemblance to those of the 6-(dihydroxypropyl)-pterins (i.e.biopterin like compounds).

6.3 Discussion

1. The Origin of the Major Folate Catabolites

In the present study quantities of p-acetamidobenzoate, p-acetamidobenzoyl-L-glutamate and $^{3}H_2O$ were found in urine samples and these are summarised in table 6.5. However since the source of the tritium label was [3', 5', 7, 9 - ^{3}H] folate and only 41% of the tritium is sited at the 3', 5' positions the actual amount of folate cleaved is greater than that indicated by the quantity of urinary catabolites recovered and appropriate corrections are given (table 6.5). Thus at the low dose of 10 CHOFA given to rats at least 14% of the dose was metabolised to scissionproducts. Folate scission reactions are thus of considerable physiological significance.

Several reports in the early literature of folate degrading enzymes exist. Silverman et al (1957) purified an enzyme from porcine liver which could generate a diazotizable amine (p-aminobenzoyl-L-glutamate) and pterins from citrovorum factor (5 CHOTHF). Detailed investigation revealed that the enzyme was N-formyl-L-glutamate : tetrahydrofolate-5-formyl-transferase (EC 2.1.2.6) which catalysed the reaction

5 CHOTHF + L-glutamate $\rightleftharpoons$ THF + N-formylglutamate

the THF generated then underwent chemical degradation yielding the observed scission products. Chicken liver extracts (Futterman and Silverman, 1957), rat liver slices (Dinning et al, 1957), rat liver cytoplasm (Connor and Vince, unpublished observations) and human red blood cells (Braganca et al, 1957) can also cleave folic acid under aerobic conditions. The enzyme responsible in each of the latter studies was probably dihydrofolate reductase and the scission products again derived by THF oxidation derived from the reduction of folate.

Whilst in vitro evidence of a specific folate cleaving enzyme in the mammal is lacking several possible folate catabolites have pre-

viously been detected in urine, although care needs to be taken in interpreting the early studies since these often failed to incorporate the use of suitable antioxidants and considerable degradation could have occurred during collection. Thus Koschara (1939) found xanthopterin and Johns et al (1961) p-aminobenzoyl-L-glutamate in human urine. The identification of metabolites of degradation products in urine is a more reliable indication of metabolic scission reactions. Blair (1957) and Fukushima et al (1972) isolated isoxanthopterin from human urine. Isoxanthopterin is the product of xanthine oxidase action on pterin in vitro (Lowry et al, 1949) and has recently been identified as a metabolite of pterin in the mouse in vivo (Knipe and McCormack, 1977). Both labelled isoxanthopterin and pterin have been isolated (albeit with extremely low yields of radioactivity) from the urine of a human subject dosed with [2-^{14}C] folic acid (Krumdieck et al, 1978). Dinnings et al (1957) summarily reported the presence of free and acetylated diazotizable amines in the urines of rats given large doses of folate and 5 CHOTHF. In none of these studies however was a catabolite with the properties of pteridine P described although a brief report (Floystrup et al, 1949) suggested that xanthopterin could be formed in situ from a non-fluorescent precursor excreted into the urine after folic acid administration.

The finding of labelled acetylated derivatives of p-aminobenzoate and p-amino- benzoyl-L-glutamate in the urine of rats dosed with tritiated 10 CHOFA in the present study and also after administration of folic acid (Connor et al, 1979) and 5 MeTHF. (Kennelly, Blair and Pheasant, unpublished observations) confirms that folate catabolism occurs in vivo since acetylation must occur prior to excretion. Interestingly although the two acetylated catabolites are very closely related chemically their urinary distributions suggest that they are metabolically discrete. Thus p-acetamidobenzoate is the dominant

catabolite in 0-6 h and 6-24 h urine samples whilst the relative amount of p-acetamidobenzoyl-L-glutamate increases with time and is most marked in the 24-48 h samples. The precedence of p-acetamidobenzoate apparently precludes its direct formation from p-acetamidobenzoyl-L-glutamate. However if the latter observation is true then the absence of labelled p-aminohippurate (i.e. the glycine conjugate) or glucuronide derivatives of p-aminobenzoate (demonstrated by the incubation of urine with β-glucuronidase) from the urine is difficult to reconcile since any p-aminobenzoate generated (a necessary step if we exclude prior acetylation) would be metabolized and excreted at least partially as the glycine or glucuronide conjugates (Tabor et al, 1951; Cohen and McGilvery, 1947).

The two conflicting pieces of data, i.e. the precedence of p-acetamidobenzoate and the absence of p-aminobenzoate metabolites from the urine can be reconciled by proposing that catabolite formation is regionalized within the body into two distinct areas. The generation of p-aminobenzoyl-L-glutamate in the tissues by either non-specific chemical oxidation or possibly enzymic degradation of the retained labile folates followed by acetylation and excretion would explain the time dependent occurrence of p-acetamidobenzoyl-L-glutamate found in the present study and also the finding of this derivative in urine 13 days after folic acid administration to rats (Murphy et al, 1976) since the half life of the retained folates is known to be quite long (Barford et al, 1977). Although the entero hepatic circulation of folate mono-glutamates is extensive (see introduction to this Chapter) and labelled folates appear in the bile within a few hours of dosing, labelled folate catabolites are absent from bile at least up to 10 h after dosing. Biliary excretion of folate catabolites would not be expected to be important in view of the low molecular weights involved and the relatively poor biliary excretion of p-aminobenzoic acid derivatives

reported in the literature (Abou-El-Makarem et al, 1967). However the persistent excess of ^{14}C over ^{3}H found in the faeces of rats dosed with mixtures of [2-^{14}C] and [3', 5', 7, 9 - ^{3}H] folate and the finding of pteridine P in faecal extracts indicates that excretion of catabolites is also occurring via the faeces. Since catabolites are absent from bile scission of the folate molecule must be occurring in the gut lumen. The products obtained from such scission reactions will depend upon several factors most importantly the mechanism of scission itself, (chemical, enzymic or gut-microfloral mediated degradation (or a combination of these) are all possible routes) and the subsequent metabolism by the intestinal cells of any catabolites formed, as the intestine itself is an important site for conjugation reactions. Intestinal metabolism of biliary excreted folate could thus account for the formation of p-acetamidobenzoate and its apparent independence of p-acetamidobenzoyl-L-glutamate. Since the folate excreted in the bile is derived largely from the free (i.e. unconjugated) tissue and plasma folate (Hillman et al, 1977) the production of p-acetamidobenzoate from radiolabelled 10 CHOFA or folic acid by the above route would be reasonably fast and would fall off as the level of labelled free folate was reduced by excretion, catabolism and tissue uptake. The latter predictions were borne out by the experimentally determined observations.

The model described above is summarised in figure 6.2. Neither the mechanism of catabolism nor the putative precursor required are known. Since catabolite production was similar after oral doses of 10 CHOFA and folic acid (Connor et al, 1979) and 5 MeTHF (J. Kennelly and J.A. Blair, unpublished observations) the precursor is probably a reduced, and thus chemically labile, folate derivative. Although this compound remains unknown the observation that 10 CHOFA, 10 CHOTHF and 5 MeTHF (13%, 19% and 52% of the biliary radioactivity respectively) make up the bulk of the labelled folate in the bile of rats up to 10 h

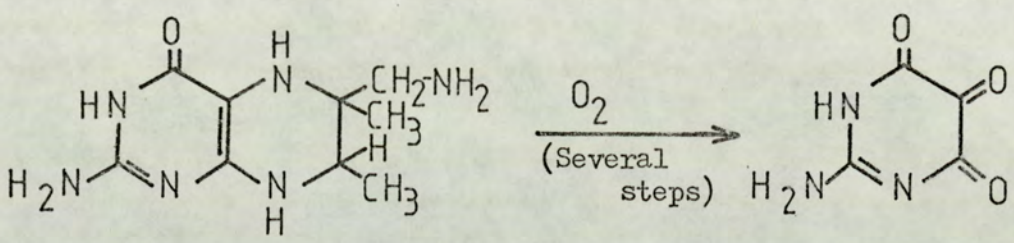
after an oral dose of folic acid (Pheasant, Connor and Blair, 1979 in preparation) suggests that the precursor is possibly 10 CHOTHF or closely related to it. Although little is known of the intestinal absorption of pterin derivatives their relatively low solubilities would suggest that they would be poorly absorbed. Confirmation of the poor absorption of reduced pterins may be inferred by the failure to detect significant increases in serum Crithidia factor levels after oral tetrahydrobiopterin administration to man (Ratanasthien, 1975). Thus any catabolites generated in the gut retaining the pteridine moiety would be lost in the faeces. The small amounts of pteridine P found in the urine may be attributed to the catabolism of the retained folates, corollary to the production of p-acetamidobenzoyl-L-glutamate.

2. The Degradation of the Pteridine Ring

Since both labelled CO_2 and urea were generated from [2-^{14}C] folate extensive degradation of the pteridine ring system must be occurring *in vivo*. The appearance of both metabolites was unexpected and such extensive catabolism has not been previously reported in the mammal. In the absence of experimental evidence of possible intermediates the mechanism of biological fragmentation of the pteridine ring system remains speculative but a brief review of two possible routes will be attempted.

Vigorous chemical oxidation of pterin-6-COOH (boiling in chlorate at acid pH) can lead to the generation of guanidine from the 2-aminopyrimidine portion of the molecule and this reaction was used in the original determination of the structure of folic acid (Stokstad *et al*, 1948). Although the oxidation of guanidine *in vivo* to urea would be possible the severity of the conditions required to form guanidine from the pteridine ring *in vitro* would suggest that this was not a suitable model for the physiological reaction. A more promising model could be provided by the observations of Argentini and Viscontini (1973) who

found that 6 and 7 substituted tetrahydropterins could undergo oxidative degradation under very mild conditions (0.1 M-phosphate buffer pH6.8 at ambient temperature) in the presence of stoichiometric amounts of a suitable electron acceptor (dimedone) to yield pyrimidine analogues. Thus 2-aminoalloxan could be generated in 90% yield under these conditions from 6-aminomethyl-6,7-dimethyltetrahydropterin via a series of quinonoid intermediates:



2-amino-alloxan

Pyrazine ring-opened intermediates, although of a more transitory nature, have also been proposed to occur during the oxidation of tetrahydrobiopterin, the phenylalanine hydroxylase cofactor, to quinonoid dihydrobiopterin in the biosynthesis of tyrosine (Hamilton, 1971) and these were confirmed in a recent study using synthetic analogues (Bailey and Ayling, 1979). If folates (or pterins derived therefrom) can undergo degradation by similar routes *in vivo* then substituted [2-^{14}C] pyrimidines would be produced from [2-^{14}C] folate. Although the catabolism of substituted pyrimidines has not been studied in detail the 2C atom of both thymine and uracil is degraded to urea (Cerecedo, 1931) and CO_2 (Fritzon and Pihl, 1957). Pyrimidine catabolism *in vivo* is extensive and intact pyrimidines are barely detectable in normal urine (Berg and Kolenbrander, 1970). Thus any [2-^{14}C] pyrimidines generated from folate catabolism *in vivo* could be quantitatively degraded to $^{14}CO_2$ and ^{14}C-urea.

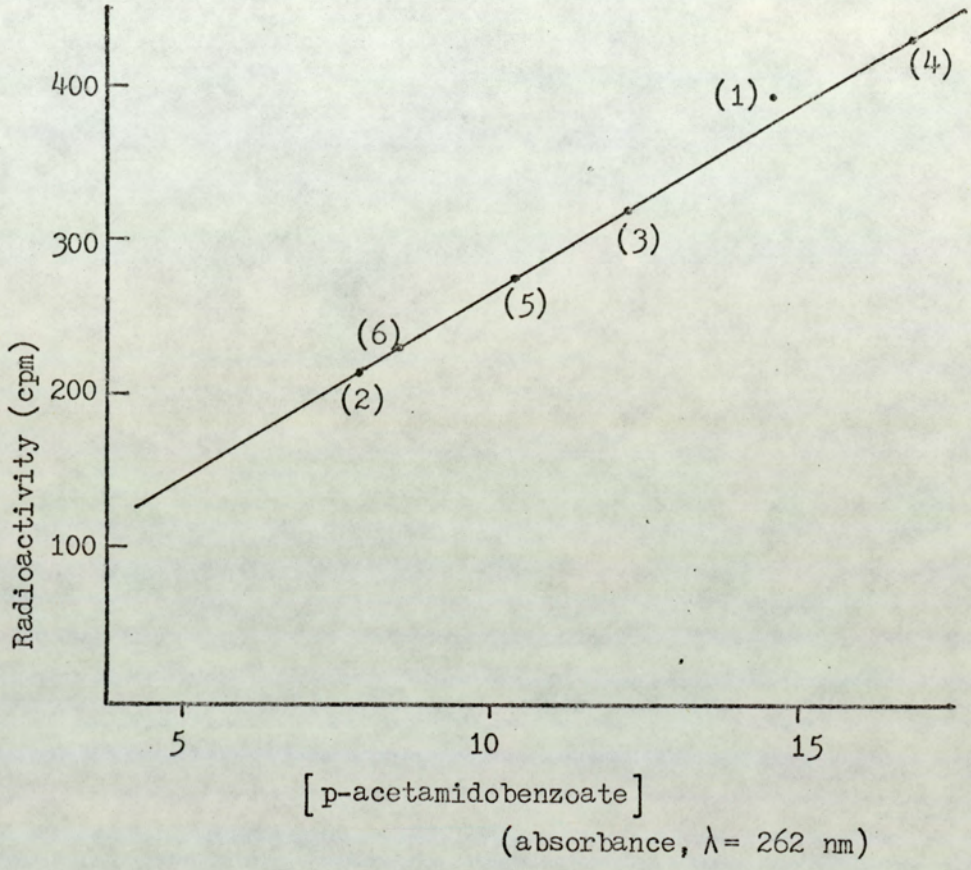


Figure 6.1 The corecrystallization of p-acetamidobenzoate and the major ^{3}H labelled urinary folate catabolite. The linear plot (r=1) of radioactivity against [p-acetamidobenzoate] i.e. constant specific radioactivity was obtained using samples of crystals from 6 recrystallizations (values in parenthesis refer to the number of recrystallizations).

Figure 6.2 Suggested routes of folate catbolism in vivo

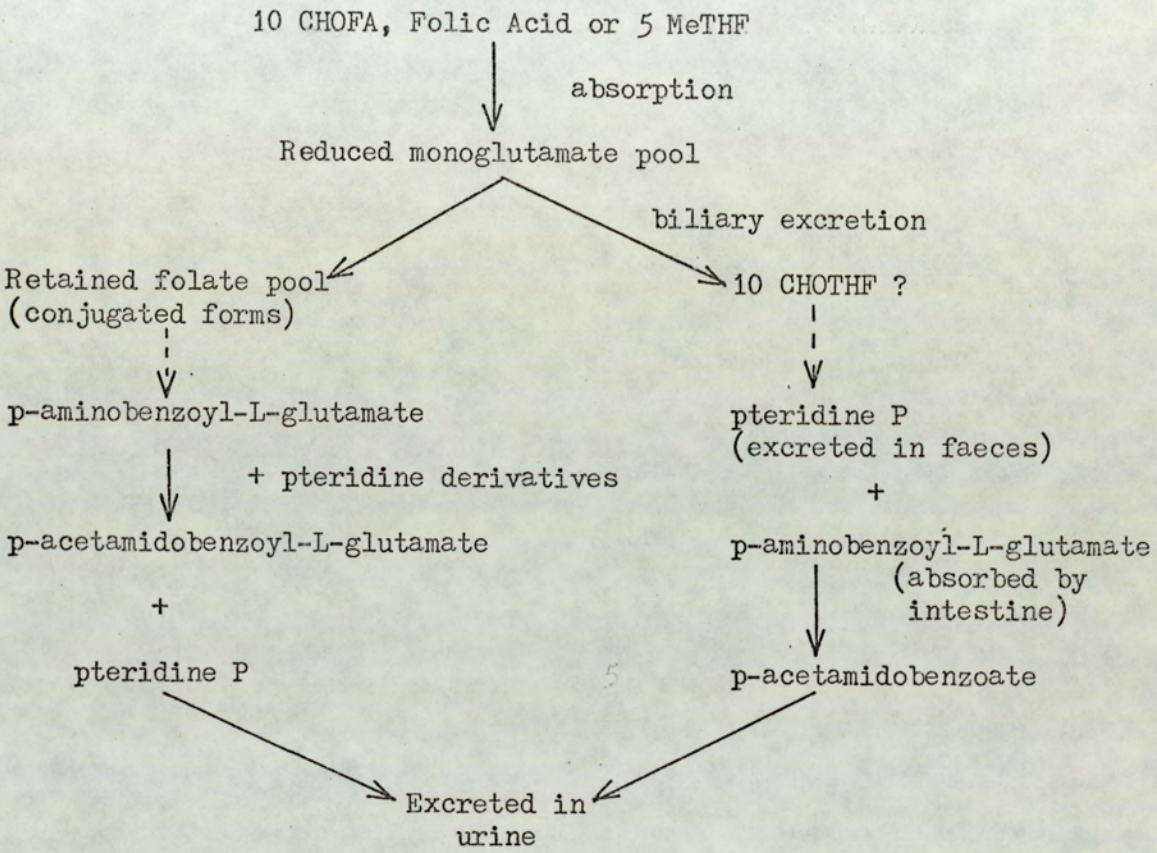


Table 6.1 Summary of the chromatographic behaviour of standard p-aminobenzoate derivatives and metabolites Q and R

	Elution position				
	DEAE-Cellulose	Sephadex -G15	Paper chromatography* 1	2	3
	NaCl	Kav	Rf	Rf	Rf
p-Acetamidobenzoate	0.45	1.73	.52	.56	.35
Metabolite Q	0.45	1.73	.52	.56	.35
p-Aminobenzoate	0.40	1.67	.39	.65	.19
Hydrolysis product of metabolite Q	0.40	-	.39	.65	-
p-Aminohippurate	-	1.51	.39	.83	.24
p-acetamidohippurate	0.35				
p-acetamidobenzoyl-L-glutamate	0.45	0.66	.28	.85	.09
Metabolite R	0.45	0.66	.27	-	-
p-aminobenzoyl-L-glutamate	0.40	0.66	.21	.85	.05

* Solvents

1 Propanol : conc. ammonia (sp.gr. = 0.880) : water (200 : 1 : 99)

2 1% acetic acid in water

3 Butanol : ethanol : ammonia : water (10 : 10 : 1 : 4)

Table 6.2 The distribution of radioactivity in the urine and faeces after the administration of [2-^{14}C] folic acid and large amounts of unlabelled folic acid to a rat over several days

Sample		% Daily dose of radioactivity	
Urines			
0- 6 h 6-24 h	day 1	17.6 62.0	
	day 2	30.0	
	day 3	80.0	
	day 4	54.0	
	Total	243.6	(48.7% total dose)
Faeces*		148.0	(29.6% total dose)

* Faeces produced on day 1 and day 2 were pooled with the faecal pellets recovered from the intestine on autopsy.

Table 6.3 Metabolites excreted in the urine of a rat given large doses of folic acid

	% daily urine sample as metabolite			
Metabolite	Day 1	Day 2	Day 3	Day 4
Folic acid	20	11.5	8.2	14.0
5 MeTHF	27.7	31.2	35.9	35.4
Folate S	6.2	9.6	11.4	7.4
Formylfolates	22.0	19.7	21.3	22.8
pterin P	7.3	12.0	7.5	8.8

Table 6.4 A summary of the products obtained from the oxidative degradation of pteridine P by different methods

Oxidation procedure	Products		
$KMnO_4$ in acid	5% Kav = 0.70 (Xanthopterin)	65% Kav = 1.03	24% Kav = 1.47
$KMnO_4$ in alkali	61% Kav = 0.07 (Xanthopterin)	39% Kav = 1.03	
Mild iodine oxidation at pH5 without the addition of ascorbate at the end of the reaction	12% Kav = 0.70 (Xanthopterin)	19% Kav = 1.47 (pterin ?)	68% Kav = 2.05 (pteridine P)
Mild iodine oxidation at pH5 with the addition of ascorbate at the end of the reaction	24% Kav = 0.70 (Xanthopterin)	36% Kav = 1.47 (pterin ?)	40% Kav = 2.05 (pteridine P)
Vigorous Iodine oxidation at pH7	> 90% Kav = 1.03	< 10% (shoulder) Kav = 1.47 (pterin ?)	

Table 6.5 The excretion of tritiated catabolites and the extent of catabolism after 10 CHOFA and 10 $CHC(glu)_4$ administration to rats.

	% Dose as Metabolite in Urine over 48 h			
Compound dosed	(a) p-acetamido benzoate	(b) p-acetamido benzoyl-L glutamate	Total (a+b)	^3H_2O
10 CHOFA				
10.5 $\mu g\ kg^{-1}$	4.3	1.5	5.8	1.7
	(10.5)	(3.7)	(14.2)	
52.4 $\mu g\ kg^{-1}$	3.2	1.1	4.3	1.4
	(7.8)	(2.7)	(10.5)	
104.7 $\mu g\ kg^{-1}$	1.7	0.2	1.9	1.2
	(4.1)	(.5)	(4.6)	
10 $CHOFA(glu)_4$				
126 $\mu g\ kg^{-1}$	8.6	3.7	12.3	1.2
	(11.0)	(4.7)	(15.7)	

Values in parenthesis refer to the estimated % of the dose catabolised.

CHAPTER 7

A PRELIMINARY STUDY OF THE METABOLISM OF [2-^{14}C] FOLIC ACID IN NORMAL MALE, NORMAL FEMALE AND IMMUNE SUPPRESSED FEMALE MICE

Neoplasm induced changes in the body folate pools have been observed both in the present study and by others. The use of the rat bearing transplantable tumours such as the Walker 256 carcosarcinoma (Barford and Blair, 1978; Connor and Blair, 1979) and the Novikoff hepatoma (Poirier, 1973; Pheasant and Blair, 1979) form useful experimental models in our understanding of such changes in more detail. However there are several evident drawbacks in the extrapolation of information derived from these animal models to that of the human diseased state. Transplantable animal tumours tend to have faster proliferation rates and less readily metastasize than human tumours. Species dependent differences in folate metabolism as well as the inherent individual characteristics of the tumours themselves complicates direct application of information derived from the rat to the human situation.

The difficulties encountered in the analysis of naturally occurring folates necessitates the use of radiotracers for in vivo studies and use of these is obviously restricted in human metabolism experiments. A more suitable animal model is now available with the development of the xenograft (Rostom et al, 1979) i.e. the transplantation of viable foreign (human) tissue into an immune suppressed host animal (usually the mouse) allowing both comparison of the impact of various human tumours on whole body metabolism and a more detailed characterisation of human tumours in situ.

Detailed studies of folic acid metabolism in the mouse are absent from the literature where the rat has long been the favoured animal. In the present Chapter the metabolism of [2-^{14}C] folic acid in normal male and female mice and in immune suppressed female mice, the xenograft host animals, is investigated.

Materials and Methods

Groups of mice, dosed orally with [2-^{14}C] folic acid (specific

radioactivity 58.2 mCi $mmol^{-1}$) were housed in pairs in small metabolism cages. Urine was collected into vessels, protected from the light, containing 3 ml of 0.05 M-sodium phosphate buffer pH7 containing sodium ascorbate (2 g 100 ml^{-1}) and dithiothreitol (5 mg 100 ml^{-1}). The first group of 9 male mice (40 g) received 72 $\mu g\ kg^{-1}$ body weight of folic acid. The second and third groups of 10 normal females (28.2 g) and 10 immune suppressed females (25 g) received 135 $\mu g\ kg^{-1}$ body weight of folic acid. The larger doses in the latter study were necessitated by the low radioactivity of urine samples which hampered metabolite identification. Urine samples were recovered after 0-6 h and 6-24 h (or 0-24 h) and 24-48 h and examined chromatographically on DEAE-cellulose and Sephadex G15 columns. After 48 h the animals were killed by cervical dislocation and the livers excised for the determination of the retained radioactivity. Qualitative examination of liver extracts prepared by the following methods was performed.

a) Hot ascorbate : as described for rat liver (Chapter 5.2) using 5 ml of extraction buffer per g of liver.

b) Isotonic sucrose : as described for rat liver (Chapter 5.2) using 5 ml of extraction buffer per g of liver but centrifuged using an ultracentrifuge (100,000 g for 30 min) to prepare a high speed supernatant.

c) 0.05 M-sodium phosphate buffer pH7 : as described for rat liver (Chapter 5.2) using 5 volumes of buffer per g of liver but with the supernatant obtained by high speed centrifugation (100,000 g for 30 min).

d) Saturated p-chloromercuribenzoate in buffer at pH7 : liver was homogenized in 5 volumes of freshly prepared saturated p-chloromercuribenzoate (pCMB) dissolved in 0.05 M-sodium phosphate buffer pH7 containing dithiothreitol (5 mg 100 ml^{-1}). High speed supernatants were obtained as described in method (b).

e) Trichloroacetic acid (TCA) : performed as described for rat liver (Chapter 5.2) using 5 volumes of TCA (10% w/v).

Results

The recoveries of radioactivity in the livers and urine of the three groups of mice are given in table 7.1; faecal radioactivity was not determined. Urinary recovery of radioactivity was similar for both groups of female mice (18.34% for group 2 and 20.75% for group 3) although the male mice (group 1) excreted slightly more of the dosed radioactivity (27.07% dose) despite the lower dose per kilogram body weight. Qualitative examination of urine samples by DEAE-cellulose ion exchange revealed a complex mixture of metabolites. Seven metabolites were present in all urine samples examined and a further three were observed periodically. The distribution of radioactivity in urine samples for each of these metabolites is given in table 7.2 and an example of DEAE-cellulose chromatography of a urine sample portrayed in figure 7.1. Both groups of female mice excreted large amounts of unmetabolised folic acid compared to the male mice, probably reflecting the difference in dose, as has been observed in rats given different doses of folic acid (Blair and Dransfield, 1971) and 10 CHOFA (Connor and Blair, 1979), rather than a sexual dimorphism in ability to reduce folic acid. The relatively lower amounts of folic acid in the urine of the male mice was compensated by a relatively larger proportion of 5 MeTHF and 10 CHOTHF the primary products of folic acid metabolism.

Of the remaining urinary metabolites pteridine P was the major derivative although unlike in the rat this occurred in relatively high amounts even in the 0-6 h and 6-24 h urine samples. Two metabolites (metabolites 1 and 2) were present in all urine samples. These early eluting species are unidentified and have no apparent parallel in rat urine. Whether these are intact folates or pterins derived therefrom is unknown since a dual label was not adminstered, however both meta-

bolites increased on storage largely at the expense of pteridine P. The latter observation combined with their early elution positions on DEAE-cellulose chromatography (eluting in similar positions to xanthopterin and pterin, although these were not compared directly) suggests that they are possibly pteridine degradation products. Metabolite 6a, observed in some samples, eluted after 5 MeTHF. The distinctive elution position of this metabolite on DEAE-cellulose is similar to that of the unknown intact folate derivative found in the urine of rats bearing implanted Novikoff hepatomas after dual labelled folic acid administration observed in this laboratory (Pheasant and Blair, 1979). Some urine samples contained a minor metabolite (metabolite 5a) identical to folate S found in rat urine (Chapter 5) and an early eluting rather diffuse peak, possibly urea (not quantified). The erratic appearance of folate S in urine samples (table 7.2) may be due to failure to resolve this component from the prominent overlapping 5 MeTHF and 10 CHOFA peaks rather than to variation in its excretion.

Qualitative analysis of extracts of liver gave similar elution profiles on Sephadex G15 to that obtained in the rat. Hot ascorbate extraction gave a major peak of high molecular weight folate (c. 90%) for each group of mice and small amounts of formylfolate and material eluting in a similar position to 5 MeTHF and pterin (table 7.3). Cold extracts of livers in buffer saturated with p-chloro-mercuribenzoate gave similar chromatographic patterns as the hot extracts. Gel filtration of the cold extracts on Sephadex G75 revealed that the high molecular weight material was not protein bound indicating that pCMB disrupts such binding. The high recovery of intact folate polyglutamate derivatives confirms the observation that liver folate conjugase is inhibited by pCMB, a phenomenon previously reported in studies using synthetic folic acid polyglutamate derivatives (Rowe et al, 1975). In the absence of pCMB cold extraction gave largely protein bound folates

(eluting at the void volume on Sephadex G75) 10 CHOTHF, 5 MeTHF and pterin (methods (b) and (c)). The reason for the presence of relatively large amounts of 5 MeTHF in such cold extracts from mouse liver compared to that observed in similar extracts of rat liver (Chapters 3 and 5) is unknown but may be due to the use of an ultracentrifuge (100,000 g for 30 min) to obtain high speed supernatant rather than the bench centrifuge used in the preparation of rat liver extracts. The former method would more effectively remove lysosomal and microsomal bodies, minimising degradation of the folate metabolising enzymes, and this would not be the case using the later method of centrifugation. Contact time with possible folate metabolising enzymes is also longer during ultracentrifugation. Extraction of livers in 10% TCA gave similar products (largely pterin and possibly dihydropterin) as observed in TCA extracts of rat liver.

Discussion

Normal male, normal female and immune suppressed female mice metabolized [2-^{14}C] folic acid in a similar manner as evidenced by examination of urinary and hepatic radioactivity. The folate-dependent biochemical pathways are thus apparently normal in the host animals used in xenograft studies.

The metabolism of folate by the mouse was markedly different from that of the rat. An increased number of urinary metabolites and enhanced excretion of pteridine P were the most obvious differences. These may reflect species specific changes in biochemical pathways or differences in physiology and anatomy. The mouse has a higher basal metabolic rate than the rat, possesses a gall bladder (in which bile may be stored) and has minor structural differences in the small intestine compared to the rat. Each of these factors could affect the handling of folate particularly if the entero-hepatic circulation of folate is as significant in the mouse as in the rat. Elucidation of

the precise origin of the difference in folate handling between the two species requires further more detailed studies of which the identification of the novel urinary metabolites is paramount.

The presence of pteridine P in the urine of both the mouse and the rat, and probably also man (Pheasant et al, 1979) demonstrates the widespread occurrence of folate catabolism reactions.

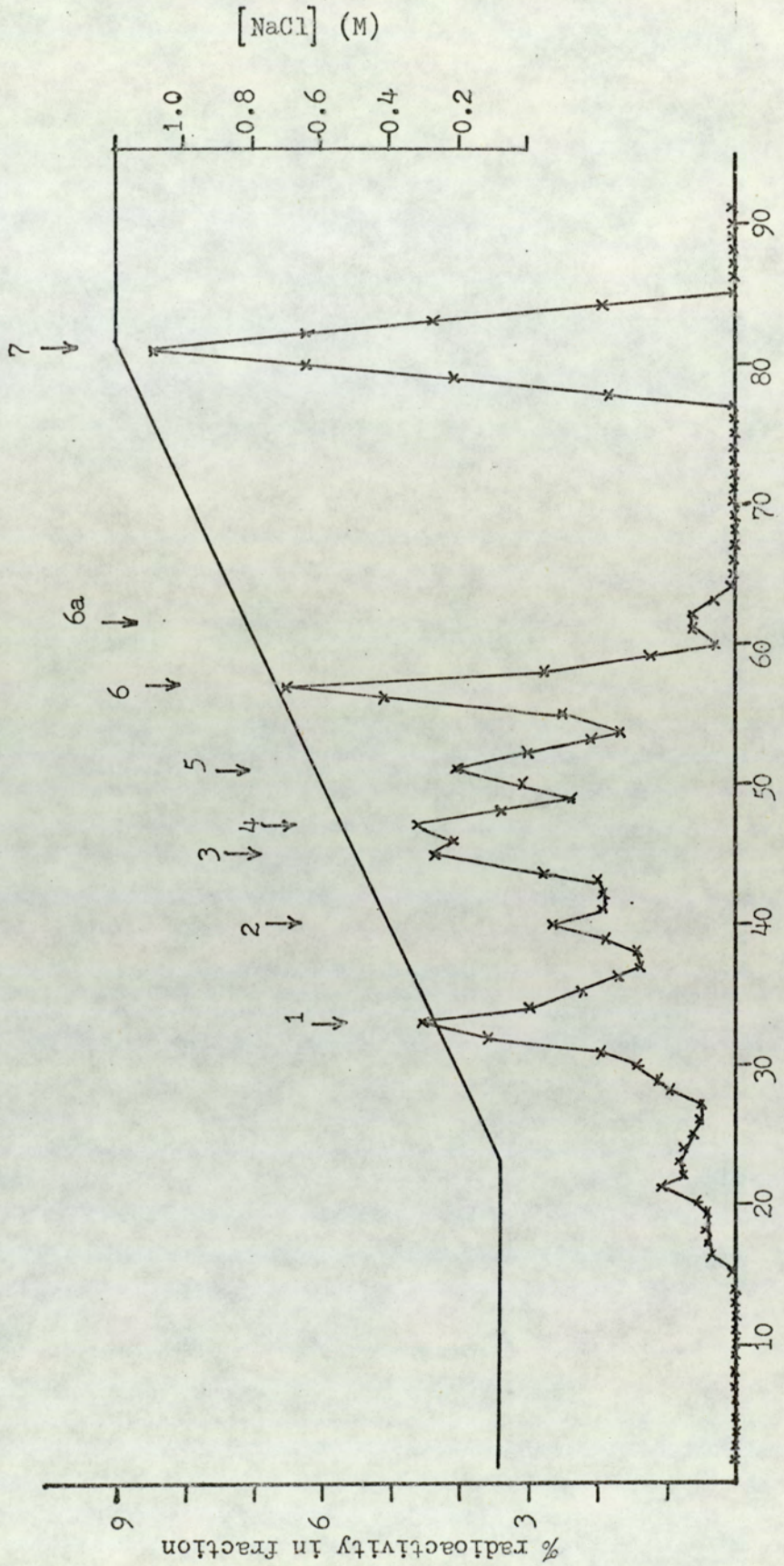


Figure 7.1 DEAE-cellulose chromatography of urine from normal female mice (group 2) collected 0-24 h after an oral dose of [2-^{14}C] folic acid (135 μg kg^{-1})

Table 7.1 Tissue retention and urinary excretion of radioactivity 48 hours after the administration of [2-^{14}C] folic acid to three groups of mice

Group	Sample		% ^{14}C
Male mice (72 μg kg^{-1})	Urine	0- 6 h	3.7
		6-24 h	19.7
		(0-24 h)	(23.4)
		24-48 h	3.7
		Total	27.1
	Intestine		2.1
	Livers	(1.9 g)	16.3
Female mice (135 μg kg^{-1})	Urine	0-24 h	17.7
		24-48 h	0.62
		Total	18.32
	Livers	(1.5 g)	15.1
Immune-suppressed female mice (135 μg kg^{-1})	Urine	0-24 h	19.7
		24-48 h	1.1
		Total	20.8
	Livers	(1.5 g)	13.8

Values were determined on pooled samples from each group of mice.

Table 7.2 Quantification of urinary metabolites in urine samples from mice dosed orally with [2-^{14}C] folic acid

Metabolite		Normal male (Group 1)			Normal Females (Group 2)	Thymus deprived females (Group 3)	
Elution Position	Identity	0- 6 h	+ 6-24 h	0-24 h	0-24 h	0-24 h	+ 24-48 h
(1) .25	?	9.0	7.2	7.5	15.2	9.6	11.0
(2) .32	?	6.0	6.2	6.2	6.0	3.5	2.9
(3) .4	10 CHOTHF	8.8	18.0	16.5	8.6	8.5	7.2
(4) .45	pteridine P	10.1	17.6	16.4	11.3	10.0	10.2
(5) .55	10 CHOFA	1.4	12.9	11.1	9.5	8.6	4.8
(5a) .61	Folate S	N.D.	N.D.	N.D.	N.D.	N.D.	4.7
(6) .65	5 MeTHF	32.7	27.7	28.5	15.3	12.7	10.5
(6a) .72	?	11.6	N.D.	1.8	1.9	1.8	6.6
(7) .95	Folic acid	9.9	4.4	5.3	26.0	42.2	41.1
	TOTAL	89.5	94.0	93.3	93.8	96.9	99.0

Values quoted are the % of the radioactivity in the urine sample as the metabolite.
Elution position refers to DEAE-cellulose chromatography and is expressed in molarity of NaCl N.D. = not detected
+ Contained a small peak (2% dose) eluting at the void volume

Table 7.3 Distribution of liver extract radioactivity amongst chromatographically separated derivatives for the 3 groups of mice

Extraction Method		Group 1	Group 2	Group 3
		% Extract radioactivity as compound		
(a) Hot ascorbate Sephadex G15	Folate$(glu)_n$	N.D.	77	85
	Formylfolate		5	5
	unidentified		15	10
(b) Isotonic sucrose Sephadex G15	Protein bound	* 17	45	49
	Formylfolate	21	16	11
	5 MeTHF + pterin	54	25	29
Sephadex G75	Protein bound	N.D	N.D.	32
	unidentified			68
(c) 0.05 M-phosphate Sephadex G15	Protein bound	* 20	38	18
	Formylfolates	45	33	44
	5 MeTHF + pterin	32	25	26
Sephadex G75	Protein bound	N.D.	N.D.	18
	unidentified			80
(d) Saturated pCMB Sephadex G15	Folate$(glu)_n$	N.D.	89	93
	Formylfolate		4	6
	unidentified		5	-
Sephadex G75	Protein bound	N.D.	3	-
	unidentified		95	100
(e) 10% TCA Sephadex G15	Folate$(glu)_n$	N.D.	6	4
	Dihydropterin (?)		60	76
	pterin		10	17

N.D. = not determined

* TCA was added to remove protein, the protein bound value thus represents folate$(glu)_h$.

CHAPTER 8

DISCUSSION

Experiments described in this thesis highlight the complexity of folate metabolism in the mammal. Folic acid and 10-formylfolate are metabolized to a variety of reduced folate monoglutamate derivatives, to high molecular weight polyglutamate forms in the tissues and ultimately to several degradation products.

The accumulated data obtained in the rat supports the hypothesis of the existence of two metabolically distinct folate pools (Blair, 1975; Barford et al, 1977 a; Krumdieck et al, 1978). Folic acid and 10 CHOFA are reduced and substituted initially to principally 5 MeTHF and 10 CHOTHF and these rapidly appear in the urine. The reduced folate monoglutamates constitute the first pool. These circulating derivatives function as a transport form and effectively act as a short term "storage" form for the second pool. Conjugation of the folate monoglutamates in the tissues to folate polyglutamate derivatives, these being the essential coenzyme form for most of the folate requiring reactions in vivo, gives rise to the second pool. Both folate pools can undergo catabolism by ostensibly chemical decomposition although the particular fragments derived from each pool may differ.

The rationale for the above propositions are outlined in the following paragraphs

a) The Disposition of the Folate Monoglutamate Pool

Despite the poor substrate qualities of both folic acid and 10-formylfolate for dihydrofolate reductase in in vitro assays both compounds are reduced in vivo and enter the body folate pools. It was proposed that this reduction is mediated in vivo by the ability to temporarily store these oxidised derivatives by reversible protein binding (Neal and Williams, 1965; Waxman, 1975) which effectively lowers their free concentration and facilitates dihydrofolate reductase action (Chapter 5). Once reduced rapid equilibration with the one-carbon substituted folate monoglutamates 5 MeTHF, 10 CHOTHF and

possibly folate S occurs. The rapidity with which these folate monoglutamates are synthesised and appear in the plasma and urine indicates that they can and do function in at least some of the reactions of one-carbon metabolism (Blair, 1975). Once formed there are several alternative routes available for their further metabolism, illustrated diagramatically in figure 8.1.

Following absorption across the intestine or biosynthesis from folic acid or 10 CHOFA a similar equilibrium between protein binding, urinary excretion and subsequent tissue metabolism is established for the reduced monoglutamates as was proposed for 10 CHOFA (Chapter 5). Two additional routes for metabolism are also available. Both 5 MeTHF and 10 CHOTHF and possibly folate S can undergo biliary excretion (Pheasant, Connor and Blair, unpublished observations). Independent studies have shown that the biliary folates are derived principally from the circulating monoglutamate pool (Hillman et al, 1977; Strum et al, 1979). Once in the intestine the derivatives are available for reabsorption. At least one of the biliary excreted folates possibly 10 CHOTHF, can also undergo a cleavage reaction yielding p-aminobenzoyl-L-glutamate and pterins. The mechanism for this remains unknown but if 10 CHOTHF is the putative precursor of the cleavage products at least one enzyme mediated step is required as 10 CHOTHF per se does not undergo spontaneous chemical degradation under the conditions usually associated with the intestinal lumen. The formation of the more unstable THF molecule (which readily undergoes scission to p-aminobenzoyl-L-glutamate and pterins (Pearson, 1974) by enzymic deformylation of 10 CHOTHF would seem the most likely route. Whether this step is performed by host enzymes or by gut microfloral metabolism is uncertain. In view of the scale of production of p-acetamidobenzoate direct catabolism of the small amounts of p-aminobenzoyl-L-glutamate or THF, which could have been excreted in the bile in concentrations below the sensitivities

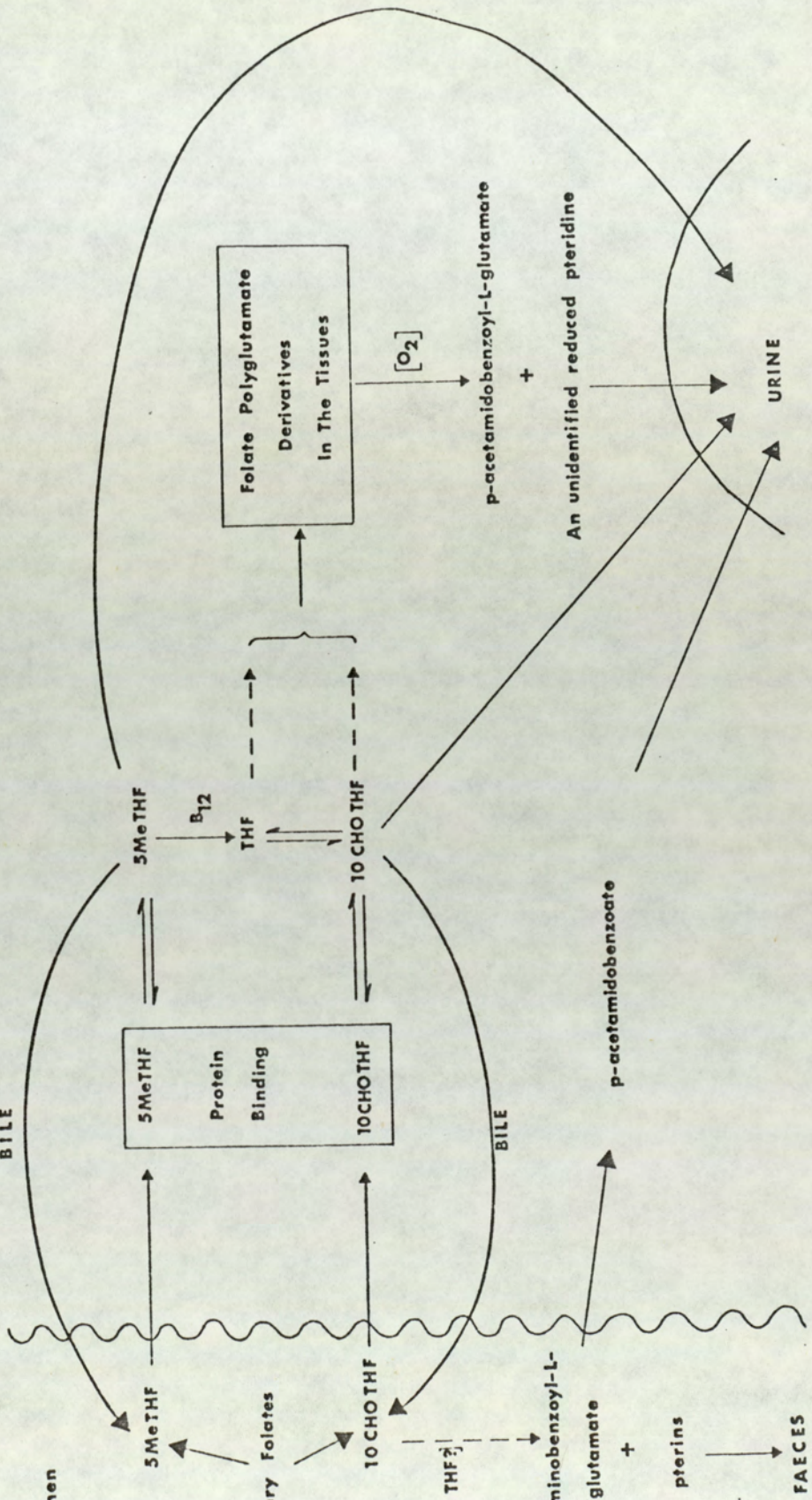


Figure 8.1 The principal routes in the distribution and fate of the major components of the folate monoglutamate pool(5MeTHF & 10 CHOTHF)

of the assay systems used in the biliary folate studies quoted above, is unlikely. More detailed information on the metabolism of 10 CHOTHF is required to answer these points.

Once formed in the intestine p-aminobenzoyl-L-glutamate is readily absorbed and undergoes intestinal metabolism to p-acetamidobenzoate. At the concentrations likely to occur in the gut essentially quantitative conversion of p-aminobenzoyl-L-glutamate to p-acetamidobenzoate would take place since even larger oral doses of p-aminobenzoyl-L-glutamate (250 mg kg^{-1}) are almost completely metabolised by this route in the rat (Pheasant and Nield, unpublished observations) and quantitative hydrolysis of the peptide bond of the closely related derivatives p-aminohippurate (i.e. p-aminobenzoyl-glycine) and p-acetamidohippurate by intestinal metabolism has been observed in man (Hulsmann and van Eps, 1967). Pteridines generated in the intestine are evidently lost in the faeces (Chapter 5). The interconversion of the principal folate monoglutamates 5 MeTHF and 10 CHOTHF occurs by several routes (Figure 1.1) and THF is a required intermediate, assuming the verity of the irreversibility of 5,10 CH_2THF reductase activity. Generation of the labile THF molecule within the rat and its subsequent chemical degradation would produce p-aminobenzoyl-L-glutamate which would then be excreted as the acetylated form in the urine. However the precedence of p-acetamidobenzoate over p-acetamidobenzoyl-L-glutamate during the time period in which radiolabelled folate monoglutamates are most plentiful (Connor et al, 1979) strongly suggests that intestinal catabolism giving ultimately p-acetamidobenzoate is the major route of degradation of the folate monoglutamate pool.

The second route available to the reduced folate monoglutamate pool not available to folic acid and 10 CHOFA is conversion to folate polyglutamate forms (i.e. the second folate pool) in the tissues. The nature and fate of the folate polyglutamate pool will be discussed in

more detail below. Available evidence suggests that in the mammal reduced folate monoglutamates are the substrates for polyglutamate synthesis. Of these THF and 10 CHOTHF are probable substrates (Spronk, 1973; McGuire et al, 1979); 5 MeTHF does not enter the folate polyglutamate pool without prior loss of the methyl group at least in man and rodents (Spronk, 1973; Lavoie et al, 1974). Since after 48 h labelled folate retained in the tissues is largely incorporated into the polyglutamate pool depletion of folate monoglutamates by this route occurs on a considerable scale. In this respect the folate monoglutamate pool acts as a storage form for the second pool. The limited routes available to 5 MeTHF, i.e. biliary or urinary excretion, protein binding and metabolism to THF by the methionine synthetase reaction (Chapter 1) supports its function as a major folate storage and transport form. 5 MeTHF metabolism is regulated by several factors especially the concentrations of homocysteine, methionine, S-adenosylmethionine and most importantly B_{12}. In B_{12} deficiency folate monoglutamate becomes trapped in its normal storage and transport form (5 MeTHF), as envisaged in the methyl trap hypothesis. Under conditions where cellular proliferation occurs at abnormally high rates, as in neoplastic growth, and in which the folate requirement for purine and pyrimidine metabolism is likely to be enhanced, incorporation of labelled folate into the tissue polyglutamate pool is increased and depletion of the storage form (5 MeTHF) occurs (Chapter 5). Recent work has revealed that some tumours contain comparatively high levels of 5,10 CH_2THF reductase activity and elevation of this enzyme in the host livers of Walker 256 tumour bearing rats occurs. It has been suggested that under such abnormal conditions the enzymic reduction of 5,10 CH_2THF to 5 MeTHF may be reversible (Albrecht, 1979). Both effects would lead to the decreased 5 MeTHF levels observed in the urine of tumour bearing rats (Barford and Blair, 1978; Connor and

Blair, 1979). The concentration of 10 CHOTHF would not be reduced so dramatically, at least in the short term under such conditions, since this will be maintained by the decrease in 5 MeTHF, although the ratio of 10 CHOTHF to 5 MeTHF would change.

b) Role and Functions of the Retained Folate Polyglutamate Pool

The present study has shown that not only do folate tracers readily enter the body folate pools, undergoing considerable dilution with endogenous folate, but that in the liver at least after 48 h they are incorporated largely into derivatives which yield 10 CHOFA$(glu)_4$ when extracted in boiling ascorbate. Since the hepatic concentration of 10 CHOFA$(glu)_4$ extracted was found to be constant in the rat, very similar amounts being found in two separate experiments despite the prior deprivation of food 24 h before the hepatic folate was determined in one of the groups of rats used, it is possible that the concentration of 10 CHOTHF$(glu)_4$ (the probable precursor of the 10 CHOFA$(glu)_4$ extracted) has a critical function in the regulation of entry of the folate monoglutamates into the folate polyglutamate pool. Confirmation of feedback inhibition of folate polyglutamate synthesis by folate polyglutamate products may be deduced from *in vitro* studies where long chain folate polyglutamates were synthesised by purified rat liver folypolyglutamate synthetase at low substrate concentrations but only short chain derivatives formed at high substrate concentrations (Taylor and Hannah, 1977; McGuire *et al*, 1979).

The formation of folate polyglutamates *in vivo* is a relatively slow process occurring by a step-wise addition ofglutamic acid (Corrocher *et al*, 1972; Shin *et al*, 1976). Once formed this second pool of retained folate persists for a relatively long time period, the biological half-life having been estimated at 12.5 and 19.3 days (Whitehead, 1973) and 8 days (Barford *et al*, 1977 a) in two separate studies of rat liver folate.

The ultimate fate of this second pool is complex. Since the urinary radioactivity of rats dosed with radiolabelled folate is associated predominantly with folate cleavage products, largely p-acetamidobenzoyl-L-glutamate and pteridine P, when the retained radioactivity is largely associated with folate polyglutamate derivatives (i.e. after 24 h) catabolism would appear the most attractive route. Similar conclusions have been drawn from more long term studies in the rat (Murphy et al, 1978) where p-acetamidobenzoyl-L-glutamate was the major urinary species found 10 days after the administration of a folic acid tracer. Although folate conjugase has a ubiquitous distribution amongst the tissues it is almost certainly lysosomal (Rowe et al, 1975; Shin et al, 1976) and endogenous folate polyglutamates may be available as a substrate for conjugase only at cell lysis. Further protection against conjugase induced hydrolysis is afforded by the folate binding proteins since protein bound folate polyglutamates are unavailable as substrates (Waxman, 1975). Since the relative concentrations of the folate monoglutamates and of p-acetamidobenzoate, the major catabolite of the folate monoglutamate pool in the rat, falls rapidly after 24 hours hydrolysis of the folate polyglutamates to monoglutamate forms either occurs at a very low rate compared to the cleavage reactions or gives a product which is not in equilibrium with the folate monoglutamate pool.

The constancy of hepatic 10 CHOTHF$(glu)_4$ levels demonstrates that it is in equilibrium with other forms. It has been suggested that 10 CHOTHF dehydrogenase has an important role in maintaining the concentration of such derivatives (Krebs et al, 1976; Scrutton and Beis, 1979). THF polyglutamates would be generated from 10 CHOTHF$(glu)_4$ and their inherent chemical instability could give rise to the observed long term catabolites p-acetamidobenzoyl-L-glutamate, pteridine P and possibly urea and CO_2. A steady state concentration of 10 CHOTHF$(glu)_4$

can be maintained by controlling the rate of entry of folate monoglutamate into the folate polyglutamate pool and its subsequent degradation by $THF(glu)_4$ formation. In situations where the folate requirement is increased, as in rats bearing implanted tumours, disruption of the equilibrium evidently occurs and polyglutamate formation increases (as found in host livers) at the expense of the monoglutamate pool. Whether this increased synthesis leads to raised 10 $CHOTHF(glu)_4$ in both absolute and relative amounts is unknown, as this was not measured in tumour bearing animals due to the requirement for large amounts of tissue in the assay system employed. Values quoted in the literature for total hepatic folate in host livers are contradictory, possibly due to variations in dietary intake (Poirier, 1973; Albrecht, 1979).

The metabolic function of the folate polyglutamate pool, despite (or perhaps because of) the enormous effort spent in studying these derivatives over the past ten years, is still controversial. Much of this literature has been covered in the introduction to this thesis (Chapter 1) and only studies pertinent to the present work will be discussed here. Measurement of the absolute concentration of hepatic polyglutamates and its inferred relation to the monoglutamate pool, the slow turnover of folate polyglutamates and the pronounced increase in hepatic uptake of folate tracers into the folate polyglutamates in tumour bearing rats, where demand for folate coenzymes is likely to be enhanced, supports the conclusion of numerous *in vitro* studies (reviewed by Hoffbrand, 1976; Rowe, 1978) that conjugated folates have a primary coenzyme function rather than as a storage pool for the folate monoglutamates. The conclusion has in fact been drawn (*vide supra*) that the folate monoglutamates effectively act as temporary circulating folate storage form for subsequent folate polyglutamate biosynthesis.

Rationalization of the exact coenzyme roles of the folate poly-

glutamates in rat liver is complicated by the failure to detect labelled 5 MeTHF polyglutamates under conditions where appreciable amounts of labelled 5 MeTHF are excreted in the urine. A simple scheme as laid out in Chapter 1 (figure 1.1)in which polyglutamyl forms are substituted for the monoglutamates portrayed is obviously untenable. Convincing unambiguous evidence for 5 MeTHF polyglutamates in mammalian tissues is in fact absent from the literature although their existence has been widely assumed. 5 MeTHF is a poor substrate for folylpolyglutamate synthetase (Spronk, 1973; McGuire et al, 1979) and 5 MeTHF polyglutamates are not synthesised directly in vivo. Mutant cell lines of Chinese hamster ovary (CHO) cells, lacking folylpolyglutamate synthetase, have an absolute requirement for adenosine, glycine and thymine for growth but no pronounced abnormal requirement for methionine (McBurney and Whitmore, 1974). Rat intestinal cells have no detectable amounts of methionine synthetase, but possess a marked ability to synthesise 5 MeTHF (Finkelstein et al, 1971; Finkelstein et al, 1978). Formation of 5 MeTHF polyglutamates in the intestine would effectively trap folate. Although only a cursory study of intestinal folate was attempted, the derivatives found behaved chromatographically in an analogous manner to hepatic folate after [2-^{14}C] folic acid administration to rats (Chapter 3), and no accumulation of a species attributable to other forms (i.e. 5 MeTHF polyglutamates) was evident. 5 MeTHF polyglutamates may not be required therefore for the methionine synthetase reaction in vivo, although synthetic 5 MeTHF$(glu)_4$ has a lower K_m (4 μM) (but also a lower V max) than 5 MeTHF (K_m = 13 μ M) in vitro (Cheng et al, 1975). The failure to detect 5 MeTHF polyglutamates in rat liver may therefore be due to their complete absence or to their occurrence only in small transitory amounts, methionine synthesis by folate requiring routes being essentially a property of the folate monoglutamate pool. Supposition of this role for 5 MeTHF considerably

simplifies the definition of the coenzyme functions of the retained folates. A simplified scheme showing the interrelationships between the major components of the folate polyglutamate pool is given in figure 8.2.

Maintenance of a steady state concentration of 10 CHOTHF$(glu)_4$ and thus those derivatives in equilibrium with it (THF$(glu)_4$ and 5,10 CH_2THF$(glu)_4$) (after Krebs et al, 1976) is possible by both end product inhibition of folypolyglutamate synthetase and by the activity of 10 CHOTHF dehydrogenase (EC 1.5.1.6) (vide supra). 10 CHOTHF dehydrogenase catalyses the reactions

$$10\ \text{CHOTHF} + \text{NADP}^+ + \text{H}_2\text{O} \rightleftharpoons \text{THF} + \text{CO}_2 + \text{NADPH} + \text{H}^+ \quad (1)$$

$$10\ \text{CHOTHF} + \text{H}_2\text{O} \overset{\text{NAD}^+}{\rightleftharpoons} \text{HCOOH} + \text{THF} \quad (2)$$

with the NAD^+ stimulated hydrolysis reaction (2) proceeding at about 21% of the rate of CO_2 formation and the dehydrogenase reaction being potently inhibited by the THF product (Scrutton and Beis, 1979). Unfortunately data is unavailable for polyglutamate substrates but from the known behaviour of similar reactions (Hoffbrand, 1976; Lewis et al, 1979) 10 CHOTHF$(glu)_n$ is probably as effective, if not better as a substrate than 10 CHOTHF. Competition between 10 CHOTHF$(glu)_4$ and 10 CHOTHF for the enzyme and the inhibitory effect of the THF$(glu)_4$ product could limit the formation of free THF. If THF is the preferred substrate for folylpolyglutamate synthetase in vivo then the equilibrium represented in equation (1) may regulate folate polyglutamate biosynthesis. Depletion of 10 CHOTHF$(glu)_4$ mediated by normal coenzyme function will reduce the competition with 10 CHOTHF for 10 CHOTHF dehydrogenase and the free THF generated become available for entry into the folate polyglutamate pool.

Confirmation of the above hypothesis awaits both further kinetic data derived from in vitro studies and a more complete picture of the folate pool in vivo. Information obtained in the present study was

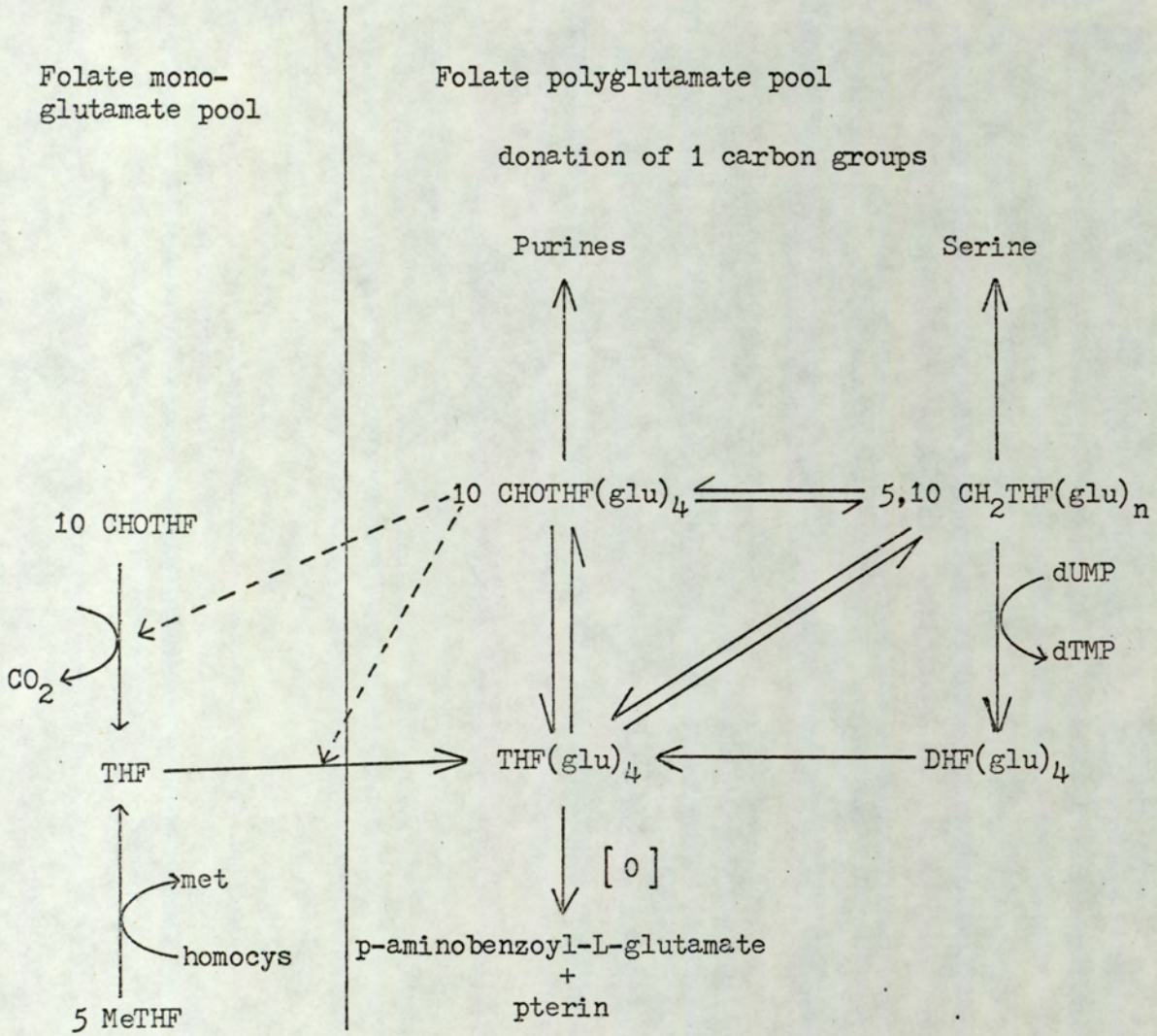


Figure 8.2 Regulatory and coenzyme roles of the folate polyglutamate pool

10 CHOTHF$(glu)_4$, the major liver folate conjugate, is in a steady state equilibrium with 5,10 CH_2 THF$(glu)_4$ and THF$(glu)_4$. Entry into the folate polyglutamate pool may be regulated by the [10 CHOTHF$(glu)_4$] either by feed-back inhibition of folylpolyglutamate synthetase or by regulation of production of its substrate (THF) by competition between 10 CHOTHF and 10 CHOTHF$(glu)_4$ for 10 CHOTHF dehydrogenase.

gained from pulse labelling experiments and allowed the quantitation of only those derivatives labelled after 48 hours. Other complications arise in the extrapolation from the *in vitro* to the *in vivo* situation, of primary concern being the competition between different pathways for folate coenzymes, from the activity of folate binding proteins and compartmentalisation of both folate and folate metabolising enzymes.

The presence of large concentrations of 10 CHOTHF$(glu)_4$ in liver (and by implication the ready formation of those folates in equilibrium with it) and its presumed role in determining the fate of the folate monoglutamate pool indicates the importance of the retained pool in pyrimidine, purine and glycine metabolism (*figure 8.2*) and its central role in the modulation of folate metabolism. Regulation of folate concentrations and of the level of folate metabolising enzymes is complex and our understanding of this is far from complete. Several folate polyglutamate derivatives have marked inhibitory properties at physiological concentrations *in vitro* and this may also be important *in vivo*; several of these reactions are tabulated (table 8.1). The facile oxidation of 10 CHOTHF to 10 CHOFA (and of the corresponding polyglutamate derivatives) observed *in vitro* has interesting connotations *in vivo*. Oxidation of 10 CHOTHF$(glu)_4$ in the cell would produce 10CHOFA$(glu)_4$ a potent dihydrofolate reductase inhibitor. End product inhibition of the thymidylate synthetase reaction by DHF is known (Dolnick and Cheng, 1978) and DHF$(glu)_n$ accumulation, resulting from 10 CHOFA$(glu)_4$ formation, could thus lead to suppression of thymidylate synthesis since DHF polyglutamate derivatives are powerful inhibitors of this enzyme at physiological concentrations (Kisliuk *et al*, 1974).

Changes in the size of the folate polyglutamate pool may thus be important in regulating thymidine biosynthesis, although this may again be complicated by the presence of binding proteins. Protein bound 5,10 CH_2THF is unavailable to thmidy-

Table 8.1 Inhibitory properties of folate polyglutamate derivatives in some folate utilizing reactions

Enzyme (Source)	Substrate	Inhibitor	Reference
DHF reductase			
(Erlich ascites)	DHF K_m = 1.3 μM	10 CHOFA K_i = 6.1 nM	Bertino et al, 1965
(Mouse leukaemia)	DHF	10 CHOFA$(glu)_4$ (7 times as inhibitory as 10 CHOFA)	Friedkin et al, 1975
(Human KB cells)	DHF$(glu)_4$	FA$(glu)_4$ K_i = .50 nM	Domin et al, 1979
Thymidylate synthetase			
(Human blast cells)	5,10 CH_2THF$(glu)_4$ K_m = 2.2 μM	DHF$(glu)_4$ K_i = 6 μM	Dolnick and Cheng, 1978

late synthetase (Rothenberg et al, 1978), although this observation was restricted to studies with monoglutamate forms and no data was presented on possible competition for binding by 10 CHOTHF. Induction of 5,10 CH_2THF reductase by folic acid, methotrexate and food deprivation (Albrecht, 1979), the fall in intracellular DHF reductase in folate depletion (Chello et al, 1977) and the pronounced changes in folate metabolising enzymes during the cell cycle (Rosenblatt and Erbe, 1973; Johnson et al, 1978) could obviously have profound effects on the parameters discussed above.

The existence of an extensive retained folate polyglutamate pool in the tissues is critical to normal cellular function. The addition of glutamic acid residues to the folate molecule both enhances substrate properties, possibly due to enhanced binding facilitated by the additional charge carried at neutral pH (Hoffbrand, 1976), and prevents the loss of folate from the cell since the polyglutamate forms are not transported across the cell wall. Thus CHO cells which fail to produce folylpolyglutamate synthetase (Taylor and Hannah, 1977) suffer folate depletion (McBurney and Whitmore, 1974). The improved binding of folate polyglutamate derivatives to enzymes may also enable finer regulatory control, since chain elongation enhances both their substrate and inhibitory properties.

c) Folate Catabolism: Function and Physiological Role

Loss of folate in vivo by degradation to structurally less complex molecules is of considerable physiological importance. What metabolic function, if in fact such a function exists, folate catabolism fulfills is obscure but some elucidation may be gained by an understanding of the mechanism of catabolism.

In Chapter 6 of this thesis it was proposed that simple chemical scission of a labile folate (probably THF) to pteridine and p-aminobenzoyl-L-glutamate derivatives was the source of the catabolites.

Given the extreme lability of folate coenzymes and the failure by several groups of workers to demonstrate the existence of folate cleavage enzymes *per se* (see Chapter 6 for references) spontaneous chemical scission of the folate molecule contrasted with enzymic hydrolysis is both the simplest and most probable mechanism. The observed catabolites are all explainable by metabolic detoxification of the products of this unavoidable folate degradation. Detoxification of the scission products is essential since both pterin fragments and p-aminobenzoate derivatives are potentially hazardous to the cell. Xanthopterin, pterin and pterin-6-aldehyde are readily generated by THF oxidation. Xanthopterin is metabolised to leucopterin (2-amino-4,6,7,-trihydroxypterin) *in vivo* and this extremely insoluble pterin can interfere with renal function by precipitating in the renal tubules (Haddow *et al*, 1972). Pterin-6-aldehyde is a powerful inhibitor of xanthine oxidase (Lowry *et al*, 1949). Pterin, which can induce epileptic seizures in the mouse, is converted to isoxanthopterin *in vivo* (Knipe and McCormack, 1977). Although isoxanthopterin has been isolated from human urine (Blair, 1958 a; Fukushima and Shiota, 1972; Krumdieck *et al*, 1978) it fulfills no known metabolic function although its use as an antimetabolite in cancer chemotherapy (Rettit *et al*, 1972) and its interference in DNA synthesis (Kokolis, 1975) infers its deleterious effect on normal metabolism. It is also conceivable that reduced pterin derivatives generated *in vivo* could act as competitors for sites on the folate and pteridine requiring enzymes. It is thus proposed that the pteridine moieties generated by cleavage are metabolised to pteridine P, urea and CO_2 by as yet undefined detoxification mechanisms and excreted in the urine, when formed in the tissues, or into the faeces when generated in the intestine.

Acetylation of aromatic amines is a well known route in the excretion of such compounds, and acetylation of p-aminobenzoyl-L-glutamate

is not therefore unexpected. The observation that loss of the glutamate residue may occur, ostensibly by intestinal metabolism (see Chapter 6) with the generation of p-aminobenzoate derivatives couldalso have metabolic consequences. p-Aminobenzoate has long been described as a B group vitamin although its function is obscure and no nutritional deficiency disorders associated with this compound are known. Both p-aminobenzoate and anthranilic acid (o-aminobenzoic acid) interfere with the respiratory chain in Streptomyces noursei, increasing the $NADH/NAD^+$ ratio in growing mycelium (Gräfe et al, 1978). That such metabolic effects may be of significance in higher life forms including man is supported by the increased oxygen uptake of rickettsial infected chicken eggs (Greiff and Pinkerton, 1948) and the successful therapy of Rocky Mountain spotted fever, a potentially lethal rickettsial infection in man, with p-aminobenzoic acid (Ross et al, 1948). Interestingly in the latter study leukopenia occurred in some cases as a toxic side effect of the therapy.

An alternative to the proposition that the observed folate catabolites are the byproducts of an unavoidable degradation route is that folate scission is a physiological requirement for the generation of unknown but metabolically essential unconjugated pteridines, (Reed et al, 1978). However the tetrahydrobiopterin derivatives which function as coenzymes in aromatic aminoacid hydroxylation reactions, the only known function of unconjugated pteridines in the mammal, are synthesised by an unrelated route from the nucleotide guanosine triphosphate (Brown 1971) and no direct interconversion between folate and tetrahydrobiopterin derivatives occurs (Fukushima and Shiota, 1972; Leeming et al, 1976; Chapter 6 of this thesis). In the absence of neither evidence for a physiological role for folate derived pteridine catabolites nor of a specific enzyme mediated cleavage reaction which would generate these the catabolic route suggested above would seem to be the most probable

one. Further studies on both the identity and metabolic status of pteridine P in the mammal are obviously important in establishing this mechanism.

The Effect of Implanted Walker 256 Tumours on Folate Metabolism in the Rat

Detailed chromatographic analysis of urine from rats bearing implanted Walker 256 carco-sarcinomas has confirmed the potential disruptive effect of tumours on folate metabolism. Changes in the 10 CHOTHF to 5 MeTHF ratio, ascribed to a raised folate requirement and increased utilization of the principal monoglutamate storage form (5 MeTHF), and the appearance of a novel urinary metabolite were observed. The evidence supports the hypothesis that the 10 CHOTHF to 5 MeTHF equilibrium is in some manner altered by proliferating tissues (Blair, 1975). The increased uptake and entry of labelled 10 CHOFA into the hepatic folate polyglutamate pool of tumour bearing rats supports the common clinical finding of folate deficiency in patients with cancer (Blakley, 1969). The quantitatively similar excretion of label following 10 CHOFA or folic acid radiotracers by normal and tumour bearing rats, despite the enhanced tissue retention in the latter animals, may suggest that analysis of urine and plasma samples (assuming a simple equilibrium between plasma and urinary folate monoglutamate exists) from subjects with neoplastic diseases does not accurately reflect the tissue folate levels and cellular folate depletion may be masked.

The relative expansion of the retained folate pool in host livers, but maintenance of a relatively normal folate monoglutamate pool size as reflected in urinary folate excretion, supports the hypothesis that changes in the normal ratio of the two folate pools (i.e. folate polyglutamates to folate monoglutamates) occur following alterations in the rate of cellular differentiation and proliferation (Whitehead, 1973).

Species Related Differences in Folate Metabolism; Folic Acid Metabolism in the Mouse; Relevance of Studies in the Rodent to Human Metabolism

Folate metabolism in the mouse differs markedly from the rat as evidenced by analysis of the urinary metabolites of oral doses of [2-^{14}C] folic acid (Chapter 7). Several additional metabolites were present in mouse urine and the rate of folate catabolism was evidently elevated. However the similarity of the retained hepatic folate in the mouse to that of the rat indicates that the folate polyglutamate pool fulfills a metabolically similar role in both rodents. Although the novel urinary metabolites were not identified they were tentatively assigned as pteridine degradation products. Increased folate catabolism in the mouse may be due to the generally high basal metabolic rate and, perhaps more importantly, the impact of anatomical differences such as the possession of a gall bladder. Stimulation of bile flow related to food intake could cause periodic flooding of the intestine with biliary folate in the mouse, whereas the flow of bile (and thus biliary folate) to the intestine in the rat is relatively constant. The extent of enterohepatic circulation of folate in the mouse is unknown but the effect of gall bladder storage on the chemical degradation of bile folate may be important. Investigation of mouse bile folate may be of more relevance to human studies than studies on rat biliary excretion, where the gall bladder is absent.

Extrapolation of experimental results obtained from studies on the rat to man can be done only with great caution, especially in the light of differences between rat and mouse folate metabolism, but general deductions can be drawn. The concentration of serum folates in the rat is an order of magnitude greater than in the human (Ratanasthien, 1975), although the major serum derivatives in both cases are 5 MeTHF and smaller amounts of 10 CHOTHF. Since the folate monoglutamate pool may act as a transient storage form for folate polygluta-

mate biosynthesis the quantitative difference in serum folate between man and the rat may simply reflect the smaller total plasma volume in the rat which would consequently limit the size of the circulating folate monoglutamate pool.

The use of radiolabels *in vivo* in man is necessarily limited although recent studies on the metabolism of mixtures of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] folic acid in normal patients and cancer patients have been reported from this laboratory (Pheasant *et al*, 1979). Metabolism of folic acid was dose dependent and at low doses (60 μg per patient) scission products (p-acetamidobenzoyl-L-glutamate and pteridine P) predominated whereas at higher doses (5 mg per patient) 5 MeTHF and folic acid were the major urinary species. Although faecal radioactivity was not determined the urinary recovery was much lower at the low dose suggesting increased tissue retention. Both these observations parallel the dose dependent metabolism of 10 CHOFA observed in the rat. A noticeable difference was the observation that at the higher dose the distribution of urinary metabolites was similar in 0-6 h, 6-12 h and 12-24 h urine samples. This slower interconversion to other folate forms may be related to an increased capacity to retain folate monoglutamates due to the larger plasma volume available. Whilst more long term studies in man are unavailable the similarities pointed out above justify the use of studies in the rat as a model for human metabolism *in vivo* since analogous processes are evident in both species.

Criticism of Methods and Suggestions for Further Work

Identification of metabolites by chromatographic methods and more especially of folate derivatives suffers many drawbacks (see Chapters 1 and 2). Although more detailed chemical and biological evidence for the rigorous identification of several metabolites was attempted, this is not practicable for routine assay of samples. Quantification of metabolites was often hamp ered due to both overlap of peaks and incom-

plete resolution of compounds caused by unavoidable minor variations during column chromatography. For example folate S, a minor derivative found in some urine samples, was often swamped by the large amounts of 5 MeTHF and 10 CHOFA which could not be completely resolved even by chromatography on two column systems. The development of refined separation techniques is needed to avoid these problems.

The use of HPLC may prove of value since a wide range of different column packings are available and these may prove capable of improved resolution. The recent application of HPLC to analysis of serum folates demonstrates its potential usefulness (Chapman et al, 1978).

The identification of 10 CHOFA$(glu)_4$ as the major labelled folate in hot ascorbate extracts of liver provides a useful basis for further studies. This derivative is intensely fluorescent and estimation of endogenous tissue folate derivatives, without the requirement for a radiolabel, by a combination of gel permeation chromatography and fluorescence detection may be feasible, although this awaits scaling down of the method to more manageable tissue amounts. Gel permeation (possibly HPLC) is suggested since although different folate derivatives do not obey simple elution behaviour on Sephadex G15 gel filtration the homologous series 10 CHOFA, 10 CHOFAglu, 10 CHOFA$(glu)_3$ and 10 CHOFA$(glu)_4$ was found to elute linearly according to molecular weight.

A major problem encountered in the present study was the finding that although the use of a double isotope was essential in whole animal studies of folate metabolism to avoid ambiguities in the assignment of metabolites the use of a mixture of [2-^{14}C] and [3', 5', 9(n) - ^{3}H] folate revealed apparent discrepancies in the handling of the individual molecules. Further work is required to identify the source of this isotope effect. The use of a true dual labelled tracer, i.e folate incorporating both ^{14}C and ^{3}H in a single molecule, is likely to be expensive and is probably unjustified since the isotope effect would

remain and could lead to considerable distortion of the folate profile obtained.

Most human tumours are slow growing and the use of the effect of rapidly proliferating animal tumours such as the Walker 256 carcosarcinoma on whole animal metabolism may not be strictly applicable as a model for human cancers. Extension of metabolism studies to the effect of slow growing tumours is therefore important. More detailed studies on folate metabolism in the mouse, using a dual label, may be fruitful since the effect of human tumours (xenografts) can be studied directly.

The present work leaves several questions outstanding and the picture of folate metabolism in the normal rat is still far from complete. The identification of pteridine P in particular and of the other unknown folate derived compounds of rat and mouse urine is essential in the elucidation of the mechanism and function of folate catabolism. Studies on hepatic folate need extending to other tissues and more information on the rate of formation and possibly regulatory significance of 10 CHOTHF$(glu)_4$ and its oxidation product 10 CHOFA$(glu)_4$ are required for complete appraisal of their function in cellular metabolism.

BIBLIOGRAPHY

Abou-el-Makarem, M.M., Millburn, P., Smith, R.L. & Williams, R.T. (1967) Biochem. J. 105,1289-1293

Albert, A. & Matsuura, S. (1962) J. Chem. Soc. 2162-2171

Albrecht, A.M. (1979) in Developments in Biochemistry (Kisliuk, R.L. & Brown, G.M. eds.), vol. 4, pp. 635-640, Elsevier North Holland Inc., New York

Albrecht, A.M., Boldizsar, E. & Hutchison, D.J. (1978) J. Bacteriol. 134, 506-513

Alt, F.W., Kellems, R.E., Bertino, J.R. & Schimke, R.T. (1978) J. Biol. Chem. 253, 1357-1370

Angier, R.B., Boothe, J.H., Hutchings, B.L., Mowat, J.H., Semb, J., Stokstad, E.L.R., Subbarow, Y., Waller, C.W., Cosulich, D.B., Fahrenbach, M.J., Hultquist, M.E., Kuh, E., Northey, E.H., Seeger, D.R., Sickels, J.P. & Smith, J.M.Jr. (1946) Science 103, 667-669

Argentini, M. & Viscontini, M. (1973) Helv. Chim. Acta 56, 2920-2924

Arly-Nelson, J., Harris, B.A., Decker, W.J. & Farquhar, D. (1977) Cancer Res. 37, 3970-3973

Bailey, S.W. & Ayling, J.E. (1977) J. Biol. Chem. 253, 1598-1605

Baker, B.R., Santi, D.V., Almaula, P.I. & Werkheiser, W.C. (1964) J. Med. Chem. 7, 24-30

Barford, P.A. & Blair, J.A. (1975) in Chemistry and Biology of Pteridines (Pfleiderer, W., ed.), pp. 413-427, Walter de Gruyter, Berlin

Barford, P.A. & Blair, J.A. (1978) Br. J. Cancer 38, 122-129

Barford, P.A., Staff, F.J. & Blair, J.A. (1977a) Biochem. J. 164, 601-605

Barford, P.A., Blair, J.A., Staff, F.J. & Malghani, M.A.K. (1977b) Biochem. Soc. Trans. 5, 1316-1318

Barford, P.A., Staff, F.J., & Blair, J.A. (1978) Biochem. J. 174, 579-583

Baugh, C.M., Braverman, E. & Nair, M.G. (1974) Biochemistry 13, 4952-4957

Baugh, C.M., May, L., Braverman, E. & Nair, M.G. (1979) in Developments in Biochemistry (Kisliuk, R.L. & Brown, G.M. eds.) vol. 4, pp. 219-224, Elsevier North Holland Inc., New York.

Beavon, J.R.G. (1973) PhD thesis, University of Aston in Birmingham, Birmingham, U.K.

Beavon, J.R.G. & Blair, J.A. (1972) Br. J. Nutr. 28, 385-390

Beavon, J.R.G. & Blair, J.A. (1975) Br. J. Nutr. 33, 299-308

Berg, C.P. & Kolenbrander, H.M. (1970) in Comparative Biochemistry of Nitrogen Metabolism (Campbell, J.W., ed.) Vol. 2, pp. 795-916, Academic Press, London and New York

Bertino, J.R., Booth, B.A., Bieber, A.L., Cashmore, A. & Sartorelli, A.C. (1964) J. Biol. Chem. 239, 479-483

Bertino, J.R., Perkins, J.P. & Johns, D.G. (1965) Biochemistry 4, 839-846

Bessey, O.A., Lowry, H.O. & Broch, M.J. (1946) J. Biol. Chem. 164, 321-329

Bird, O.D., McGlohon, V.M. & Vaitkus, J.W (1965) Anal. Biochem. 12, 18-35

Blair, J.A. (1958a) Biochem. J. 68, 385-387

Blair, J.A. (1958b) Nature, 181, 996

Blair, J.A. (1975) in Chemistry and Biology of Pteridines (W. Pfleiderer, ed.), pp. 373-405, Walter de Gruyter, Berlin.

Blair, J.A. & Dransfield, E. (1971) Biochem. J. 123, 907-914

Blair, J.A. & Matty, A.J. (1974) Clinics in Gastroenterol, 3, 183-197

Blair, J.A., Pearson, J.A. & Robb, A.J. (1975) J. Chem. Soc., Perkin Trans. 2, 18-21

Blakley, R.L. (1959) Biochem. J. 72, 707-715

Blakley, R.L. (1969) in Frontiers in Biology (Neuberger, A. & Tatum, E.L., eds.) vol. 13, North Holland, Amsterdam

Braganca, B.M., Aravindakshan, I. & Ghanekar, D.S. (1957) Biochim. Biophys. Acta 25, 623-634

Brown, G.M. (1971) Meth. Enzym. 18B, 761-765

Brown, J.P., Scott, J.M., Foster, F.G. & Weir, D.G. (1973) Gastroenterology 64, 223-232

Brown, J.P., Davidson, G.E. & Scott, J.M. (1974) Biochim. Biophys. Acta. 343, 78-88

Burgen, A.S.V. & Goldberg, N.J. (1962) Br. J. Pharmacol. 19, 313-320

Butterworth, C.E.Jr., Santini, R. & Frommeyer, W. (1963) J. Clin. Invest. 42, 1929-1939

Calvert, A.H., Bondy, P.K. & Harrap, K.R. (1977) Cancer Treat. Rep. 61, 1647-1656

Cerecodo, L.R. (1931) J. Biol. Chem. 93, 269-274

Chanarin, I., Mollin, D.L. & Anderson, B.B. (1958) Proc. Roy. Soc. Med. 51, 757-763

Chapman, S.K., Greene, B.C. & Streiff, R.R. (1978) J. Chromatogr. 145, 302-306

Chello, P.L., McQueen, C.A., DeAngelis, L.M. & Bertino, J.R. (1977) Cancer Treat. Rep. 61, 539-548

Cheng, F.W., Shane, B. & Stokstad, E.L.R. (1975) Can. J. Biochem. 53, 1020-1027

Chippel, D. & Scrimgeour, G. (1970) Can. J. Biochem. 48, 999-1009

Cohen, P.P. & McGilvery, R.W. (1947) J. Biol. Chem. 171, 121-133

Connor, H., Newton, D.J., Preston, F.E. & Woods, H.F. (1978) Postgrad. Med. J. 54, 318-320

Connor, M.J. & Blair, J.A. (1979) in Developments in Biochemistry (Kisliuk, R.L. & Brown, G.M. eds.) vol. 4, pp. 531-532, Elsevier North Holland Inc., New York

Connor, M.J., Blair, J.A. & Barford, P.A. (1977) Biochem. Soc. Trans. 5, 1319-1320

Connor, M.J., Pheasant, A.E. & Blair, J.A. (1979) Biochem. J. 178, 795-797

Cook, R.J. & Blair, J.A. (1979) Biochem. J. 178, 651-660

Corrocher, R., Bhuyan, B.K. & Hoffbrand, A.V. (1972) Clin. Sci. Mol. Med. 43, 799-813

Corrocher, R., Sandre, D., Paeor, M. & Hoffbrand, A.V. (1974) Clin. Sci. Mol. Med. 46, 551-554

Curthoys, N.P., Scott, J.M. & Rabinowitz, J.C. (1972) 247, 1959-1964

Dabrowska, W., Kazenko, A. & Laskowski, M. (1949) Science 110, 95

Dev, I.K. & Harvey, R.J. (1978a) J. Biol. Chem. 253,4242-4244

Dev, I.K. & Harvey, R.J. (1978b) J. Biol. Chem. 253, 4245-4253

Dinning, J.S., Sime, J.T., Work, P.S., Allen, B. & Day, P.L. (1957) Arch. Biochem. Biophys. 66, 114-119

Dinovo, E.C., Lynn, J.K., McIntosh, M.E., Johnson, M. & Bauer, C. (1978) Proc. West.Pharmacol. Soc. 21, 395

Dolnick, B.J. & Cheng, Y. (1978) J. Biol. Chem. 253, 3563-3567

Domin, B.A., Cheng, Y. & Hakala, M.T. (1979) in Developments in Biochemistry (Kisliuk, R.L. & Brown, G.M. eds.) vol. 4, pp. 395-399, Elsevier North Holland Inc., New York

Donaldson, K.O. & Keresztesy, J.C. (1959) J. Biol. Chem. 234, 3235-3240

Drury, H.F. (1948) Arch. Biochem. 19, 455-466

Earle, W.R. (1935) Am. J. Cancer 24, 566-612

Farnworth, E.R. & Hill, D.C. (1977) Nutr. Rep. Int. 16, 565-572

Finkelstein, J.D. & Mudd, S.H. (1967) J. Biol. Chem. 242, 873-880

Finkelstein, J.D., Kyle, W.E. & Harris, B.J. (1971) Arch. Biochem. Biophys. 146, 84-92

Finkelstein, J.D., Martin, J.J., Kyle, W.E. & Harris, B.J. (1978) Arch. Biochem. Biophys. 191. 153-160

Fischer, C.D., da Costa, M. & Rothenberg, S.P. (1975) Blood 46, 855-867

Floystrup, T., Schou, M.A. & Kalckar, H.M. (1949) Acta Chem. Scand. 3, 985-990

Francis, K.T., Thompson, R.W., & Krumdieck, C.L. (1977) Amer. J. Clin. Nutr. 30, 2028-2032

Friedkin, M. (1973) in Advances in Enzymology (Meister, A. Ed.) pp. 279-281, John Wiley, New York

Friedkin, M., Crawford, E.J. & Mistra, D. (1962) Fed. Proc. 21, 176

Friedkin, M. Plante, L.T., Crawford, E.F. & Crumm, M. (1975) J. Biol. Chem. 250, 5614-5621

Fritzon, P. & Pihl, A. (1957) J. Biol.Chem. 226, 229-235

Fukushima, T. & Shiota, T. (1972) J. Biol. Chem. 247, 4549-4556

Futterman, S. (1963) Meth. Enzym. 6, 801-802

Futterman, S. & Silverman, M. (1957) J. Biol. Chem. 224, 31-40

Gapski, G.R., Whiteley, J.M. & Huennekens, F.M. (1971) Biochemistry 10, 2930-2934

Goldman, I.D., (1977) Cancer Treat. Rep. 61, 549-558

Goodfriend, T.L. & Kaufman, S. (1961) J. Clin. Invest. 40,1743-1750

Gräfe, U., Bocker, H. Reinhardt, G. & Thrum, H. (1978) Z. allgemeine Microbiol. 18, 479-486

Greiff, D. & Pinkerton, H. (1948) J. Expt. Med. 87, 175-197

Hachey, D.L., Palladino, L., Blair, J.A., Rosenberg, I.H. & Klein, P.D. (1978) J. labelled compd. 14, 479-486

Haddow, A., Ross, W.C.J. & Timmis, G.M. (1972) Perspect. Biol. Med. 15, 177-217

Halpern, R., Halpern, B.C., Stea, B., Dunlap, A., Conklin, K., Clark, B., Ashe, H. Sperling, L., Halpern, J.A., Hardy, D. & Smith, R.A. (1977) Proc. Natl. Acad. Sci. USA. 74, 587-591

Halsted, C.H., Reisenauer, A., Beck, C. & Gotterer, G.S. (1976) J. Nutr. 106, 485-492

Hamilton, G.A. (1971) in Progress in Biorganic Chemistry (Kaiser, E.T. & Kezdy, T.J., eds.) vol. 1, pp. 83-157, Wiley Interscience, New York

Hepner, G.W. (1969) Br. J. Haematol. 16, 241-249

Herbert, V. & Zalusky, R. (1962) J. Clin. Invest. 41, 1263-1276

Herbert, V., Larrabee, A.L. & Buchanan, J.M. (1962) J. Clin. Invest. 41, 1134-1138

Hillman, R.S., McGuffin, R. & Campbell, C. (1977) Trans. Assoc. Am. Physicians 90, 145-156

Hoffbrand, A.V. (1976) Progress Haematol. 9, 85-105

Houlihan, C.M. & Scott, J.M. (1972) Biochem. Biophys. Res. Commun. 48, 1675-1681

Hulsmann, W.C. & Van Eps, L.W.S. (1967) Clin. Chim. Acta 15, 233-239

Hutchings, B.L., Stokstad, E.L.R., Boothe, J.H. Mowat, J.H., Walker, C.W., Angier, R.B., Semb, J. & Subbarow, Y. (1947) J. Biol. Chem. 68, 705-710

Itoh, R. (1975) Int. J. Biochem. 1, 617-623

Iwai, K. Oe, H. & Kobashi, M. (1979) in Developments in Biochemistry (Kisliuk, R.L. & Brown, G.M. eds.) vol. 4, pp. 659-664, Elsevier North Holland Inc., New York

Jackson, R.C. & Harrap, K.R. (1973) Arch. Biochem. Biophys 158, 827-841

Jackson, R.C. & Neithammer, D. (1977) Eur. J. Cancer 13, 567-575

Jackson, R.C., Neithammer, D. & Hart, L.I. (1977) Arch. Biochem. Biophys. 182, 646-656

Jacobs, S.A., Stroller, R.G., Chabner, B.A. & Johns, D.G. (1976) J. Clin. Invest. 57, 534-538

Jacobs, S.A., Derr, C.J. & Johns, D.G. (1977) Biochem.Pharmacol. 26, 2310-2313

Johns, D.G. & Plenderleith, I.H. (1963) Biochem. Pharmacol. 12, 1071-1074

Johns, D.G., Sperti, S. & Burgen, A.V. (1961) J. Clin. Invest. 40, 1684-1695

Johnson, L.F., Fuhrman, C.L. & Wiedemann, L.M. (1978) J. Cell. Physiol. 97, 397-406

Kazenko, A. & Laskowski, M. (1948) J. Biol. Chem. 173, 217-221

Keresztesy, J.C. & Silverman, M. (1951) J. Am. Chem. Soc. 73, 5510

Kesavan, V. & Noronha, J.M. (1978) J. Physiol. 280, 1-7

Kisliuk, R.L., Gaumont, Y. & Baugh, C.M. (1974) J. Biol. Chem. 249, 4100-4103

Kokolis, N. (1975) Folia Biochem. Biol. Graeca 12, 11-17

Koschara, W. (1936) Z. Physiol. Chem. 246, 127-151

Knipe, J.O. & McCormack, J.J. (1977) Biochem. Pharmacol 26, 2217-2224

Krebs, H.A., Hems, R. & Tyler, B. (1976) Biochem J. 158, 341-353

Krumdieck, C.L., Boots, L.R., Cornwell, P.E. & Butterworth, C.E.Jr. (1976) Am. J. Nutr. 29, 288-294

Krumdieck, C.L., Fukushima, K., Fukushima, T., Shiota, T. & Butterworth, C.E.Jr. (1978) Am. J. Clin. Nutr. 31, 88-93

Kutzbach, C.A. & Stokstad, E.L.R. (1971) Method. Enzymol. 18B, 793-798

Lavoie, A. & Cooper, B.A. (1974) Clin. Sci. Mol. Med., 46, 729-741

Lavoie, A., Tripp, E. & Hoffbrand, A.V. (1974) Clin. Sci. Mol. Med. 47, 617-630

Leeming, R.J., Blair, J.A., Melikian, V. & O'Gorman, D J. (1976) J. Clin. Pathol. 29, 444-451

Lepage, R., Poirier, L.A., Poirier, M.C. & Morris, H.P. (1972) Cancer Res. 32, 1099-1103

Leslie, K.I. & Rowe, P.B., (1972) Biochemistry 11, 1696-1703

Lewis, G.P. & Rowe, P.B. (1977) Proc. Australian Biochem.Soc. 10, 48

Lewis, G.P. & Rowe, P.B. (1979) in Developments in Biochemistry (Kisliuk, R.L. & Brown, G.M. eds.) vol.4, pp. 253-254,Elsevier North Holland Inc., New York

Lewis, G.P., Salem, M.E. & Rowe, P.B. (1979) ibid., pp. 441-442

Lewis, K.F., Randolph, V.M., Nemeth, E. & Frisell, W.R. (1978) Arch. Biochem. Biophys. 185, 443-449

Lowry, O.H., Bessey, O.A. & Crawford, E.J. (1949) J. Biol. Chem. 180, 389-398

Lowry, O.H. & Crawford, E.J. (1949) J. Biol. Chem. 180, 399-410

Maclean, F.I., Forrest, H.S. & Hoare, D.S. (1966) Arch. Biochem. Biophys. 117, 54-58

Magnus, E.M. (1967) Cancer Res. 27, 490-497

Maruyama, T., Shiota, T. & Krumdieck, C.L. (1978) Anal. Biochem. 84, 277-295

Mathews, C.K. & Huennekens, F.M. (1963) J. Biol. Chem. 238, 3436-3442

McBurney, M.W. & Whitmore, G.F. (1974) Cell 2, 173-812

McGuire, J.J., Kitamoto, Y., Hsieh, P., Coward, J.K. & Bertino, J.R. (1979) in Developments in Biochemistry (Kisliuk, R.L. & Brown, G.M., eds.) vol. 4, pp. 471-476, Elsevier North Holland Inc., New York

McIntyre, L.J. & Harding, N.G.L. (1977) Biochim. Biophys. Acta 482, 261-271

Mengel, R., Pfleiderer, W. & Knappe, W.R. (1977) Tetrahedron lett. 32, 2817-2820

Moran, R.G., Werkheiser, W.C. & Zakrewski, S.K. (1976) J. Biol. Chem. 251, 3569-3575

Murphy, M., Keating, M., Boyle, P., Weir, D.G. & Scott, J.M. (1976) Biochem. Biophys. Res. Commun. 71, 1017-1024

Murphy, M., Boyle, P.H., Weir, D.G. & Scott, J.M. (1978) Br. J. Haematol. 38, 211-218

Nakamura, K. & Kozloff, L.M. (1978) Biochim. Biophys. Acta 540, 313-319

Neal, G.E. & Williams, D.C. (1965) Biochem. Pharmacol 14, 903-914

Nixon, P.F. & Bertino, J.R. (1972) New Eng. J. Med. 286, 175-179

Nixon, P.F., Slutsky, G., Nahas, A. & Bertino, J.R. (1973) J. Biol. Chem. 248, 5932-5936

Noronha, J.M. & Silverman, M. (1962) in Eur. Symp. Vit. B_{12} Intrinsic Factor (Heinrich, H.C., ed.) pp. 728-736, F. Enke, Stuttgart

Oertel, J. (1976) Exp. Pathol. 12, 218-222

Osifo, B.O.A. (1978) Enzyme 23, 116-120

Parke, D.V. (1968) The Biochemistry of Foreign Compounds, 1st ed., pp. 77-81, Pergamon Press, London

Paukert, J.L., D'Aristrauss, L. & Rabinowitz, J.C. (1976) J. Biol. Chem. 251, 5104-5111

Pearson, A.J. (1974) Chem. Indust. 233-239

Perry, J. & Chanarin, I. (1977) Br. J. Haematol. 35, 397-402

Pettit, G.R., Houghton, L.E., Rogers, N.H., Coomes, R.M., Berger, D.F., Reucroft, P.R., Day, J.F., Hartwell, L. & Wood, H.B. (1972) Experientia 28, 381-382

Pfiffner, J.J., Calkins, D.G., Bloom, E.S., & O'Dell, B.L. (1946) J. Am. Chem. Soc. 68, 1392

Pheasant, A.E. & Blair, J.A. (1979) in Developments in Biochemistry (Kisliuk, R.L. & Brown, G.M., eds.) vol. 4, pp. 577-579, Elsevier North Holland Inc., New York

Pheasant, A.E., Blair, J.A. & Allan, R.N. (1979) ibid. 327-328

Piper, W.N. & Van Lier, R.B.L. (1977) Mol.Pharmacol. 13, 1126-1135

Poirier, L.A. (1973) Cancer Res. 33, 2109-2113

Pollock, R.J. & Kaufman, S. (1978) J. Neurochem. 31, 115-123

Rao, N. & Noronha, J.M. (1977a) Biochim. Biophys. Acta 481, 594-607

Rao, N. & Noronha, J.M. (1977b) Biochim. Biophys. Acta 481, 608-616

Ratanasthein, K. (1975) PhD thesis, University of Aston in Birmingham

Reed, B., Weir, D.G. & Scott, J.M. (1977) Clin. Sci. Mol. Med. 52, 83-86

Reed, B., Weir, D.G. & Scott, J.M. (1978) Clin. Sci. Mol. Med. 54, 355-360

Rembold, H., Chandrashekar, V. & Sudershan, P. (1971) Biochim. Biophys. Acta 237, 365-368

Retief, F.P., Heyns, A.P., Oosthuizen, M. & van Reenen, O.R. (1976) Br. J. Haematol. 33, 415-424

Robinson, D.R. (1971) Method. Enzymol. 18B, 716-725

Rose, D.P. (1966) J. Clin. Pathol. 19, 29-32

Rosenberg, I.H. & Godwin, H.A. (1971) Gastroenterology 60, 445-463

Rosenberg, I.H. & Neumann, H. (1974) J. Biol. Chem. 249, 5126-5130

Rosenblatt, D.S. & Erbe, R.W. (1973) Biochem. Biophys. Res. Commun. 54, 1627-1633

Ross, S., McLendon, P.A. & Davis, H.J. (1948) J. Paedriatr. 2, 163-174

Rostom, A.Y., Thomas, J.M., Peckham, M.J. & Steel, G.G. (1978) Lancet 428-429

Rothenberg, S.P., Fischer, C.D. & DaCosta, M. (1978) Biochim. Biophys. Acta 543, 340-348

Rowe, P.B. (1971) Method. Enzymol. 18B, 733-735

Rowe, P.B. (1978) in the Metabolic Basis of Inherited Disease (Stanbury, J.B., Wyngaarden, J.B. & Frederickson, D.S., eds.) 4th edition, pp. 430-457, McGraw-Hill, New York

Rowe, P.B., Silink, M. & Reddel, R. (1975) in Chemistry and Biology of Pteridines (W. Pfleiderer, ed.) pp. 85-92, Walter de Gruyter, Berlin

Santini, R., Brewster, C. & Butterworth, C.E.Jr. (1964) Am. J. Clin. Nutr. 14, 205-210

Sawada, F., Kanesaka, Y. & Irie, M. (1977) Biochim. Biophys. Acta 479, 188-197

Scrutton, M.C. & Beis, I. (1979) Biochem. J. 177, 833-846

Shin, Y.S., Williams, M.A. & Stokstad, E.L.R. (1972) Biochem. Biophys. Res. Commun. 47, 35-43

Shin, Y.S., Chan, C., Vidal, A.J., Brody, T. & Stokstad, E.L.R. (1976) Biochim. Biophys. Acta 444, 794-801

Shiota, T., Disraely, M.N. & McCann, M.P. (1964) J. Biol. Chem. 239, 2259-2266

Silverman, M., Keresztesy, J.C. & Koval, G.D. (1954) J.Biol.Chem. 211, 53-61

Silverman, M., Keresztesy, J.C., Koval, G.D. & Gardiner, R.C. (1957) J. Biol. Chem. 226, 83-94

Silverman, M., Law, L.W. & Kaufman, B. (1961) J. Biol. Chem. 236, 2530-2533

Sotobayashi, H., Rosen, F. & Nichol, C.A. (1966) Biochemistry 5, 3878-3883

Spector, R. Fosburg, M., Levy, P. & Abelson, M.T. (1978) J. Neurochem. 30, 899-901

Spronk, A.M. (1973) Fed. Proc. 32, 471

Stahl, P.D. & Touster, O. (1972) Meth. Enzym. 28, 814-819

Stea, B., Backlund, P.S., Berkey, P.B., Cho, A.K., Halpern, B.C., Halpern, R.M. & Smith, R.A. (1978) Cancer Res. 38, 2378-2384

Stocks-Wilson, R. (1971) PhD thesis, University of Aston in Birmingham, Birmingham, U.K.

Stokstad, E.L.R., Hutchings, B.L., Mowat, J.H., Boothe, J.H., Waller, C.W., Angier, R.B., Semb, J. & Subbarow, Y. (1948) J. Am. Chem. Soc. 70, 5-9

Stokstad, E.L.R., Webb, R.E. & Shah, E. (1966) J. Nutr. 88, 225-232

Stout, R.W., Cashmore, A.R., Coward, J.K., Horvath, C.G & Bertino, J.R. (1976) Anal. Biochem. 71, 119-124

Strum, W.B., Liem, H.H. & Muller-Eberhard, U. (1979) in Developments in Biochemistry (Kisliuk, R.L. & Brown, G.M. eds.) vol. 4, pp. 615-618, Elsevier North Holland Inc., New York

Tabor, C.W., Freeman, M.V., Bailey, J. & Smith, P.K. (1951) J. Pharmacol. Exp. Ther. 102, 98-102

Tan, L.U.L., Drury, E.J. & Mackenzie, R.E. (1977) J. Biol. Chem. 252, 1117-1122

Taylor, R.T. & Hanna, M.L. (1977) Arch. Biochem. Biophys. 181, 331-344

Thorndike, J. & Beck, W.S. (1977) Cancer Res. 37, 1125-1132

d'Urso Scott, M. & Makulu, D.R. (1973) Fed. Proc. 32, 471

d'Urso Scott, M., Uhoch, J. & Bertino, J.R. (1974) Proc. Natl. Acad. Sci. USA. 71, 2736-2739

Uyeda, K. & Rabinowitz, J.C. (1963) Anal. Biochem. 6, 100-108

Valerino, D.M. (1972) Res. Commun. Chem. Pathol. Pharmacol. 4, 529-542

Valerino, D.M., Johns, D.G., Zaharko, D.S. & Oliverio, V.T. (1972) Biochem. Pharmacol. 21, 821-831

Waller, C.W., Goldman, A.A., Angier, R.B., Boothe, J.H., Hutchings, B.L., Mowat, J.H. & Semb, J. (1950) J. Am. Chem.Soc. 72, 4630-4633

Wang, F.K., Koch, J. & Stokstad, E.L.R. (1967) Biochem Z. 346, 458-466

Watson, B.M., Schlesinger, P. & Cotton, R.G.H. (1977) Clin. Chim. Acta 78, 417-423

Waxman, S. (1975) in Chemistry and Biology of Pteridines (W. Pfleiderer, ed.) pp. 165-178, Walter de Gruyter, Berlin

Waxman, S. & Schreiber, C. (1973) Blood 42, 281-290

White, W.E., Yielding, K.L. & Krumdieck, C.L. (1976) Biochim. Biophys. Acta 429, 689-693

Whitehead, V.M. (1973) Clin. Res. 21, 571

Whiteley, J.M., Drais, J., Kirchner, J. & Huennekens, F.M. (1968) Arch. Biochem. Biophys. 126, 954-957

Whittle, E.L., O'Dell, B.L., Vanderbilt, J.M. & Pfiffner, J.J. (1947) J. Am. Chem. Soc. 69, 1786-1792

Wong, P.W.K., Justic, P., Ruby, M.H., Weiss, E.B. & Diamond, E. (1977) Pediatr. 59, 749-756

Wright, B.E. (1955) J. Am. Chem. Soc. 77, 3930-3932

Wright, B.E. & Anderson, M.L. (1957) J. Am. Chem. Soc. 79, 2027-2028

Zalusky, R. & Herbert, V. (1962) Lancet 1, 108

Zakrewski, S.F. (1960) J. Biol. Chem. 235, 1780-1784

Zakrewski, S.F., Evans, E.A. & Phillips, R.F. (1970) Anal. Biochem. 36, 197-206

Zamierowski, M. & Wagner, C. (1974) Biochem. Biophys. Res. Commun. 60, 81-87