Potential Inhibitors

of

Dihydrofolate Reductase :

Synthesis and NMR Spectroscopy

by

YAP CHEE HOONG

A Thesis submitted for the Degree of Doctor of Philosophy

at

The Department of Molecular Sciences (Chemistry)

The University of Aston in Birmingham

December 1985

The University of Aston in Birmingham

Potential Inhibitors of Dihydrofolate Reductase: Synthesis and NMR Spectroscopy

by

YAP Chee Hoong

for the Degree of Doctor of Philosophy
December 1985

SUMMARY

Our aim was to investigate differences in the manner various dihydrofolate reductases bind folic acid analogues. These studies were hoped to eventually lead to the design of antifolates capable of discriminating between closely related enzymes on the basis of small differences in their primary structures.

Several folates were synthesized and assayed against bacterial and vertebrate DHFRs to determine reduction rates and K_is. NMR spectra were recorded to determine how minor modifications at N(10) and the benzene ring affected proton and ^{13}C chemical shifts. Analysis of primary structure and X-ray crystallography of DHFRs containing bound antifolate have suggested the presence of potential nucleophiles in the binding pockets of many reductases. Folates with reduced electron density at the benzene ring were hoped to bind preferentially to these enzymes and discriminate against others.

NMR spectroscopy suggested a 'bent' conformation for folates in $^{2}H_6$-dimethylsulphoxide. Benzene and pteridine rings are thought to be in close proximity in space, π-cloud interactions presumably stabilizing the molecule. Small modifications have produced large chemical shift changes, these mainly attributable to conformational change. Since they could not be separated into 'through-bond' (from the substituents) and 'through-space' (from the anisotropic benzene and pteridine rings) effects, we were unable to correlate the electronic properties of substituents with changing electron density at the benzene ring. We have speculated on possible reasons behind different reduction rates and K_is.

Benzene-ring fluorinated folates were synthesized with several studies in mind including:-
(i) DHFR-folate interaction in solution using ^{19}F NMR, (ii) folate metabolism by analysing urine samples, and (iii) positron emission tomography using ^{18}F.

Time limitations, non-reducibility of fluorofolates, publication of a paper (Clore *et al.*, 1984) describing some of the intended ^{19}F NMR experiments and inability to prepare ^{18}F-folate have forced the cancellation of these studies.

Key words: Dihydrofolate reductase (DHFR), nuclear magnetic resonance (NMR) spectroscopy, antifolates, folic acid analogues and nucleophiles.

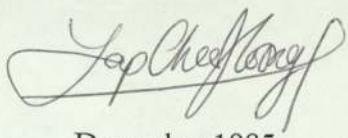
ACKNOWLEDGEMENTS

I am indebted to The Cancer Research Campaign, The Overseas Research Studentships Awards Scheme and The University of Aston in Birmingham for the financial assistance which has made this project possible. I am grateful to Dr. Susan Whitburn and Professor John A. Blair for their supervision, patience and continual guidance.

I also wish to thank all members of The Folate Group for their kind help and many stimulating discussions. Thanks are also extended to everyone in the NMR laboratory.

Finally, I would like to express appreciation for the moral support and encouragement shown by my family in Kuala Lumpur throughout the duration of this project.

No part of the work described in this thesis was done in collaboration unless specifically stated. This work has not been submitted for any other academic award.



December 1985

CONTENTS

CONTENTS

CONTENTS

CONTENTS

LIST OF GRAPHS* AND TABLES

LIST OF GRAPHS* AND TABLES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

A HISTORICAL

Work on pteridines was first begun by Hopkins (1889,1891) who investigated butterfly wing pigments. 29 years elapsed before pure pigments were isolated from brimstone and cabbage butterfly wings by Wieland and Schöpf (1925,1926). They named these compounds xanthopterin and leucopterin, respectively, as an indication of their source and colour (Greek xanthos, yellow; leukos, white; and pteron, wing). Another 14 years followed before Purrman (1940a and b) was able to characterise these compounds (figure A I).

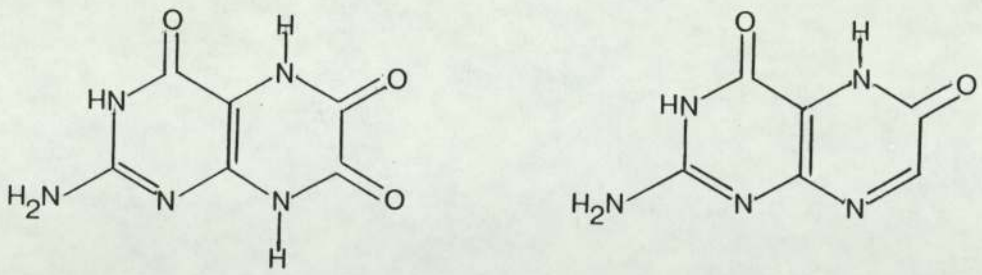


Figure A I Structures of leucopterin (left) and xanthopterin (right)

The term "pteridine" was coined by Wieland (Schöpf and Reichert, 1941) for these and other compounds bearing the same bicyclic nitrogenous ring system. The numbering system (figure A II), proposed by Kuhn and Cook (1937), was based on

rules applied to all aza derivatives of naphthalene. This has been approved by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB).

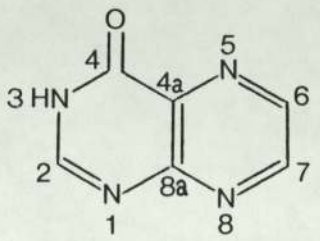


Figure A II IUPAC and IUB approved numbering system for pteridines

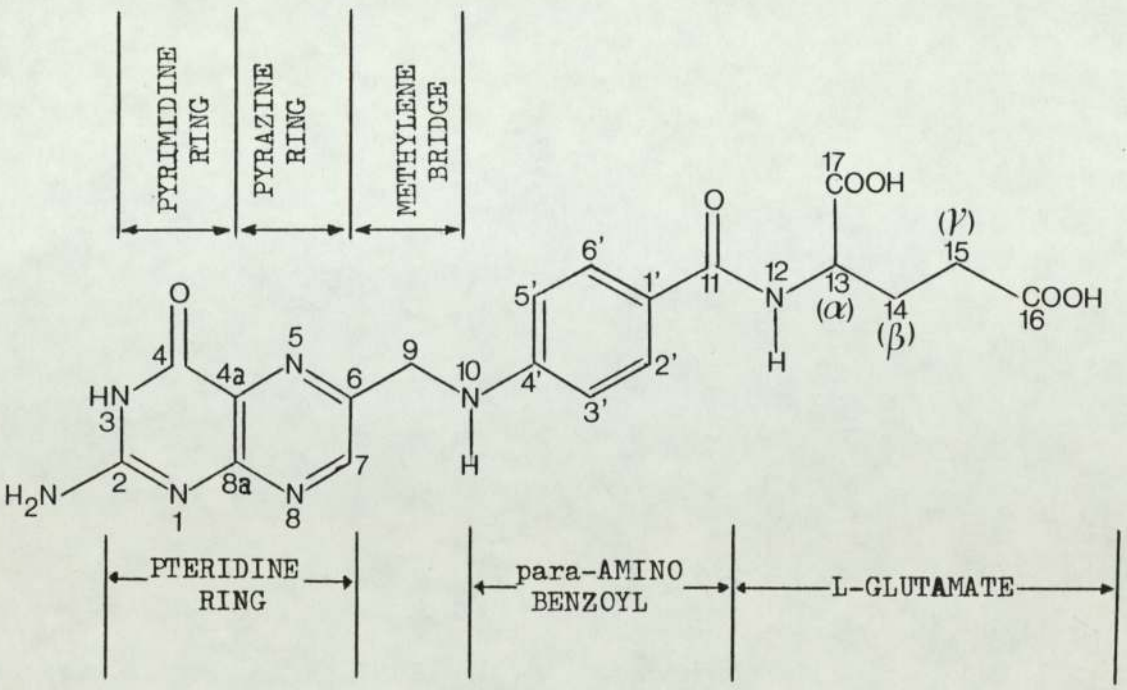


Figure A III Structure of folic acid illustrating the parts of the molecule and the numbering system

Folic acid was discovered after it was found that yeast and liver extracts were effective in treating tropical macrocytic anaemia (Wills, 1931). A factor was isolated which was later shown to comprise linked pteridine and benzoylglutamate moieties (Mowat *et al.*, 1946 and 1948) (figure A III).

B ROLES OF FOLATE AND DIHYDROFOLATE REDUCTASE (DHFR) IN METABOLISM

Folic acid is an artefact formed during the isolation of the growth factor in yeast and liver extracts. Reduced folates not stabilized by 5-methyl substitution are readily oxidised in air to a number of compounds, some of which possess the pteroylglutamate skeleton (Blakley, 1957; Zakrzewski, 1966). The major dietary form of folates is 5-methyltetrahydrofolate (5-Me-THF). This compound and the 5-formyl analogue are both absorbed by cells (Goldman, 1971; Bender, 1975). Some important folate metabolic pathways are illustrated in figure A IV.

The role of dihydrofolate reductase (DHFR, tetrahydrofolate: $NADP^+$ oxidoreductase; tetrahydrofolate dehydrogenase; EC 1.5.1.3) is shown in pathway 1 of figure A IV. It catalyses the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF). The importance of THF lies in the reaction where 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) is converted to DHF (pathway 2) under the influence of thymidylate synthetase (TS). TS simultaneously catalyses the synthesis of thymidylate from deoxyuridylate (Werkheiser, 1969). This reaction depletes the cellular pool of THF more rapidly than it can be replenished by

tetrahydropteroylglutamate methyltransferase (pathway 6, figure A IV). In order to

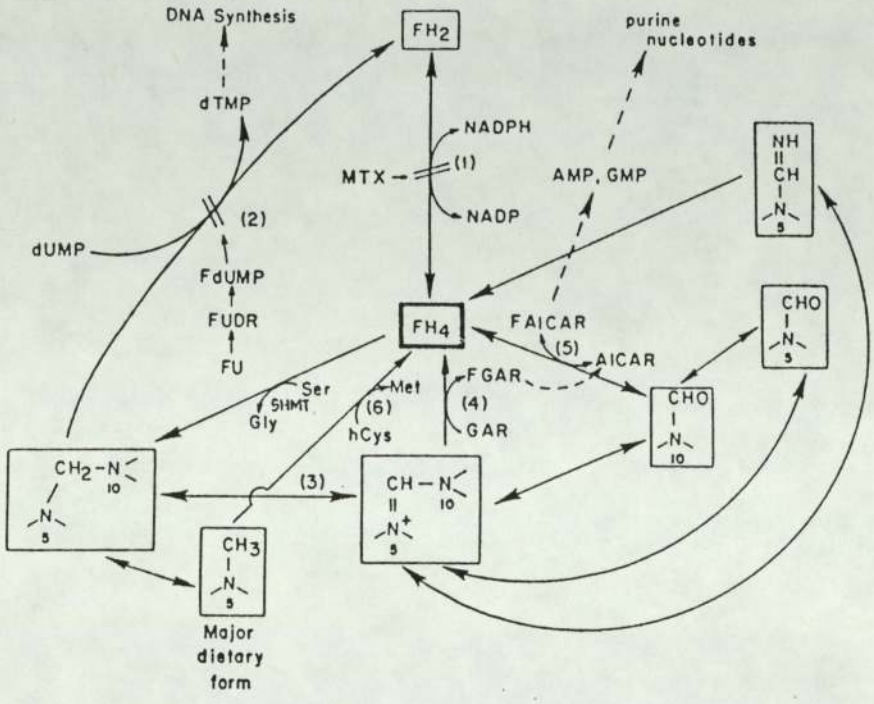


Figure AIV Some important folate metabolic pathways, illustrating the role of dihydrofolate reductase (1). (1) Dihydrofolate reductase (tetrahydrofolate dehydrogenase, EC 1.5.1.3: (2) thymidylate synthetase (EC 2.1.1.45); (3) methylene tetrahydrofolate dehydrogenase (EC 1.5.1.5); (4) phosphoribosylglycinamide formyltransferase (EC 2.1.2.2); (5) phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3); (6) tetrahydropteroylglutamate methyltransferase (EC 2.1.1.13); (5>N–CH_2–N<)=5,10-methylenetetrahydrofolate; (5>N–CH_3–=5-methyltetrahydrofolate; (5>N^+=CH–N<)=5,10-methenyltetrahydrofolate; (5>N–CHO)=5-formyltetrahydrofolate (citrovorum factor, CF); 5>N–CH=NH)= 5-formiminotetrahydrofolate; (10>N–CHO) = 10-formyltetrahydrofolate; hCys = homocysteine; FGAR = formylglycinamide ribonucleotide; GAR = glycinamide ribonucleotide; AICAR = aminoimidazolecarboxamide ribonucleotide, FAICAR = 5-formamidoimidazolecarboxamide ribonucleotide; dUMP = deoxyuridylate; AMP = adenylic acid; GMP = guanylic acid; dTMP = thymidylate; FU = 5-fluorouracil. FUDR = 5-fluorodeoxyuridine; FdUMP = 5-fluorodeoxyuridylate

maintain the cellular pool of tetrahydrofolate derivatives, THF must be regenerated via the DHFR catalysed reaction (pathway 1). DHFR inhibitors halt thymidylate production by this pathway. Cells which are rapidly synthesizing DNA will be depleted of THF most rapidly. The consequent thymidylate deficiency results in disruption of nucleic acid synthesis. As THF is also an essential cofactor in the biosynthesis of glycine, methionine and purines (Blakley, 1969), DHFR inhibition will decrease the production

of these compounds as well, further upsetting nucleic acid syntheses. 'Thymineless death' (Werkheiser, 1961; Cohen, 1971) occurs in bacteria - where the mechanism for thymine uptake does not exist - when DNA synthesis is blocked out but RNA and protein synthesis continues. Thymine starvation not only halts cell proliferation but also leads to irreversible cell damage including DNA degradation. The above reasons provide the biochemical basis for the antifolate class of drugs (Bertino, 1971) like methotrexate (MTX), aminopterin (AMT), pyrimethamine (PYT) and trimethoprim (TMP). These have found widespread clinical applications in the treatment of neoplastic and microbial diseases (Livingstone and Carter, 1970).

C TECHNIQUES FOR DESIGNING DHFR INHIBITORS

The aim of this project is to design conservatively-modified folates capable of exhibiting significantly different binding properties towards bacterial and mammalian dihydrofolate reductases. It was hoped that work along these lines would lead eventually to the preparation of compounds capable of discriminating between enzymes on the basis of small changes in amino acid sequences especially at or near the active sites.

Problems associated with the design of selective DHFR inhibitors can be tackled in a number of different ways. The following methods combined theoretical studies and calculations with results obtained from DHFR assays to determine binding modes and strengths.

Qualitative Structure-Activity Relationships involved the study of a large number of closely related compounds with the aim of linking certain properties of the compounds

with increased or decreased binding strengths. Quantitative Structure-Activity Relationships (QSAR) is a very similar but more rigorous method. It uses regression analysis to separate and delineate electronic, steric, hydrophobic and other effects of substituents on the potencies of inhibitors (Martin, 1978).

Another method attempts to study steric factors involved in enzyme-ligand interactions. Minimal Topological Difference (MTD) analysis aims to define the MTD for a molecule with respect to a standard (S) - usually a substrate or the most potent inhibitor - which is presumed to be close to an ideal fit for the receptor (Simon, 1977; Stephan, 1977). A related technique is Molecular Shape Analysis, initiated by Hopfinger and his colleagues (Hopfinger, 1980 and 1983).

Other theoretical studies include Distance Geometry in Quantitative Structure-Activity Relationships (Ghose and Crippen, 1983; Crippen and Havel, 1978) and molecular graphics methods (Blaney, 1982; Smith, 1982; Hansch, 1982, and references therein). The latter is extremely useful as it allowed the user to view enzymes and complexes from various perspectives, 'dock' ligands to enzymes, superimpose various ligands, examine van der Waals and other surfaces of ligands, enzymes or complexes, etc. We have utilised some of the results obtained from these theoretical studies of DHFR inhibitors - especially those pertaining to steric and electronic requirements of the pteroylglutamate binding pocket - for the interpretation of data obtained from binding studies of our inhibitors.

X-ray crystallography, analysis of amino acid sequences, calorimetric and kinetic studies, infrared, Raman, ultraviolet, nuclear magnetic resonance and other spectroscopic studies of DHFR and its complexes with NADPH, NADH, inhibitors and substrates have also been used in the design of specific DHFR inhibitors. As we have

only been able to synthesize and test a small number of antifolates and enzymes, we have had to rely on these data for our studies. A discussion of these techniques have been included in the Conclusions chapter.

D THE PTEROYLGLUTAMATE BINDING POCKET

Attempts at rational design of dihydrofolate reductase inhibitors have been greatly helped by results from X-ray crystallographic studies of DHFR crystals containing bound inhibitors and/or cofactor (Matthews *et al.*, 1977; Baker *et al.*, 1981a and b; Matthews and Volz, 1982; Beddell, 1984). There is a considerable body of data suggesting that average solution structures of proteins were indeed similar to their crystal structures (Dobson, 1977; Poulsen *et al.*, 1980; Nagayama and Wuthrich, 1981). Indeed some success in rationally designed DHFR inhibitors has already been obtained by Birdsall *et al.* (1984) and Kuyper *et al.* (1982 and 1985). They have tailored inhibitors to interact with specific amino acid side chains in the binding pocket of *Lactobacillus casei* and *Escherichia coli* enzymes.

I TERTIARY STRUCTURE OF DIHYDROFOLATE REDUCTASE

Dihydrofolate reductase is a very roughly pear-shaped molecule (Blakley *et al.*, 1981). The carboxy terminus forms the apex and a loop forms an extension from the surface. Its polypeptide chain is folded into an eight stranded β sheet (figure A V). DHFR contained only one antiparallel strand - strand H. This is inserted between βF and βG and led to the carboxy terminus. The enzyme also has four α helices - αB, αC, αE

and αF - adjacent to the β sheets. Remaining residues formed loops joining elements of secondary structure. X-ray crystallographic studies have also revealed a large number of solvent molecules, many of them fixed in similar positions even for enzymes from different sources. These water molecules are thought to play an important role in stabilizing overall enzyme structure through hydrogen bonding.

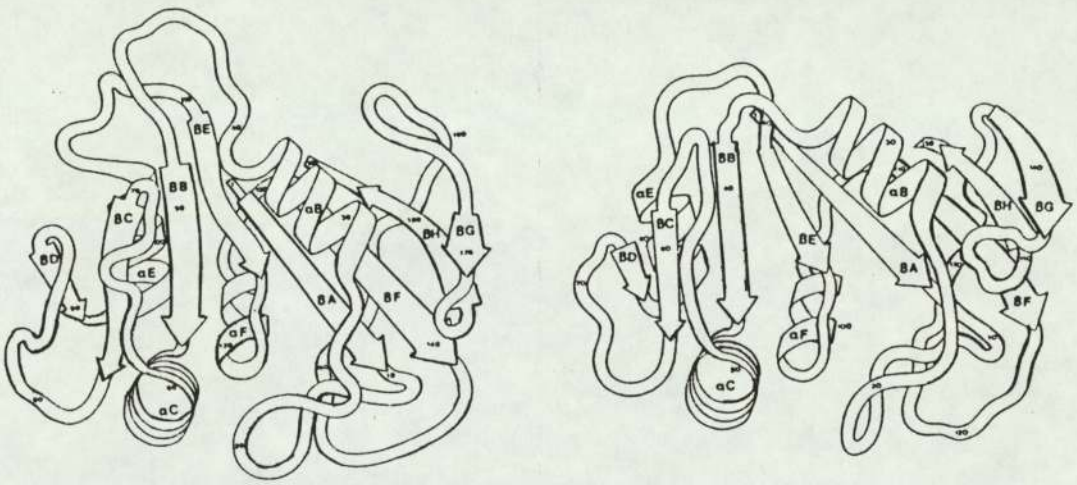


Figure A V Schematic illustration of folding in chicken liver (left) and *E. coli* (right) dihydrofolate reductases. Note the overall similarity in tertiary structures despite differences in amino acid sequences.

Comparative studies of bacterial and vertebrate dihydrofolate reductases have revealed that overall folding of polypeptide chains in these enzymes is very similar despite differences in their amino acid sequences (Volz *et al.*, 1982; Bolin *et al.*, 1982).

Structural similarity has been preserved mainly by the replacement of amino acids in one enzyme by homologous residues in another. In addition, the thirty or so extra residues present only in vertebrate enzymes have been placed in loops connecting elements of regular secondary structure (figure A V). In view of the high degree of sequence homology amongst vertebrate enzymes (Freisheim and Matthews, 1984), it would not be unreasonable to assume that polypeptide chains of other vertebrate enzymes are folded in manners very similar to that of the chicken liver reductase.

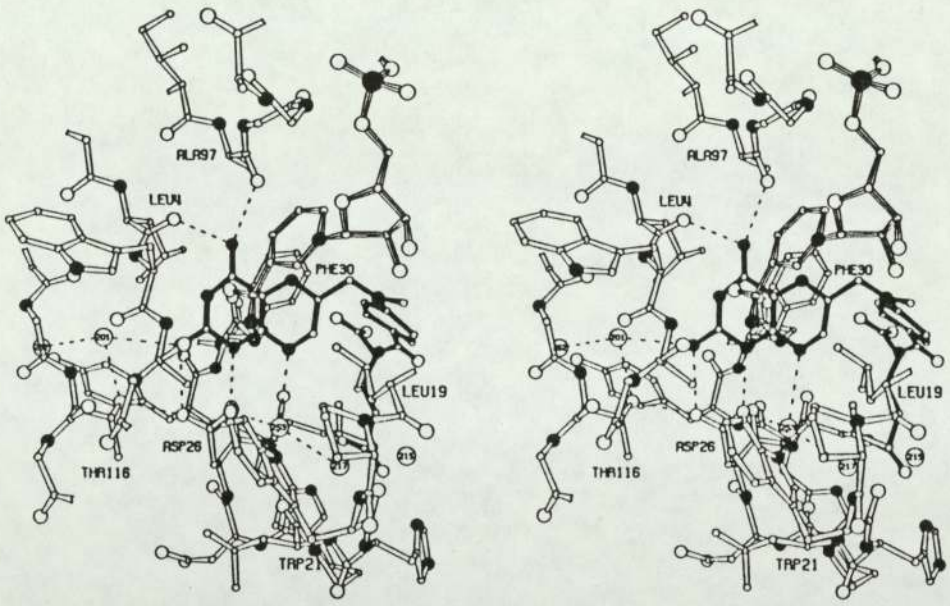


Figure A VI Conformation of methotrexate and NADPH when bound to L. casei DHFR (from Bolin *et al.*, 1982). Methotrexate is represented by solid bonds, protein by open bonds and NADPH by striped bonds. Carbon atoms are indicated by small open circles, oxygen atoms by large open circles, nitrogen atoms by blackened circles and fixed solvent (water) molecules by large numbered circles. Dashed lines represent hydrogen bonds.

The ligand binding pocket of DHFR is crescent-shaped, built up by the β strands A, B, C, E and F and the carboxyl end of αF. It cuts halfway across the base of the molecule and most of the way up the side. αB and the preceding connecting strand from βA made up one side of this cleft whilst the other side comprised of αC and the subsequent strand connecting to βC.

In ternary complexes comprising enzyme, cofactor and inhibitor, reduced nicotinamide adenine dinucleotide phosphate (NADPH) sits in an extended conformation on one side of the binding pocket (Matthews *et al.*, 1978; and, Filman *et al.*, 1982). The reduced nicotinamide moiety is placed near the middle of the cleft with the adenine portion in an outermost position. Inhibitors were bound close to the nicotinamide ring on the other side of the cleft (figure A VI).

II THE PTERIDINE BINDING POCKET

Bolin *et al.* (1982) have described structures of *E. coli* and *L. casei* DHFR crystals refined to 1.7Å resolution. Comparison of the pteridine binding pockets in these enzymes with the corresponding part in chicken liver reductase containing bound TMP and phenyltriazines has revealed that the 2,4-diaminopyrimidine and 2,4-diaminotriazine rings were bound in an almost identical manner (Matthews *et al.*, 1985a and b; Freisheim and Matthews, 1984). Aspartic acid-26 lc[1] is hydrogen bonded to the

1 lc represents the residue in *L. casei* DHFR, ec is for *E. coli* and cl is for chicken liver enzyme. Amino acids have been numbered according to Volz *et al* (1982).

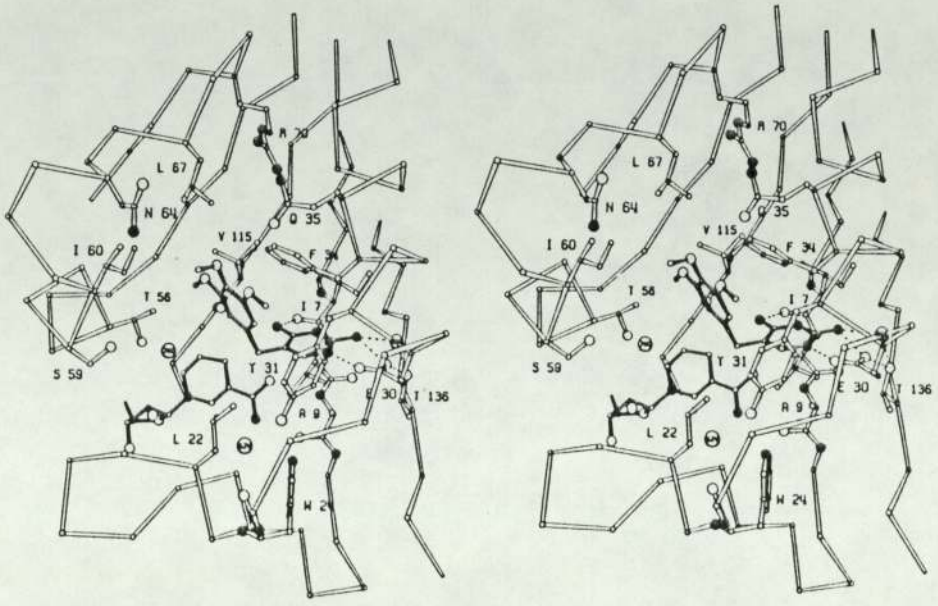


Figure A VII The trimethoprim (TMP) and NADPH binding site in chicken liver DHFR. Although overall conformation of the 2,4-diaminopyrimidine ring in TMP is very similar to the corresponding moiety in MTX and TMP bound to E. coli DHFR, note that TMP here has been inserted about 1Å closer to αB on the right hand side of the cleft (residues 27-39 make up αB). From Matthews *et al.* (1985). TMP is indicated by solid bonds, all other representations are the same as in figure A VI.

2-amino group and N(1) of methotrexate via the two oxygen atoms of its side chain, 0δ1 and 0δ2, respectively, (figure A VII). Evidence from a variety of studies including calorimetric (Subramanian and Kaufman, 1978), theoretical (Perault and Pullman, 1961) and spectroscopic (Cocco *et al.*, 1981a and b) have suggested that N(1) of DHFR-bound MTX is protonated. Bolin *et al.* (1982) have assumed that Asp-26 to be anionic and have proposed an ionic interaction between its side chain and protonated N(1) of MTX.

The 2-amino group also forms a second hydrogen bond with the enzyme. However, its interaction with the side chain of Thr-116 lc is mediated by a fixed water molecule. Thr-116 is hydrogen bonded to 0δ1 in Asp-26 as well. This triad of hydrogen bonds - involving Thr-116, Asp-26 and the fixed solvent molecule on one hand and the three protons of the 2-amino group and protonated N(1) on the other - forms a tightly constrained polar pocket whose constituents complement one another in both geometry and charge distribution. Both hydrogen atoms of the 4-amino group form hydrogen bonds too. However, these involved the backbone carboxyl oxygens of Leu-4 and Ala-97 lc.

Unlike the pyrimidine ring, the pyrazine ring of methotrexate is not directly hydrogen bonded to the enzyme. N(8) formed a weak hydrogen bond linkage to a structurally conserved fixed water molecule whilst N(5) did not form any hydrogen bonds at all.

Binding of the pteridine ring of MTX (and corresponding moieties in other inhibitors) has been further enhanced by van der Waals interactions with hydrophobic residues at the active site. These include Phe-30, Ala-6, the backbone amide of Trp-5 and Ala-6 and Leu-19 (all lc).

III THE PARA-AMINOBENZOYL BINDING POCKET

The bulk of interactions between the enzyme and the para-aminobenzoyl ring are hydrophobic (Blakley, 1981). Some difference was observed between the two bacterial enzymes. In *E. coli* DHFR, the interactions involved side chains from Leu-28, Phe-31, Ile-50, Leu-54 and Ile-94 which were all in van der Waals contact with the ring. The

carboxyl oxygen of the benzoyl moiety participated in a set of hydrogen bonds involving the side chain nitrogen (Nρ) of Arg-52, a fixed water molecule, the carboxyl oxygen of Ile-50 and another side chain nitrogen (Nε) of Arg-52. In the *L. casei* reductase, these hydrophobic interactions involved side chains from Pro-50, Phe-49 and Leu-54. The side chain of the corresponding arginine residue (Arg-52 lc), however, does not hydrogen bond to the carboxyl oxygen in the benzoyl ring. Instead, the latter bonds to two fixed water molecules.

IV THE GLUTAMATE BINDING POCKET

A pair of hydrogen bonds to the guanidium side chain of Arg-57 (ec and lc) has been used to secure the α-carboxylate group of MTX in the binding pocket (Gready, 1980). This ion pair is placed in a narrow hydrophobic crevice near the enzyme surface. Studies of the enzyme-TMP complex has revealed that this arginine residue - strictly conserved in all reductases - is held in the same conformation even in the absence of ion pairing interactions with the ligand. This observation underlines the importance of this residue in the binding of pteroylglutamates by DHFR.

Interactions between the γ-carboxylate and the enzyme are less specific and are weaker than interactions involving the α-carboxylate group. Differences have been observed between the two bacterial enzymes in this case as well. In *L. casei* DHFR, the γ-carboxylate of MTX is very loosely hydrogen bonded to His-28 but in the *E. coli* enzyme, it hydrogen bonds to several water molecules instead.

Component of methotrexate	Contact in *L. casei* DHFR	Contact in *E.coli* DHFR	Sequence conservation[1]
6-methyl-pteridine	Leu-4 side chain Trp-5 main chain Ala-6 N Cα Cβ Leu-19 side chain Leu-27 side chain Phe-30 side chain Ala-97 Cα Cβ Nicotinamide ring	Ile-5 side chain Ala-6 main chain Ala-7 N Cα Cβ various bound waters Leu-28 side chain Phe-31 side chain Ile-94 Cα Cβ various bound waters	Conserved except *E. coli* (Met) Conserved
N(10)-methyl	Leu-19 side chain Ser-48 side chain Water-439	various bound waters Ser-49 side chain various bound waters	except *E. coli* (Met) except *S. faecium* (Ala)
para-aminobenzamide	Leu-27 side chain Phe-30 side chain Phe-49 side chain Pro-50 side chain Leu-54 side chain	Leu-28 side chain Phe-31 side chain Ile-50 side chain Leu-54 side chain	Conserved Conserved
L-glutamate	Leu-27 side chain Phe-30 side chain Arg-31 Cβ Cγ Cδ Leu-54 side chain	Leu-28 side chain Phe-31 side chain Lys-32 Cβ Cγ Cδ Leu-54 side chain	Conserved Conserved

Table I Hydrophobic and van der Waals interactions between methotrexate and dihydrofolate reductase (from Bolin *et al.*, 1982)

1 Residues are listed as conserved only in cases where they have been conserved in all known generic sequences (according to the alignment in Volz *et al.*, 1982). Exceptions are listed for those residues that have been identically conserved in all but one species.

Component of methotrexate	Contact in *L. casei* DHFR	Contact in *E.coli* DHFR	Description
Pteridine, N 1 and 2-amino	Asp-26 carboxylate	Asp-27 carboxylate	Two hydrogen bonds; ionic; always Asp or Glu
2-amino group	Water-201	Water-405; (Water-639)[1]	Water also bound by conserved Thr-116lc
N-8	Water-253	Water-403; (Water-603) (long contact)	Water also bound by Asp-26lc and conserved Trp-211c
4-amino group	Leu-4 and Ala-27 carbonyl groups	Ile-5 and Ile-94 carbonyl groups	
Benzoylcarbonyl		Arg-52 guanidium, Water-566; (Water-685)	Water also bound by Arg-52ec and carbonyl 50ec
L-glutamate, α-carboxylate	Arg-57 guanidium	Arg-57 guanidium	Conserved Arg
γ-carboxylate	His-58 imidazole	various waters	

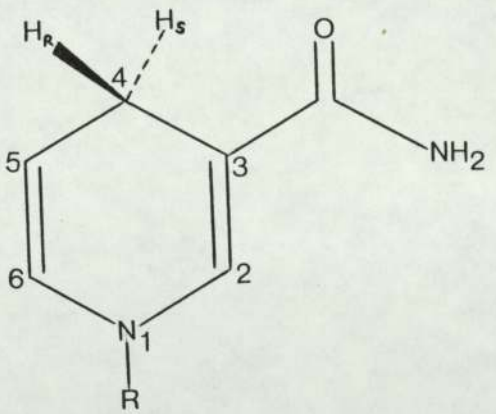
Table II Hydrogen-bonding interactions between methotrexate and dihydrofolate reductase (from Bolin et al., 1982).

1 Solvent molecules in both forms of *E.coli* DHFR have been listed.

A summary of the various enzyme-inhibitor interactions have been included in table I (hydrophobic and other van der Waals interactions) and table II (hydrogen bond interactions) which have been reproduced from Bolin *et al.* (1982).

V THE NADPH BINDING POCKET

Comparison of the *E. coli* DHFR.MTX binary complex with the ternary *L. casei* DHFR.MTX-NADPH complex has shown that binding of NADPH did not result in major conformational change in either the enzyme or the inhibitor (Matthews *et al.*, 1977 and 1978). For simplicity, we have described below only the nicotinamide portion of the NADPH binding pocket. The remainder of the site is of lesser relevance to our studies.



R = Adenine Dinucleotide Phosphate

Figure A VIII Structure of NADPH. The A side of NADPH is above the plane of the page. The carboxamide group has been rotated by 180° from the minimum energy conformation for NADPH in solution.

Most of the residues forming the NADPH binding pocket are invariant. The A side of the nicotinamide ring (figure A VIII) interacts with the pyrazine ring of bound methotrexate and side chains of Leu-19, Trp-21 and Ser-49 (all lc). This part of the pocket also contained a water molecule which appears to be isolated from an external solvent. This interacts with Oγ of Ser-48 and the 2'-hydroxyl in the NMN ribose of NADPH.

All enzyme-nicotinamide interactions on the B side are hydrophobic. The residues here include Ile-13, Phe-103, the cis-peptide bond between Gly-98 and Gly-99 and a few atoms belonging to Trp-5 and Met-128 (all lc).

The carboxamide of bound NADPH is coplanar with the nicotinamide ring and has been rotated by 180^{o} from the minimum energy conformation for NADPH in solution (figure A VIII). A pair of hydrogen bonds joins this moiety to the backbone nitrogen and carboxyl oxygen of the strictly conserved Ala-6 lc. The amide nitrogen, N(7), forms an additional hydrogen bond to the carboxyl oxygen of yet another strictly conserved residue - Ile-13 (lc). Both hydrogen bonds involving N(7) are linear and coplanar with the nicotinamide ring.

A very important feature of the nicotinamide binding pocket are the interactions between C2, C4, C6 and the carboxyl oxygens of Ile-13 and Ala-97 and Oδ1 of Thr-45 (all lc), respectively. Distances between each pair of atoms are a little too short for non-bonded contacts and all six atoms lie in approximately the same plane. Residues Ile-13 and Thr-45 are strictly conserved and adjacent amino acids show a high degree of sequence homology in other enzymes. Filman *et al.* (1982) argued that these features and the conformation of the NADPH carboxamide group are vital in stabilizing the transition state for hydride transfer during catalysis of the substrate by DHFR.

E A MODEL FOR BOUND SUBSTRATE

This model is based on a comparison of spectroscopic and X-ray crystallographic studies of bound inhibitors and substrate. At the time of writing, no X-ray crystallographic studies have been performed on dihydrofolate reductases containing bound 7,8-dihydrofolate or other 2-amino-4-hydroxypteroylglutamates. However, with the use of spectroscopic, model-building and other studies, Bolin *et al.* (1982) have managed to construct a model of the possible conformation of DHFR-bound DHF.

Stereochemical studies of products from the DHFR reduction of DHF (Charlton *et al.*, 1979; Hitchings *et al.*, 1982) have demonstrated the absolute configuration at C(6) in the pteridine rings of the products - 5,6,7,8-THF - to be (S). X-ray diffraction studies by Fontecilla-Camps *et al.* (1979) on 5,10-methenyltetrahydrofolate derived from the enzymic reduction of the biologically-active diastereomer of 5-formyl-THF have also demonstrated the same configuration for C(6). Clearly, these results could only be obtained if pteridine rings in folic acid and DHF were rotated by 180^{o} about the C(6)-C(9) bond with regard to the corresponding ring of bound MTX.

Another study vital for the correct placement of the pteridine ring in the binding pocket was concerned with the tautomeric form of the pteridine ring in bound 2-amino-4-hydroxypteroylglutamates. Studies of dihydrofolate when in solution (Pfleiderer *et al.*, 1960; Brown and Jacobsen, 1961) or when bound to DHFR have demonstrated that the preferred form of the substrate is the 4-keto tautomer.

Taking these two observations into account, Bolin *et al.* (1982) have proposed the conformation for enzyme-bound DHF shown in figure A IX. This model was so constructed to ensure that protein conformation and solvation of the enzyme-substrate complex was unchanged with respect to the enzyme-inhibitor complex. In accordance

with results from spectroscopic studies by Ozaki *et al.* (1981), the para-aminobenzoyl moiety in the substrate was maintained in the same conformation as in the inhibitor.

This model predicts quantitatively that, as a result of the net loss of a single hydrogen bond - due to N(8) in DHF replacing the 4-amino group of MTX - dihydrofolate should be bound less tightly to the enzyme than MTX. This prediction was borne out by spectroscopic results obtained by Dann *et al.* (1976), Hood and Roberts (1978) and Cocco *et al.* (1981b).

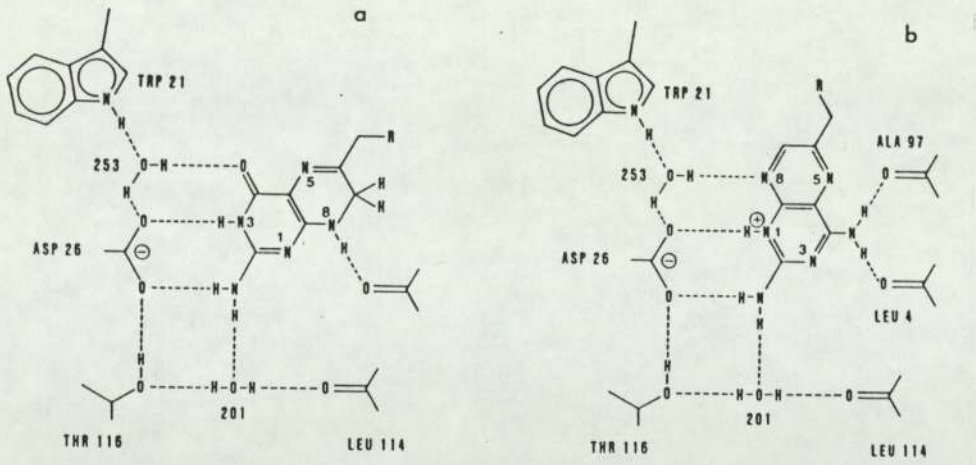


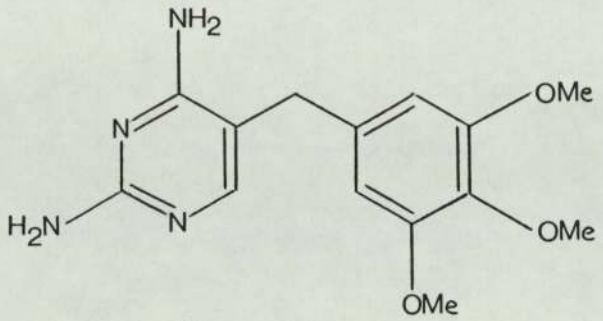
Figure A IX Schematic representation of hydrogen-bonding interactions between pteridine moieties of
a) DHF, and
b) MTX
and amino acid side chains in the binding pocket of DHFR (from Bolin *et al.*, 1982).

If we also assumed folic acid to be bound as the 4-keto tautomer and in the same conformation as DHF, the model would predict a large association constant for dihydrofolate relative to folic acid because N(8) in the latter does not have the ability to hydrogen bond to the carboxyl oxygen of Leu-4 lc (Ala-97 in *E. coli*). The relative magnitudes of binding constants for folic acid and dihydrofolate in binary complex with *L. casei* (Dann *et al.*, 1976) and *E. coli* DHFRs (Greenfield *et al.*, 1972; Cayley *et al.*, 1981) bears out this assumption. However, circular dichroic spectra for folic acid and DHF in ternary complex with DHFR and NADPH (Reddy *et al.*, 1978) seem to reveal differences in the binding modes of these two compounds.

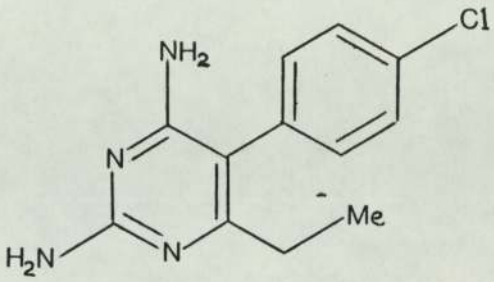
F DESCRIPTION OF THE AIMS OF THIS PROJECT

Our work is an exploratory look at one aspect of the differences between bacterial and vertebrate dihydrofolate reductases namely, the manner in which they bind closely related folic acid analogues.

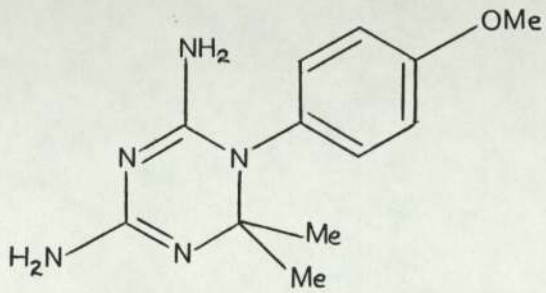
Differences between these types of enzymes have already been well-exploited by the use of antibacterial compounds like trimethoprim (TMP) which binds some three thousand times more strongly to *E. coli* DHFR than to vertebrate enzymes (Baccanari *et al.*, 1982). Other antifolates exhibiting differential binding properties to vertebrate and invertebrate DHFRs include pyrimethamine (PYT) -widely used in the treatment of malaria and toxoplasmosis (Hitchings, 1960) - and the phenyltriazines (Burchall, 1973) (figure A X).



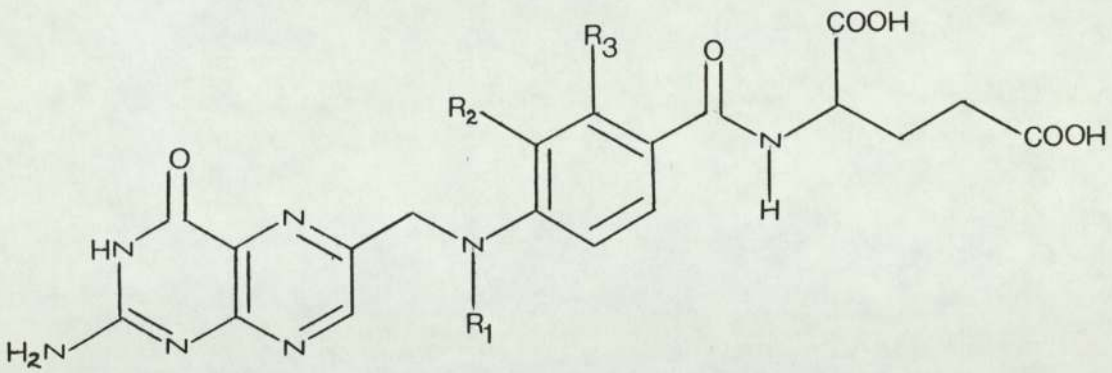
TRIMETHOPRIM (TMP)



PYRIMETHAMINE (PYT)



6,6-DIMETHYL-4'-METHOXYPHENYLTRIAZINE



"CLASSICAL" ANTIFOLATE STRUCTURE. Positions of modification have been labelled R_1, R_2 and R_3.

Figure A X Structures of a "classical" antifolate, trimethoprim, pyrimethamine and a phenyltriazine. Note the structural similarities, ie the 2,4-diaminopyrimidine and triazine rings.

TMP is a potent inhibitor of bacterial DHFRs but only weakly inhibitory towards vertebrate enzymes. Phenyltriazines, however, behave in the opposite manner, being weak inhibitors of bacterial enzymes but strongly inhibitory towards vertebrate DHFRs. Matthews *et al.* (1985a and b) have studied chicken liver and *E. coli* DHFRs in ternary complex with NADPH, TMP or phenyltriazines in an attempt to uncover the molecular basis for differences in binding strengths of these two classes of antifolates towards vertebrate and bacterial reductases. They have found that the 2,4-diaminopyrimidine ring in TMP has been inserted about 1Å deeper into the binding pocket of chicken liver DHFR than the corresponding moieties of the phenyltriazines. This has resulted in the loss of the hydrogen bond between the 4-amino group of TMP and the carboxyl oxygen of Val-115 in the vertebrate enzyme. The consequent reduction in binding energy has been cited by Matthews *et al.* (1985b) as one of the main reasons for weaker binding of TMP to vertebrate enzymes compared to the less-deeply bound phenyltriazines. A similar reason was advanced for the lowered affinity of phenyltriazines towards bacterial DHFRs when compared to the 2,4-diaminobenzylpyrimidines.

The source of differences in binding behaviour between benzylpyrimidines (like TMP) and phenyltriazines towards bacterial and vertebrate reductases, according to Matthews *et al.* (1985b), were small differences in the active sites of the enzymes and structural differences between the two classes of antifolate (specifically, the presence of a methylene bridge in the benzylpyrimidines). Our antifolates all shared a common structure with only very small modifications (figure A X). Therefore, any differences in binding properties would be due to the direct result of differences in architecture of the active sites between bacterial and vertebrate DHFRs.

We have synthesized several folates modified at the benzene ring, at N(10) and two

compounds modified at both positions. These 2-amino-4-hydroxylpteroylglutamates were tested against dihydrofolate reductases from various sources including *L. casei*, bovine liver, rat liver, mouse sarcoma (M5) and mouse plasma cytoma (PC6). Using published amino acid sequences and results from X-ray crystallographic studies of DHFR crystals, attempts were made to link the performance of these antifolates as inhibitors and/or substrates to the three-dimensional structure of the enzymes and to the properties of substituents on the folates. The behaviour of a particular compound towards various reductases was studied. We have also examined the behaviour of various antifolates towards a single enzyme.

During our attempts to correlate antifolate activity with the three-dimensional enzymic structure determined by X-ray crystallography, we have always borne in mind that, in solution, enzymes, like any other molecule, are not rigid. Proteins have been demonstrated to undergo a variety of significant structural fluctuations about their average conformations (Karplus and McCammon, 1981 and 1983). Some of these fluctuations may contribute to differences in binding strengths and reduction rates.

It has been noticed for some time that poorly reduced folic acid analogues were often good inhibitors of dihydrofolate reductase. Studies by Morales and Greenberg (1964) and Bertino *et al.* (1965) using DHFR from sheep liver and Ehrlich ascites cells have demonstrated this phenomenon on a number of compounds including N(10)-formylfolate and pteroate. We hope to uncover during the course of this study the reasons behind this behaviour. A better understanding of the factors governing reducibility and binding strengths of folates could be achieved by studying a large number of enzymes and closely related compounds, especially those poorly-reduced folates which were weak inhibitors of DHFR.

INTRODUCTION

There were three reasons for synthesizing benzene-ring fluorinated folates. Firstly, we were interested in studying the effects of electron-withdrawing substituents at the benzene ring on the binding properties of folic acid analogues. Secondly, we wanted to discover whether 2' and 3' were bound differently to the same enzyme. As fluorine is a sterically undemanding substituent, comparison of the inhibition data from 2'- and 3'-fluorofolates with data from 3'-iodofolate and folates unsubstituted at the benzene ring would be ideal for assessing the steric and electronic contributions the benzene ring makes to the overall binding energies of folates. It could also give us a picture of the structure of the DHFR active site. Finally, we had also planned to study the folate binding pocket by direct methods, namely by using ^{19}F NMR spectroscopy. ^{19}F NMR spectra of solutions containing DHFR, NADPH and various molar equivalents of 2'- and 3'-fluorofolates could be extremely informative because of the sensitivity of the fluorine-19 chemical shifts to the chemical environment of the ^{19}F nucleus. Variable temperature experiments could be used to determine the rate of exchange between bound and free ligand. It could also reveal whether the benzene ring rests immobile at the active site or flips about its N(10)-C(11) axis. NMR spectra of solutions containing enzyme, cofactor, fluorofolate and a substrate or another inhibitor could also be useful. Unfortunately, numerous problems were encountered during the synthesis of 2'- and 3'-fluorofolates. Consequently, there was insufficient time to conduct these experiments. A similar study using 3',5'-difluoromethotrexate and *L. casei* DHFR was recently reported by Clore *et al.* (1984). For the binary complex, they found that the benzene ring flipped about its symmetry axis at a rate of 7.3×10^3 s^{-1} at 298K. Addition of NADPH changed the ^{19}F chemical shift and increased the flip rate by 2.6 to 2.8 fold.

The ^{19}F nucleus has been widely used in biological NMR because of the reasons mentioned above. Recently, the positron-emitting ^{18}F nucleus has become increasingly popular for biological studies through its use in positron emission tomography (PET) (Ter-Pogossian *et al.*, 1980). Suitably labelled folates should be extremely useful for studies of *in vivo* distribution of folates in neoplastic and other human tissues. With this objective in mind, we attempted the preparation of benzene ring ^{18}F substituted folates. The compound had to be prepared by simple reactions which gave relatively good yields. Due to the short half-life of ^{18}F, it was imperative that the synthesis and purification steps took as short a time as possible, preferably less than a day. We attempted to react folates and its benzene ring nitrated and iodinated analogues with nucleophilic fluorine from various sources but were unable to displace those rather poor leaving groups.

CHAPTER 2 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

A BRIEF THEORY

The nuclear magnetic resonance phenomenon was discovered by two groups of physicists working independently - Purcell *et al.* (1946) and Bloch *et al.* (1946). NMR is observable because nuclei with odd mass numbers possess spin and behave like bar magnets. Nuclei possessing both spin and charge are conferred a magnetic moment (μ) proportional to the magnitude of the spin (equation 2.1)

$$\mu = \gamma \hbar I \qquad (2.1)$$

where γ is the magnetogyric constant (measured in radians s^{-1} $Tesla^{-1}$). $\hbar$ is Planck's constant divided by 2π and I is the value of the spin (in multiples of 1/2).

Application of a steady magnetic field B_o to a nucleus results in interaction between the magnetic moment $\vec{\mu}$ and the field (equation 2.2)

$$\mathcal{H} = -\vec{\mu} B_o \qquad (2.2)$$

where $\mathcal{H}$ is the Hamiltonian. When the magnetic field is in the z direction, this interaction takes the form of equation 2.3

$$\mathcal{H} = -\gamma \hbar B_o I_z \qquad (2.3)$$

where I_z is the allowed component of the nuclear spin in the z direction. For nuclei like

^{1}H, ^{13}C and ^{19}F, I_z has the values of $\pm 1/2$. The lower level (m=+1/2) corresponds to the situation where magnetic field (B) and nuclear moment ($\vec{\mu}$) are parallel. The upper, higher energy level (m=-1/2) refers to the situation where they are antiparallel.

Application of an oscillating magnetic field to the system induces transitions between these two nuclear spin levels. The frequency of radiation associated with absorption, ν, can be calculated from $E=h\nu$. Consequently, in a given magnetic field, a particular nucleus can have only one characteristic resonance frequency namely, the Larrnor frequency, ν (equation 2.4)

$$\nu = \gamma B_o / 2\pi \qquad (2.4)$$

A net absorption of radiation occurs during resonance because, at equilibrium, the ground state contain more nuclei than the excited state. Thus, a slightly greater number of nuclei will be promoted from the lower level than there are stimulated to fall from the higher one. NMR signals disappear as this excess of nuclei tend to zero. This phenomenon is termed saturation. Relaxation maintains the excess of nuclei in the lower level. There are two relaxation mechanisms - spin-lattice and spin-spin relaxation. The former involved energy exchange between spin system and the lattice whilst the latter is related to lifetimes of the excited state.

The exact frequency of the NMR absorption line of a particular nucleus is dependent on the nature of the molecule bearing the nucleus. Frequency differences between nuclei are termed chemical shifts. They arise because the magnetic field experienced by a nucleus (B) is not equal to the applied field (B_o). B_o induces motion in electrons surrounding the nucleus, producing secondary fields which modify B (equation 2.5)

$$B = B_0(1-\sigma) \qquad (2.5)$$

where σ is the 'magnetic shielding' factor or screening constant. σ comprises both intra and intermolecular components. The former include diamagnetic and paramagnetic screening, delocalized electron screening and interatomic shielding. The intermolecular contributions to σ include bulk susceptibility screening, van der Waals screening, electric field screening, anisotropy in the molecular susceptibility of the solvent and specific screening.

Chemical shifts between two nuclei i and j is strictly defined as the difference between their screening constants (equation 2.6)

$$\delta_{ij} = \sigma_i - \sigma_j \qquad (2.6)$$

Since it is impractical to measure screening constants directly, chemical shifts are generally measured in terms of differences between resonant frequencies (or fields) of the relevant absorptions. A compound possessing suitable properties is usually chosen as an arbitrary reference. We have used tetramethylsilane (TMS) as reference in ^1H and ^{13}C experiments. Chemical shifts are quoted in dimensionless parts per million (ppm) units (equation 2.7)

$$\delta = 10^6 \times (\nu_{sample} - \nu_{standard}) / \nu_{standard} \qquad (2.7)$$

where ν represents resonance frequencies of the respective nuclei.

In addition to influencing chemical shifts in each other, interaction between magnetic fields of adjacent nuclei also lead to spectral fine structure. This is termed spin-spin coupling and is characterised by the coupling constant J. Coupling operates through two

mechanisms - a direct through-space interaction which depends on internuclear distances and an indirect mechanism transmitted through bonding electrons. Magnetically equivalent nuclei do not couple with one another. Coupling constants are independent of temperature and applied magnetic field strengths. J decreases with increasing internuclear distance but increases as atomic numbers of coupled nuclei increase.

There are two types of NMR spectrometers - Fourier transform (FT) and continuous wave (CW) instruments. Pulse FT-NMR spectroscopy involves the application of a short, powerful pulse at one end of the spectrum. This pulse, which behaves like a spread of frequencies, simultaneously excites all nuclei whose resonance frequencies are covered by its range. In CW-NMR spectroscopy, a radio frequency wave is swept from one end of the spectrum to the other. This sequential excitation of nuclei is extremely time-consuming. CW-NMR spectrometers, although cheaper, are less sensitive than FT instruments.

B FOURIER-TRANSFORM NMR SPECTROSCOPY

Where there is a large collection of nuclei under the influence of an applied magnetic field B_o, marginally fewer nuclei will be aligned against the field (down) than with it (up). Vector addition leads to a net macroscopic magnetization M in the same direction as B_o. Application of a radio frequency field B, for a short time t_p, rotates the nuclear magnetization vector through θ radians (equation 2.8)

$$\theta = \gamma B_1 t_p \qquad (2.8)$$

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

When a 90^{o} pulse is applied at the resonance frequency along the X' axis in the rotating frame, the magnetization M is brought to lie entirely along the Y' axis (figure B I). Since detection coils are normally placed along the Y' axis, the strength of the observed signal is determined by the magnitude and time-dependent variations of M_{xy} (figure B II). Since the nuclei precessess freely without perturbation from the rf field, this signal is known as the free induction decay (FID). This magnetization decays with a time constant known as the transverse relaxation time T_z.

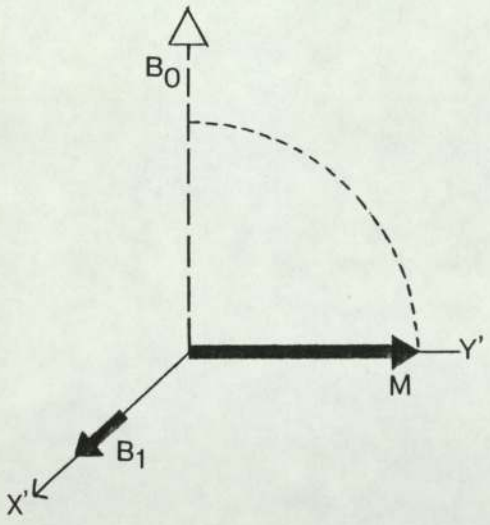


Figure B I Result of a 90^{o} pulse applied along X'

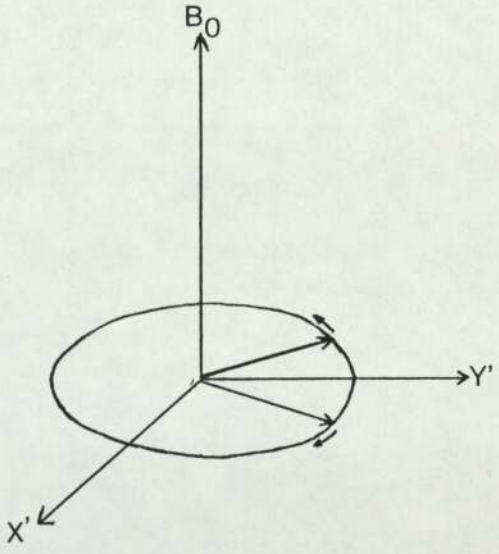


Figure B II Decrease of M_{xy} due to transverse relaxation

FIDs following a 90^{o} pulse or a series of $n\theta$ pulses provide the basic spectral information required in Fourier-transfrom NMR spectroscopy. Measurement of this FID yields the magnitude and other characteristics of the magnetization. When there are more than one line present in the FID, their modulation frequencies interfere with each other.

This produces an interferogram which is a regular beat pattern. Fourier-transform of this yields the NMR spectrum.

Fourier transformation is a mathematical process usually requiring the use of a computer. FIDs must be stored in the computer prior to Fourier-transformation. Therefore, digitization of the analogue signal is necessary. An analog-digital converter (ADC) is used. FIDs are sampled at regular intervals, using as many data points as there are available computer words (N). These N samples of the time domain fk are transformed into N points of the frequency domain F_j. Acquisition rates of data are determined by the sampling theorem. Resolution can be enhanced by using longer acquisition times, increasing computer size N or decreasing the frequency range of the spectrum.

C THE JEOL FX90Q FOURIER-TRANSFORM NMR SPECTROMETER

The JEOL FX90Q has a proton resonance frequency of 89.60 MHz, a ^{13}C resonance frequency of 22.53 MHz and a ^{19}F resonance frequency of 84.30 MHz. This instrument uses a digital quadrature detection (DQD) system and has light pen control (LPC) and autostacking software.

I THE PROBE AND MAGNET

The spectrometer has a probe system capable of detecting nuclei in five different frequency ranges. Its computer memory contains 24K words of which 8K are used for the program and 16K for data.

The magnet is encased in a compact console to protect against sudden temperature changes. It generates a field of 2.11 Tesla. A regulated voltage and current supply system ensures a stable excitation current for the magnet.

Shim coils were placed in the probe in between the pole faces. Current flowing through them can be adjusted using controls on the spectrometer table. These coils are used to improve magnetic field homogeneity. The manufacturers specify the resolution of the ^{13}C line to be less than 0.3 Hz. As magnetic field stability is 0.1 Hz/hour, signal broadening would appear in experiments left to run for a long time. This was overcome by using the in-built autoshim unit. It corrects for small shifts in magnetic field homogeneity.

II TRANSMITTER, RECEIVER AND DATA SYSTEMS

The basic transmitter, receiver and data systems of the FX90Q are illustrated in figure B III. The transmitter has three channels - observation, irradiation and lock oscillator units. A reference frequency of 44 MHz for the lock oscillator is provided by a master clock. The OFFSET components of the observation RF output is generated by the observation oscillator unit using a four-phase generator. This oscillator's gate is operated by a PG20 pulse programmer which generates the required rf pulse sequence.Two IF reference signals, 0^{o} and 90^{o} out of phase, are taken to the OBSIF amplifier unit to be used in the DQD system. The signal is then adjusted to the selected nucleus at the wideband local oscillator unit. At the next stage, the rf output is amplified using the RF power amplifier unit. When the rf signal reaches the probe, most of the energy generated at the transmitter coil is absorbed by the sample. The FID produced by

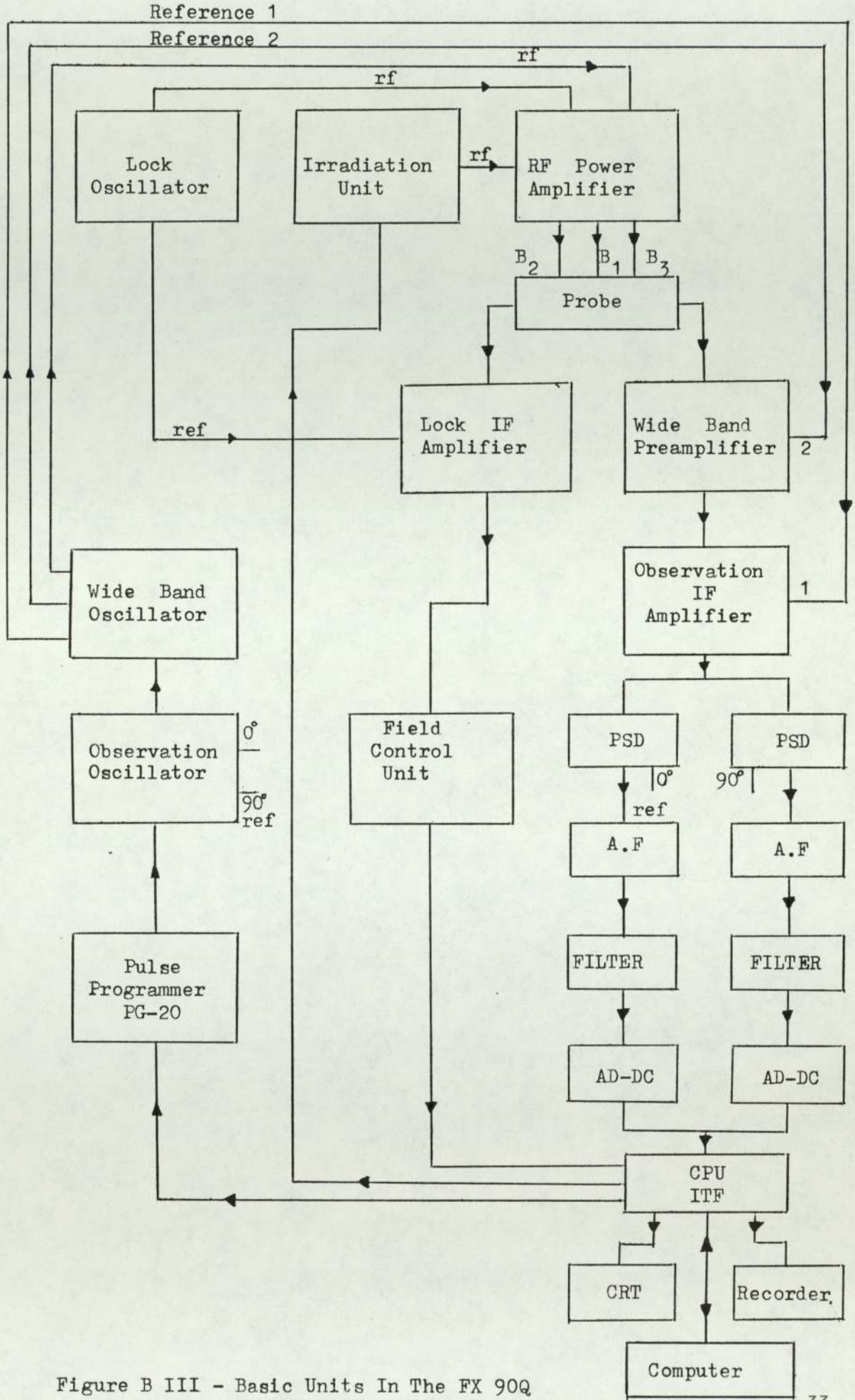


Figure B III - Basic Units In The FX 90Q NMR Spectrometer.

the rf pulse is detected by the receiver coil and amplified in the wide band pre-amplifier unit. Here, a reference signal from the wideband local oscillator unit is mixed in to reduce signal level. Another amplification and frequency reduction stage occur at the observation IF amplifier. Further 0^{o} and 90^{o} reference signals from the OBS OSC unit are used to get the audio frequencies at 0^{o} and 90^{o} out of phase. Note that, at this stage, the detection system contains two phase sensitive detectors instead of one. This is required by the digital quadrature detection technique. The DQD system is advantageous in that it allowed Fourier-transform NMR experiments to be conducted with the excitation pulse placed in the center of the observation width. Consequently, the observation band width can be reduced to only one half of that required in single phase detection (SPD). This produces a $\sqrt{2}$ fold improvement in signal to noise (S/N) ratio. Moreover, as the RF pulse is given at the center of the spectrum, RF power efficiency has been enhanced four times compared to SPD. This allowed for more accurate relaxation time determinations when several signals are being considered.

The AD-DA unit digitizes filtered signals. These are transferred to the computer via the CPU (Controller). The information is stored here until required whereupon the FID is transformed to obtain the signal in digital form. The DA converter changes information into analogue signals which can be recorded or displayed on the oscillator screen.

III LIGHT PEN CONTROL

Conversation between operator and computer is through the light pen unit which links the computer and the spectrometer units via the CPU unit. An order is transmitted

to this unit by pointing the light pen to the desired function on the screen. Pulse and irradiation modes, observation and irradiation frequencies, etc, are set using the light pen.

IV THE LOCK

The lock signal is obtained in the same way as a continuous wave spectrometer. The lock oscillator unit generates an rf signal at the appropriate resonance frequency which is amplified in the RF power amplifier. A sawtooth signal produced by the field control unit modulates the magnetic field, allowing observation of the resonance signal. This signal is phase sensitive detected at the LOCK IF amplifier unit which receives a reference signal from the LOCK OSC unit.

The lock signal is also used for monitoring and adjusting the resolution of the magnet. The rf oscillator for the lock signal can be used with two nuclei - ^{2}H and ^{7}Li. An external ^{7}Li lock fitted to the instrument has allowed us to run spectra of samples containing neither ^{7}Li or ^{2}H. This external lock is also convenient when one has to run a large number of samples for it is no longer necessary to obtain the lock each time a new sample is introduced into the probe.

V THE IRRADIATION UNITS

The RF irradiation oscillator units can be selected as desired to give noise irradiation or a single RF output. Irradiation power in both cases has to be amplified to different levels. This is controlled by the irradiation selector unit which is linked to the RF power

amplifier. Noise irradiation modulation widths can be adjusted to 0.5, 1, 2.5 or 5 kHz.

Several irradiation modes are possible. Gated modes allow selected experiments. As these modes are identified by binary codes, they can be combined using the extension facilities (EXTNS).

VI THE PROBE

This is placed between the poles of the magnet. It comprises several modules including:-

(a) Permabody. This is fixed and houses replaceable modules (OBS, IRR and insert) placed inside a double-walled dewar for variable temperature work. Irradiation coils and a thermocouple are mounted on it. A spinner mechanism and a photosensor (for detecting spinning rates) are placed on top. Air is used to spin the sample tube and water from an insulated reservoir is used to maintain constant temperature.

(b) rf tunable module. This allowed for easy selection of nuclei. It has five frequency ranges and five independent impedance matching channels. Optimum sensitivity is maintained by fine tuning facilities.

(c) Irradiation module. It enables the irradiation circuit to be tuned. Tuning is necessary to ensure that most of the irradiation energy is used in the coil to produce the maximum field. This is indicated by a minimum in the Standing Wave Ratio (SWR) meter.

(d) Sample insert. The insert is exhangeable for several sample tube sizes. It holds the sample coil and a LOCK coil wound around a glass tube. A plug at the bottom of the insert establishes electrical contact between the coils and the instrument.

VII THE VARIABLE TEMPERATURE CONTROLLER

The spectrometer used a variable temperature controller of type NM-VTS2 with an operation range of -110 to +180^{o}C . Desired temperatures are obtained by the passage of hot or cold air into the sample area. In the high temperature range (+30 to +180^{o}C) air from the compressor is heated and the temperature automatically controlled as the heating mechanism is linked to the thermocouple in the probe. Air flow instabilities have made the medium range (+33 to 50^{o}C) difficult to operate when set at 30^{o}C. The majority of our experiments were conducted at ambient probe temperature (27.5^{o}C). It was noticed that decoupling experiments (for example in ^{13}C studies) in ionic solvents could cause sample temperatures to rise by as much as 2^{o}C.

Ethylene glycol was used for temperature calibration. The chemical shift difference ($\Delta\nu_o$) between the two signals was measured in Hz. The sample temperature (in K) was obtained using equation 2.9.

$$T = 446.23 - 1.13890\Delta\nu_o \qquad (2.9)$$

In variable temperature studies, samples were left in the probe for fifteen minutes prior to commencing each experiment to allow them to equilibrate at the new temperature.

VIII THE AUTOSTACKING PROGRAM

The spectrometer used a JEC-980B computer which has a 24K word memory. The different operations which could be performed by the computer are contained in the autostacking program which comprised:-

(a) Normal program. This was used in routine measurements. It has several command patterns including observation and irradiation frequency offset (OBSET and IRSET, respectively), irradiation mode and frequency, pulse width setting, pulse repetition, observation frequency, frequency range and number of scans.

The FID is adjusted at the end of the accumulation using mathematical parameters. These modify the final spectrum in terms of S/N, resolution, line shape, etc. Line widths and chemical shifts could also be measured on the screen using the light pen. Spectra were recorded on conventional chart paper and data was printed out on silvered paper.

(b) Stacking program. Automatic measurements according to preset orders could be made using this facility with resultant data being stored on cassette tape.

The stacking program contained three 'RUN' parameter tables. Since each table could have a completely different set of acquisition parameters, data storage modes and decoupling modes, this facility allowed programming of the spectrometer to carry out different experiments. For example, the computer could be instructed to measure nuclear Overhauser enhancements in RUN #1. In RUN #2, spectrometer conditions could be changed to measure relaxation times by the progressive saturation method.

While in RUN #3, relaxation times could be measured by the inversion recovery method. Upon completion of these runs, all sets of data were stored on cassette tape and could be processed at a later time.

(c) Analysis program. Relaxation times were calculated using this program by the least squares method. It enabled the user to obtain T_1 values for each line in a spectrum and to plot observation points which defined a straight line. Measured T_1s and error (variance) are indicated on the visual display unit as well.

D EXPERIMENTAL

The majority of our NMR spectra have been recorded in $^2H_6$-dimethylsulphoxide (Gold Label, 99.9% atom ^2H, Aldrich). Deuteriated sodium hydroxide (Gold Label, 99+ atom % ^2H, Aldrich) in deuterium oxide (99.8 atom % ^2H; Gold Label, Aldrich) was used for compounds that were extremely insoluble in $^2H_6$-DMSO. 10 ±0.01 mm diameter silica tubes (Loxford Equipment Company Ltd) were used for all experiments. With the exception of variable temperature spectra, all experiments were conducted at the ambient temperature of the magnet (27.5°C). Samples were dried overnight in vacuo at room temperature over phosphorus pentoxide and silica gel prior to use. The amount dissolved in the solvent varied from compound to compound, this being dependent upon differences in solubility and availability. No folate spectra were recorded at a concentration above 70mM. Internal tetramethylsilane (Special For Spectroscopy, BDH) was used as a reference in proton and ^{13}C spectra. In ^{19}F experiments, chemical shifts

were referred to the center of the spectrum. An internal deuterium lock was used for the vast majority of the NMR spectra. The lock irradiation rf level was set at LOW 7 and the lock amplifier set at around 20. An external ^{7}Li lock was used in all the other experiments. In these instances, the irradiation level was set at minimum and the amplifier level increased to around 22.

In some of the proton and most of the ^{13}C experiments, use was made of the 'overflow' (Block-averaging) technique in the data handling program of the FX90Q. 'Overflow' enhances the intensities of small signals in a spectrum which would otherwise be dominated by one or more very intense signals. In proton spectra, the strongest signal is usually $^{2}H_{2}O$ or $^{2}H^{1}HO$ from the hydrated pteridines. For ^{13}C spectra, the interfering signal is the septet arising from ^{2}H-^{13}C coupling in the methyl groups of dimethylsulphoxide. In the 'overflow' technique, the region of interest was initially specified by inputting the relevant data points into the computer. A block of spectra was then accumulated in the frequency domain. This was transformed and stored in the computer memory. A second block was then accumulated, Fourier-transformed and added to the first block. Signal intensities in the specified region were allowed to increase until a maximum is reached. Now, as more blocks are added into the computer memory, the intensities of the smaller signals will begin to increase. The process was allowed to continue until favourable S/N for the smaller peaks are reached.

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

I PROTON NMR SPECTRA

^{1}H NMR spectra were observed at 89.6047 MHz using a spectral width of 1 kHz. 8K data points were used to cover this region. A pulse width of 17μs and pulse repetition time of 4.3s was selected. Trapezoidal window parameters T1, T2, T3 and T4 were set at 0, 0, 1024, and 4032, respectively. An exponential factor of 0.11 was also set prior to Fourier-transformation. Spectra were recorded under non-decoupling conditions. A few selective irradiation experiments were also attempted, mainly for structure-determination purposes. In these experiments, the magnitude of decoupling rf level had to be carefully chosen; especially in situations where other signals were present in close proximity with the irradiated signal. The position of the irradiating pulse was roughly set using the computer. Exact positioning of this pulse was obtained only after fine adjustments (±5Hz) using the manually-operated vernier on the spectrometer console.

II ^{13}C NMR SPECTRA

^{13}C spectra were recorded at 22.533 MHz. A spectral width of 5 kHz was used. The pulse width was set at 17μs and pulse repetition time at 0.84s. In ^{13}C-^{1}H completely decoupled spectra, the irradiation pulse was offset to 54.5 kHz. Off-resonance decoupled spectra employed irradiation pulses offset to 53.0 or 56.0 kHz. Noise modulation of 1 kHz was used to decouple protons from the ^{13}C nuclei. Data points and trapezoidal window parameters were the same as those used in proton spectra. However, the exponential factor was increased to 0.58. Due to the highly

dilute nature of these solutions and the intensity of the solvent signal, the majority of ^{13}C spectra were recorded overnight using the 'overflow' technique.

III ^{19}F NMR SPECTRA

^{19}F NMR spectra were recorded at 84.3022 MHz. Although a spectral width of 5 kHz was used, as well, 16K data points were required to cover this region. This was necessary due to the sharpness of the signal. In ^{1}H-^{19}F decoupled spectra, the irradiation pulse was offset to 54.5 kHz. In order to completely decouple protons from the ^{19}F nucleus, noise modulation of 5 kHz was required. Pulse widths of 100μs and pulse repetition times of 1.66s were used. Trapezoidal window parameters for the time domain spectra - T1, T2, T3 and T4 - were set at 0, 0, 2048 and 8064, respectively. An exponential factor of 0.58 was included prior to transformation into the frequency domain. ^{19}F-^{1}H coupled spectra were also recorded to assist in structural determination studies. Splitting patterns were very complicated in some spectra and interpretation quite difficult.

IV ^{2}H NMR SPECTRA

Spectral widths of 1 kHz were used to study ^{2}H NMR spectra at 13.7548 MHz. Both ^{1}H-^{2}H coupled and decoupled spectra were examined. In the former, the irradiation pulse was offset to 54.5 kHz. Noise modulation was set at 1 kHz. Undecoupled spectra were not informative. These signals had very broad linewidths as a result of unresolved ^{2}H-^{1}H coupling. A pulse width of 24μs and pulse repetition time

of 4.3s was used. Data points and manipulation of time domain spectra were identical to ^{1}H NMR spectra.

CHAPTER 3 EXPERIMENTAL

A GENERAL EXPERIMENTAL

I ULTRAVIOLET SPECTROSCOPY

Ultraviolet spectra were recorded on three instruments - Shimadzu UV240 Recording Spectrophotometer, Phillips Pye Unicam PU8800 and Unicam SP1700 ultraviolet/visible spectrophotometers. Spectra of all compounds were normally recorded between 200 and 400 nm using quartz cells with path lengths of 1 cm. Experiments were conducted at 37°C unless otherwise stated. 0.05M sodium phosphate buffer was used for spectra recorded at pH 7.0 whilst dilute hydrochloric acid and dilute sodium hydroxide were used when pHs of 1.0 or 13.0, respectively, were required. Spectra were recorded immediately after pH determination. Samples deviating from the required pH by more than 0.1 unit were discarded. In experiments to determine extinction coefficients, ε_{max}, a small amount of folate (up to 0.3±0.01 mg) was weighed out on an analytical balance (Sartorius) and dissolved by vigorous mechanical shaking or sonication (Semat Ultrasonic Bath) in 10 or 20 ml of the appropriate media.

II INFRARED SPECTROSCOPY

Infrared spectra were recorded on a Perkin-Elmer 599B Infrared Spectrophotometer to which was attached a Perkin-Elmer Model 3600 Data Station. Spectra were manipulated using the computer program to reduce noise levels (smoothening), to flatten

curved base lines and to make the strongest absorbance signal represent 100% transmittance (thereby increasing the intensities of the smaller absorbances). Air was used as reference in spectra recorded using potassium bromide discs, Nujol or liquid films. In solution spectra, usually in chloroform (Analar grade, from BDH, Poole, Dorset), pure solvent was used in the reference cell. Spectra were scanned from 4000 to 250 cm^{-1} using normal slitwidths and scan times of 12 minutes.

III COLUMN CHROMATOGRAPHY

a Ion-exchange chromatography

Anion-exchange chromatography experiments used Whatman pre-swollen microgranular DEAE cellulose (DE52, from Pharmacia, Hounslow, Essex). New gel was prepared by allowing it to swell in 0.05M sodium phosphate buffer (pH 7.0) for about three hours. Fines were decanted away and the gel washed five times with buffer before resuspension in buffer containing 5% (w/v) DL-dithiothreitol (Sigma, Poole, Dorset). This suspension was degassed in vacuo for two hours and packed into a 1.5 x 45 cm glass column. In view of the sensitivity of folates to photolytic decomposition, chromatographic columns were wrapped in aluminium foil. All experiments were conducted overnight in a dark room. A solution of the sample dissolved in a minimal amount of phosphate buffer was introduced at a dropwise rate from the top of the column by using a peristalic pump (LKB Bromma 1200 Varioperpex, setting 8 (x10)). A linear gradient of 0-1.2M sodium chloride generated by a LKB Bromma 11300 Ultrograd gradient mixer over a period of eight hours was used to develop each column. Solution eluting from the bottom of the column was passed through an ultraviolet

detector (LKB Bromma 11300 Uvicord II) which was connected to a chart recorder (LKB Bromma Chopper Bar Recorder 6520-8). One hundred 5 ml fractions were collected on a LKB Bromma 7000 Ultrarac fraction collector. Fractions containing the desired compounds were worked-up immediately to minimise further breakdown of the folates in solution. Using the print-out of absorbances versus fraction numbers, tubes containing the major fractions were pooled. After cooling in ice for 30 minutes, the solution was acidified slowly (dropwise addition of dilute acid) to reprecipitate the folate. Products were collected by bench centrifugation (MSE Minor Centrifuge), washed with cold water or dilute hydrochloric acid (pH 3.0) and dried overnight at room temperature in a vacuum dessicator. Exact details of the work-up procedures for each folate are included in the appropriate preparative sections of these compounds.

b Gel-filtration chromatography

Gel filtration chromatography was used in an attempt to purify compounds that could not be chromatographed on DE52. It was found that folates containing strongly electron-withdrawing functionalities (eg. the formylated fluorofolates) adhered extremely tightly to DEAE cellulose and could not be eluted off even with 0.1M sodium hydroxide.

Sephadex G15 (Pharmacia, Hounslow, Essex) was allowed to swell overnight at room temperature in distilled water. G15 was selected because previous experiments have demonstrated that it had a better resolving ability for folates than Sephadex G10. Distilled water was used in preference to buffer because the former gave better resolved fractions. Swollen gel was washed five times with distilled water before resuspension in 5% aqueous dithiothreitol. After a two-hour degassing, the gel was packed into a 2.5x

35 cm column using a peristalic pump (LKB Bromma 1200 Varioperpex) working at maximum speed. This column was then washed with about 500 ml of water. Gaps that appeared were filled with gel. Solutions of folates in water or dilute sodium hydroxide were introduced from the bottom of the column and chromatographed against gravity. Peristalic pump speed was set at 8 (x10). Effluents from the column were passed through an ultraviolet detector linked with a chart recorder. Fifty 5 ml fractions were collected on a LKB Bromma 2112 Redirac fraction collector. Using the print-outs from the chart recorder, tubes containing the desired products were pooled. Attempts to reprecipitate the folates by slow acidification with dilute hydrochloric acid were almost always unsuccessful. Consequently, solutions had to be frozen in round-bottomed flasks using cardice-acetone and lyophilized in a freeze-drier (Virtis 10-030 Bench Top Freeze-Drier). In none of these gel filtration experiments were we able to obtain anything other than partially-purified samples. Although they could not be used for enzymic studies, these samples were of adequate purity to be used for recording NMR spectra where signals belonging to impurities could easily be identified on the basis of their relative intensities and chemical shifts. We have assumed that chemical shifts from the nuclei of our folates were not greatly perturbed by the impurities. Nonetheless, we were still vigilant in guarding against "extraordinary" chemical shifts. Impurities were expected to exert a greater influence on enzymic assays than NMR spectra because small molecules, especially pteroylglutamate fragments, could sometimes bind extremely well to DHFR and thus upset determinations of inhibition constants and other binding properties of our compounds.

c Silica gel column chromatography

Silica gel 100 for column chromatography (0.2-0.5 mm, 35-70 mesh ASTM) was purchased from Fluka AG (distributed in Britain by Flurochem Limited, Glossop, Derbyshire). A 2x30 cm column was normally used but large scale experiments used a 4x40 cm column instead. Columns were packed and run under gravity. Solvents used include 95% ethanol, sodium-dried diethyl ether and n-hexane. The latter two were redistilled prior to use. Products were collected in round-bottomed flasks. Solvents were removed using a rotary evaporator (Buchi Rotavapor R110).

IV THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) was carried out on plastic sheets pre-coated with either a 0.1 mm thickness of MN300 cellulose impregnated with a fluorescent indicator (Polygram Cel 300 UV_{254} purchased from Camlab, Cambridge) or a 0.2 mm thickness of silica gel (Silica gel 60, UV_{254} from Camlab).

In preparative TLC experiments, glass plates were coated with a 0.1 cm thickness of cellulose MN300G/UV_{254} (Camlab) containing the fluorescent indicator. Plates were laid using a suspension of cellulose power in methanol and allowed to dry for an hour at 100^{o}C.

All chromatograms were developed in the dark. For cellulose plates, five eluting solvents were used - 3% aqueous ammonium chloride, 5% aqueous acetic acid, 4:1:5 n-butanol/acetic acid/water (nBAW), pH 7.0 0.05M sodium phosphate buffer and pH 7.6 0.5M Tris (hydroxymethyl) aminomethane ("Tris", from Sigma)/hydrochloric acid

buffer. With the exception of nBAW, all four solvent systems developed quickly and gave reasonably good resolution. The former gave better resolution but chromatograms took a longer time to develop.

A variety of organic solvents were used to develop silica gel TLC plates. These included chloroform, ethyl acetate, ethanol, methanol, acetone, diethyl ether and mixtures of two or more solvents in varying proportions. General purpose grade reagents were used throughout as impurities were not found to present significant problems.

Chromatograms were dried under a stream of warm air and viewed under an ultraviolet lamp (Camag Universal UV Lampe) at 254 and 366 nm. For chromatograms of amines, a 2% w/v solution of ninhydrin (Spectrophotometric Grade, Gold Label, Aldrich, Dorset) in butan-1-ol was sprayed onto the dried plates. These were left at 110^{o} for 10 minutes before viewing under the ultraviolet lamp.

V MISCELLANEOUS

Microanalyses were performed in this department by Ms. C. Jakeman on a Carlo Erba Elemental Analyser Model 1106. Melting points (uncorrected) were recorded on a Gallenkamp MF-370 melting point apparatus using 1 mm diameter glass capillaries. Ultracentrifugation experiments were run on a MSE Superspeed 50 using the 8x50 ml rotor. Samples were spun at $0^{o}C$ to minimise thermal breakdown of folates.

pHs were determined on a Kent Model 7020 Laboratory ph meter. The instrument was calibrated at pH 7.0 with a solution of Cambridge Buffer tablets (Burroughs-Wellcome, London) in water prior to use.

B SYNTHESIS

Due to the sensitivity of folates to light and heat, all experiments were conducted in the dark or in subdued lighting. Solutions containing folates were not allowed to stand for long periods because of the instability of these compounds in solution. Purified folates were stored in tightly sealed light-proof containers in a freezer at -18^{o}C. All ultraviolet spectroscopic data and nuclear magnetic resonance (NMR) chemical shifts (^{1}H, ^{13}C and ^{19}F) have been tabulated in the Results chapter (Chapter 4).

I SYNTHESIS OF N(10)-FORMYLFOLIC ACID (N(10)-CHO FA)

N(10)-formylfolic acid was prepared by direct formylation of folic acid according to the method of Blakley (1959).

4.0g of folic acid (Crystalline, approximately 98%, from Sigma) was dissolved in 160 ml of formic acid (90%, BDH, Poole, Dorset) and left to stand in the dark over a period of 65 hours. This solution was poured slowly into an excess of sodium-dried diethyl ether (500 ml). A creamy white precipitate was formed. This was collected by vacuum filtration, thoroughly washed with more dry ether and sucked dry to yield 3.99 g (94%) of crude material.

N(10)-CHO FA was purified by anion-exchange chromatography on DE52 (diethylaminoethyl cellulose) as described in Section 3AIIIa. Pure material was eluted between fractions 30 and 40. These were pooled and left to stand in an ice-bath for about 15 minutes before addition of dilute hydrocholric acid in a slow, dropwise manner. The solution became cloudy around pH 3.0. After standing in the ice bath for a

further 30 minutes, the solution yielded a white precipitate. This was collected by centrifugation on a bench centrifuge (MSE Minor Centrifuge, samples spun for 15 minutes at top speed in room temperature). The residue was washed with dry ether (3x30 ml) and left overnight in a vacuum dessicator over phosphorus pentoxide (crystalline, approximately 90%, from Sigma). Yield of dry product 53 mg (53%).

Thin layer chromatography on cellulose-coated plastic sheets using 3% aqueous ammonium chloride (3% aq NH_4Cl), 5% aqueous acetic acid (5% ag AcOH) and 4:1:5 n-butanol/acetic acid/water (nBAW) all demonstrated the presence of one blue, strongly-fluorescent spot (R_f 0.77, 0.78 and 0.35, respectively) and a minor, weakly fluorescent spot (R_f 0.46, 0.51 and 0.16, respectively) which probably belonged to a breakdown product of N(10)-formylfolate. Ultraviolet spectroscopy (pH 7.0 0.05M sodium phosphate buffer) gave λ_{max} 245, 267 and 351 nm. cf. Blakley (1969) λ_{max} 245, 269 and 348 nm.

II SYNTHESIS OF N(10)-NITROSOFOLIC ACID (N(10)-NO-FA)

N(10)-nitrosofolic acid was prepared from folic acid by direct nitrosation according to the method of Cosulich and Smith (1949).

Folic acid (4.4 g) was dissolved in concentrated hydrochloric acid (50 ml) and cooled in an ice bath to 2°C. Sodium nitrite (0.7 g), dissolved in a minimal amount of water, was added to this acid solution at a slow, dropwise rate using a Pasteur pipette.

A yellow precipitate appeared towards the end of the addition. This was collected by vacuum filtration, washed very thoroughly with ice-cold distilled water and left to dry overnight in a vacuum dessicator over phosphorus pentoxide. Yield of crude product 3.1 g (66%).

Crude N(10)-NO FA (1 g) was finely-powdered and dissolved in sodium hydroxide (5M, 25 cm^3). This yellow solution was clarified by shaking with activated charcoal and filtered. Cooling of the brown coloured filtrate precipitated the bright yellow coloured sodium salt which was collected by bench centrifugation (top speed, 15 minutes, room temperature). The residue was dissolved in a little distilled water. To this solution was added glacial acetic acid at a slow, dropwise rate in order to reprecipitate the free dicarboxylic acid. N(10)-nitrosofolate reappeared from solution in the form of a gel which could be most conveniently collected by centrifugation. This residue was left to dry overnight in vacuo over phosphorus pentoxide. Yield of partially purified product 0.72 g (72%).

Further purification of N(10)-NO FA was again effected by anion exchange chromatography on DEAE cellulose. Conditions used were the same as those used in the purification of N(10)-CHO FA. Pure compound was eluted between fractions 66 and 76. Slow addition of dilute hydrochloric acid to the ice-cold pooled fractions reprecipitated the product which had a solubility minima around pH 1.8. The whitish-yellow gelatinous product was cooled in an ice bath and harvested by centrifugation on a bench centrifuge (maximum speed, 15 minutes, room temperature). The residue was washed down with ice-cold distilled water (3x30 ml) and left to dry overnight in vacuo over phosphorus pentoxide.

TLC on cellulose-coated plastic sheets using 5% aq. AcOH (R_f 0.62), 3%aq.

NH_4Cl (R_f 0.69) and 4:1:5 nBAW (R_f 0.56) all gave a single dark spot when viewed with ultraviolet light at 254 nm. Microanalytical results also agreed well with calculated values. Obtained C 46.2, N 23.2 and H 4.0%. Calculated for $C_{19}H_{18}N_8O_7.H_20$ C 46.7, N22.9 and H 4.1%. Ultraviolet spectroscopy (in pH 7.0 0.05M sodium phosphate buffer) gave λ_{max} 232sh, 276 and 342 nm.

III SYNTHESIS OF N(10)-METHYLFOLIC ACID (N(10)-MeFA)

N(10)-methylfolic acid was prepared by selective partial alkaline hydrolysis of methotrexate as described by Charlton and Young (1982).

Methotrexate (500 mg) was dissolved in nitrogen-saturated dilute sodium hydroxide (1M, 50 ml) and refluxed for 4.5 hours in the dark under a slow stream of nitrogen. The reaction mixture was allowed to cool before dilution with deaerated water (500 ml). Sodium chloride (2 g) was added to this solution and the pH adjusted to 3.5 by dropwise addition of hydrochloric acid (5M). A dull yellow precipitate was deposited as the solution became acidic. This suspension was left under nitrogen and cooled in the dark in an ice bath for 5 hours. Aggregated precipitate was collected by ultracentrifugation ($3x10^4$ rpm, 0^oC, 5 mins.). Residue was washed with ice cold deaerated hydrochloric acid (0.001M, 80 ml) and dried overnight in vacuo over phosphorus pentoxide. Yield of crude product 0.39 g (78%).

Some of this material (61 mg) was powdered and shaken in degassed sodium phosphate buffer (pH 7.0, 0.05M, 25 ml) for 2 hours prior to introduction into a DEAE cellulose anion-exchange column. Purification conditions were identical to those used

for N(10)-CHO FA. The product was eluted between fractions 49 and 57. These were collected and cooled in an ice bath. Slow addition of hydrochloric acid (2M) to bring the pH to 3.5 reprecipitated the product. This suspension was stood in an ice bath for about 15 minutes. Aggregated product was collected by ultracentrifugation ($3x10^4$ rpm, 50 minutes). Residue was thoroughly washed with ice-cold degassed hydrochloric acid (0.001M, 30 ml) and dried overnight in a vacuum dessicator over phosphorus pentoxide and sodium hydroxide pellets. Yield of dry compound 16 mg (29%).

TLC of this purified material on cellulose-coated plastic sheets using 3% aq. NH_4Cl (R_f 0.71), 0.05M pH 7.6 Tris/hydrochloric acid buffer (R_f 0.85) and 0.05M pH 7.0 sodium phosphate buffer (R_f 0.85) as eluting solvents all demonstrated the presence of a single dark spot when viewed with ultraviolet light at 366 nm. 5% aq. AcOH and 4:1:5 nBAW were not used as eluting solvents because previous tests have shown that these two solvent systems were incapable of resolving methotrexate and N(10)-Me FA from each other.

Microanalytical results were in good agreement with theoretical values calculated for $C_{20}H_{21}N_7O_6.H_2O$ - C 48.9, H 5.1 and N 19.9%. Found C 49.0, H 4.8 and N 19.8%. These values also agreed closely with those from Gupta and Huennekens (1967) which were obtained from a sample prepared by a different method (C 50.3, H 5.1 and N 20.7%).

Ultraviolet spectral data of our compound were almost identical to the data of Charlton and Young (1982). In 0.05M pH 7.0 sodium phosphate buffer λ_{max} 282 (shoulder) and 303 nm (cf 282 and 303 nm).

IV SYNTHESIS OF 3',5'-DINITROFOLIC ACID

(3',5'-$(NO_2)_2$-FA)

3',5'-Dinitrofolic acid was synthesized by direct nitration of folic acid according to the method of Cosulich *et al.* (1953).

Folic acid (4.9 g) was added in small portions to ice-cold concentrated sulphuric acid (98%, 30 ml). This slow procedure was necessary in order to minimise the formation of a thick, highly insoluble, resinous material. When all the folic acid had dissolved, ice-cold nitric acid (70%, 1.87 ml) was added slowly, using a Pasteur pipette. Care was taken to ensure that the temperature of the reaction mixture did not exceed 5°C. Nitration was allowed to proceed at around 2°C for approximately 10 minutes before quenching the reaction by pouring the solution into flaked ice (200 g). A bright yellow solid was formed but this redissolved when the solution was warmed to 40°C. The solution was clarified with activated charcoal. After filtration to remove the charcoal, pH of the filtrate was adjusted to 1.0 by slow addition of sodium hydroxide pellets. This neutralization was done in an ice bath to avoid heating up the solution. Further cooling reprecipitated a yellow solid. This was collected by vacuum filtration and thoroughly washed with a large volume of ice-cold distilled water (≈ 200 ml) to remove inorganic salts. The residue was allowed to dry overnight in vacuo over calcium chloride. Yield of dry solid 2.9 g (49%).

Crude 3',5'-$(NO_2)_2$FA (0.6g) was suspended in water (530 ml). A minimal amount of sodium hydroxide (3M) was added to dissolve the solid. This solution was heated in a steam bath and poured gently into hot (≈ 70°C) aqueous acetic acid (30%, 265 ml). After clarification with activated charcoal, the solution was slowly cooled. It

deposited a fine, yellow precipitate. Partially purified product was collected by bench centrifugation (top speed, 15 minutes, room temperature), washed thoroughly with ice-cold distilled water and dried overnight in vacuo over phosphorus pentoxide. Yield of dry product 0.4g (67%).

TLC of recrystallised 3',5'-dinitrofolate on cellulose-coated plastic sheets demonstrated the presence of fluorescent impurities. Attempts to purify the product by anion-exchange chromatography (DEAE cellulose) failed. It adhered very strongly to the gel and could not be removed even with 0.1M sodium hydroxide. Preparative TLC on cellulose-coated glass plates (0.1 cm thickness) failed to yield 3',5'-$(NO_2)_2$FA uncontaminated with impurities.

Elemental analysis of a crude sample isolated by preparative TLC gave C 44.3, H 3.3 and N 24.4%. Calculated for $C_{19}H_{17}N_9O_{10}$ - C 42.9, H3.4 and N 23.7%. Ultraviolet spectroscopy (in dilute sodium hydroxide, pH 13.0) λ_{max} 370, 254 and minima at 234 nm (cf Cosulich *et al.*, 1953) λ_{max} 367, 258 and minima at 312 nm).

Due to the impure state of the product, proton and ^{13}C NMR spectra gave a very large number of resonances and only a partial assignment of peaks were possible. Brief enzymic studies on *Lactobacillus casei* and bovine liver dihydrofolate reductases seemed to indicate that 3',5'-dinitrofolate neither inhibits nor is reduced by the bacterial and mammalian enzymes.

V SYNTHESIS OF N(10)-ACETYLFOLIC ACID (N(10)-Ac FA)

N(10)-acetylfolic acid was prepared by direct acetylation of folic acid according to the method of Temple *et al.*, (1982).

Folic acid (11.95 g) was slowly added in small portions to a mechanically-stirred solution of freshly redistilled N,N-dimethylacetamide (DMAC, 125 ml).[1] Freshly redistilled acetyl chloride (6.1 g) in DMAC (50 ml) was added, under vigorous mechanical stirring, to this solution at a slow, dropwise rate. A gel was formed initially but this disappeared as more acetyl chloride was added. The reaction was allowed to proceed overnight (about 18 hours) at room temperature and in the dark.

The colour of the reaction mixture changed from orange to dark brown. Solvent was removed on a rotary evaporator to leave a greenish-brown syrup which was left overnight over phosphorus pentoxide.

This syrup was poured into distilled water (750 ml). Sodium hydroxide pellets were slowly added to bring the pH to 3.6. The solution was heated to boiling, filtered and allowed to cool slowly over three hours. An orange gel was formed. To this was added distilled water (350 ml) followed by calcium hydroxide to bring the pH to 7.1. Finally, absolute ethanol (95%, 1.3 L) was added in order to precipitate a creamy, yellow solid. Crude N(10)-Ac FA was harvested by vacuum filtration, washed with more ethanol and dried overnight in vacuo over phosphorus pentoxide. Yield of dry product 7.0 g (48%).

1 DMAC was dried overnight with barium oxide and redistilled under reduced pressure.

This crude material was further purified by anion-exchange chromatography on DEAE cellulose. Powdered N(10)-acetylfolate was suspended in pH 7.0 0.05M sodium phosphate buffer and basified with dilute sodium hydroxide. After shaking for 90 minutes, undissolved solids were removed by bench centrifugation before introducing the supernatant into the column. Pure N(10)-Ac FA appeared between fractions 28 to 32. These were collected and cooled in ice. Slow acidification with hydrochloric acid (2M) to pH 2.9 yielded a greyish-green gel which coagulated after standing in ice for 30 minutes. Product was collected by bench centrifugation (top speed, 10 minutes, room temperature), washed with ice-cold dilute sulphuric acid and dried overnight in vacuo over phosphorus pentoxide and sodium hydroxide pellets. TLC on cellulose-coated plastic sheets using 5% aq. AcOH (R_f 0.92), 3% aq. NH_4Cl (R_f 0.89) and 0.05M pH 7.0 sodium phosphate buffer (R_f 0.91) all gave a single blue fluorescent spot when observed under ultraviolet light at 366 nm.

Elemental analysis of this material gave C 35.4, H 3.5 and N 13.8%. Calculated for $C_{21}H_{21}N_7O_7.3/2H_20$ - C 35.7, H 4.0 and N 13.7%. Ultraviolet spectroscopy (in pH 13.0 dilute sodium hydroxide) λ_{max} 224 (shoulder), 253 and 364 nm (cf. Temple *et al.*, 1982) λ_{max} 255 and 365 nm).

VI SYNTHESIS OF α–DEUTERIOMETHOTREXATE (α-^{2}H-MTX)

α-Deuteriomethotrexate was synthesized by the reaction of concentrated deuteriosulphuric acid ($^{2}H_2SO_4$) with methotrexate.

0.05 g of methotrexate (gift from Lederle Labs Division of The American Cyanamid Company, Pearl River, New York, USA, 91.3% purity, expired on November 1983) was dissolved in a minimal amount of concentrated $^{2}H_2SO_4$ (about 0.5 ml, Gold Label, 98% solution in $^{2}H_2O$, Aldrich Chemical Company) under a stream of dry nitrogen and allowed to stand in the dark at room temperature for 65 hours. Reaction was quenched by pouring the solution into flaked ice (10 g). Sodium hydroxide (3M) was gradually added to this solution to bring the pH to 13. Insoluble material were removed by vacuum filtration. Reacidification to pH 3.0 by slow addition of sulphuric acid (2M) precipitated the crude product. This suspension was allowed to stand in an ice bath for 30 minutes. Aggregated product was collected by bench centrifugation (5 minutes, top speed, room temperature). The residue was thoroughly washed with ice-cold dilute sulphuric acid (pH 3.0) and dried overnight in vacuo over phosphorus pentoxide. Yield of dry compound 0.048 g (96%).

A little portion of this dry material was shaken for an hour in degassed sodium phosphate buffer (0.05M, pH 7.0) and loaded onto an anion-exchange column (DEAE cellulose). Elution conditions were similar to those used to purify the other folates. α-$^{2}H_2$-MTX was eluted between fractions 58 to 66. These were collected and cooled to

2°C. Acidification to pH 3.0 by slow, dropwise addition of hydrochloric acid reprecipitated the product. This suspension was left to stand in ice for 15 minutes to allow the solids to aggregate. Products were collected by bench centrifugation (top speed, 15 minutes, room temperature), thoroughly washed with ice-cold dilute hydrochloric acid (pH 3.0, 2x20 ml) and dried overnight in a vacuum dessicator over phosphorus pentoxide and sodium hydroxide pellets.

TLC on cellulose using aq. NH_4Cl (R_f 0.78), 5% aq. AcOH (R_f 0.69) and pH 7.6 0.5M Tris/hydrochloric acid buffer (R_f 0.61) all gave a single dark spot comigrating with the starting material. Elemental analysis yielded C 46.8, H 4.65 and N 22.0%. Calculated for $C_{20}H_{21}{}^2HN_8O_5.HCl.H_2O$ C 47.1, H 4.7 and N 21.9%. Ultraviolet spectroscopy (pH 13.0 dilute sodium hydroxide) λ_{max} 222, 258, 301 and 368 nm (cf. Seeger *et al.*, 1949, 257, 302 and 370 nm). This spectrum was similar in every respect to that from the starting material at pH 7.0 and 1.0 as well. Proton, ^{13}C and ^2H NMR spectra all indicate deuteriation at the α position of the glutamate side chain in methotrexate. Details have been tabulated in the NMR section of the Results chapter.

Attempts to determine the enantiomeric composition of the sample were unsuccessful because of large errors involved in the measurements of $[\alpha]_D$. $[\alpha]_D{}^{22}$ of MTX (from Lederle), α-^{2}H-MTX, N^{10}-methylfolic acid and folic acid (from Sigma) were -39, -36, -34 and -32°, respectively. All had errors of ±3°. Determinations were made at pH 13.0 in dilute sodium hydroxide. We used a cell with a path length of 5 cm and solutions of concentrations around 40 mM. Since these samples were extremely

strongly coloured, accurate determinations of α were very difficult. This was the main source of errors. Cramer *et al.* (1984) has shown, using chiral high performance liquid chromatography, that methotrexate obtained from Lederle Laboratories may be contaminated by up to 7% of the D-enantiomer.

VII SYNTHESIS OF 3'-IODOFOLIC ACID (3'-I FA)

3'-iodofolic acid was synthesized according to the method of Cosulich *et al* .(1953) by reacting folic acid with iodine monochloride.

Folic acid (2.45 g) was dissolved in a solution of concentrated hydrochloric acid (≈10M, 22ml) and water (22 ml) and cooled to 15°C. To this was added, at a dropwise rate, 1.63 g (0.526 ml) of iodine monochloride (GPR, 98% ICI minimum, Hopkin & Williams Limited, Essex) from a freshly opened bottle. The reactants were warmed gently to 30°C in a steam bath and left to stand at room temperature. A white precipitate appeared after a short time. On further standing, the mixture completely solidified into a gel. This was left overnight in the dark at room temperature.

The yellow-brown coloured gel was centrifuged (Bench Top centrifuge, maximum speed, 15 minutes, room temperature). The dark brown coloured supernatant was discarded. After washing with ice-cold hydrochloric acid (5M, 2x10 ml), the residue was dissolved in concentrated hydrochloric acid (15 ml, ≈ 10M). Addition of water to this solution produced a thick slurry which was allowed to cool in ice. The precipitate was collected by centrifugation and the steps described above were repeated. Residue from the second centrifugation was slurried in ice-cold water (125 ml). Sodium hydroxide pellets were gradually added to adjust the solution to pH 2.5. After standing

for 30 minutes in ice, the precipitate was collected by bench centrifugation and washed with ice-cold distilled water (2x15 ml). This material was left to dry in vacuo for 48 hours at room temperature over silica gel. Yield of dry product 1.964 g (62%).

0.45 g of 3'-iodofolate was suspended in water (5 ml). Sodium hydroxide (2M) was gradually added to dissolve the solid and to adjust the pH to 11. Slow acidification of this brown solution with glacial acetic acid afforded a light-yellow gel. An equal volume of acetone was added, followed by enough hydrochloric acid (4M) to give a clear, brownish-black solution. Addition of sodium hydroxide (2M) to bring the solution to pH 3.5 precipitated a bright yellow solid. This was collected by bench centrifugation, washed with ice-cold water (2x8 ml) and acetone (3x8 ml) and left to dry overnight in vacuo over silica gel and phosphorus pentoxide. Yield of dry product 0.379 g (84%).

A small amount of this powder was vigorously shaken in degassed sodium phosphate buffer (0.05M, pH 7.0) for 30 minutes. The bright yellow solution was loaded onto a DEAE cellulose anion-exchange column. Under conditions used to purify the other folates, 3'-I-FA was eluted between fractions 73 and 98. These were collected and cooled to $2^{o}C$ in an ice bath. Slow, dropwise addition of hydrochloric acid (1M) resulted initially in a cloudy solution. A yellow precipitate was deposited after this solution was allowed to stand in ice for 30 minutes. The product was collected by bench centrifugation (maximum speed, 10 minutes, room temperature), thoroughly washed with ice-cold distilled water (3x20 ml) and left to dry overnight in a vacuum dessicator over phosphorus pentoxide and silica gel.

TLC on cellulose using 3% aq. NH_4Cl (R_f 0.23), 5% aq. AcOH (R_f 0.0) and

0.05M pH 7.0 sodium phosphate buffer (R_f 0.59) all yielded a single dark spot. Elemental analysis gave C 38.2, H 3.0 and N 18.8%. Calculated for $C_{19}H_{18}N_7O_6I.1/2H_20$ - C 39.6, H 3.3 and N 17.0. Ultraviolet spectroscopy (in dilute sodium hydroxide, pH 13.0) λ_{max} 225 sh, 254, 281 and 364 nm (cf. Cosulich *et al.*, 1953, λ_{max} 255, 280 and 365 nm).

VIII SYNTHESIS OF 2'- AND 3'-FLUOROFOLATES (2'- AND 3'-F-FAs)

The method used in the syntheses of 2'- and 3'-fluorofolates was a modification of the recipé for preparing the corresponding aminopterins as described by Henkin and Washtien (1983). The procedure is illustrated in figure C I.

a Preparation of pterin-6-carboxaldehyde (6-CHO-pterin)

Pterin-6-carboxaldehyde was prepared by oxidative degradation of folic acid by bromine and aqueous hydrobromic acid according to the method of Thijssen (1973).

Folic acid (4.4 g) was added in small portions to a vigorously stirred solution of 8 g of bromine (SLR, 99% minimum, Fisons, Loughborough, Leicestershire) in 20 ml of aqueous hydrobromic acid (SLR, about 48%, Fisons). When all solid had dissolved,

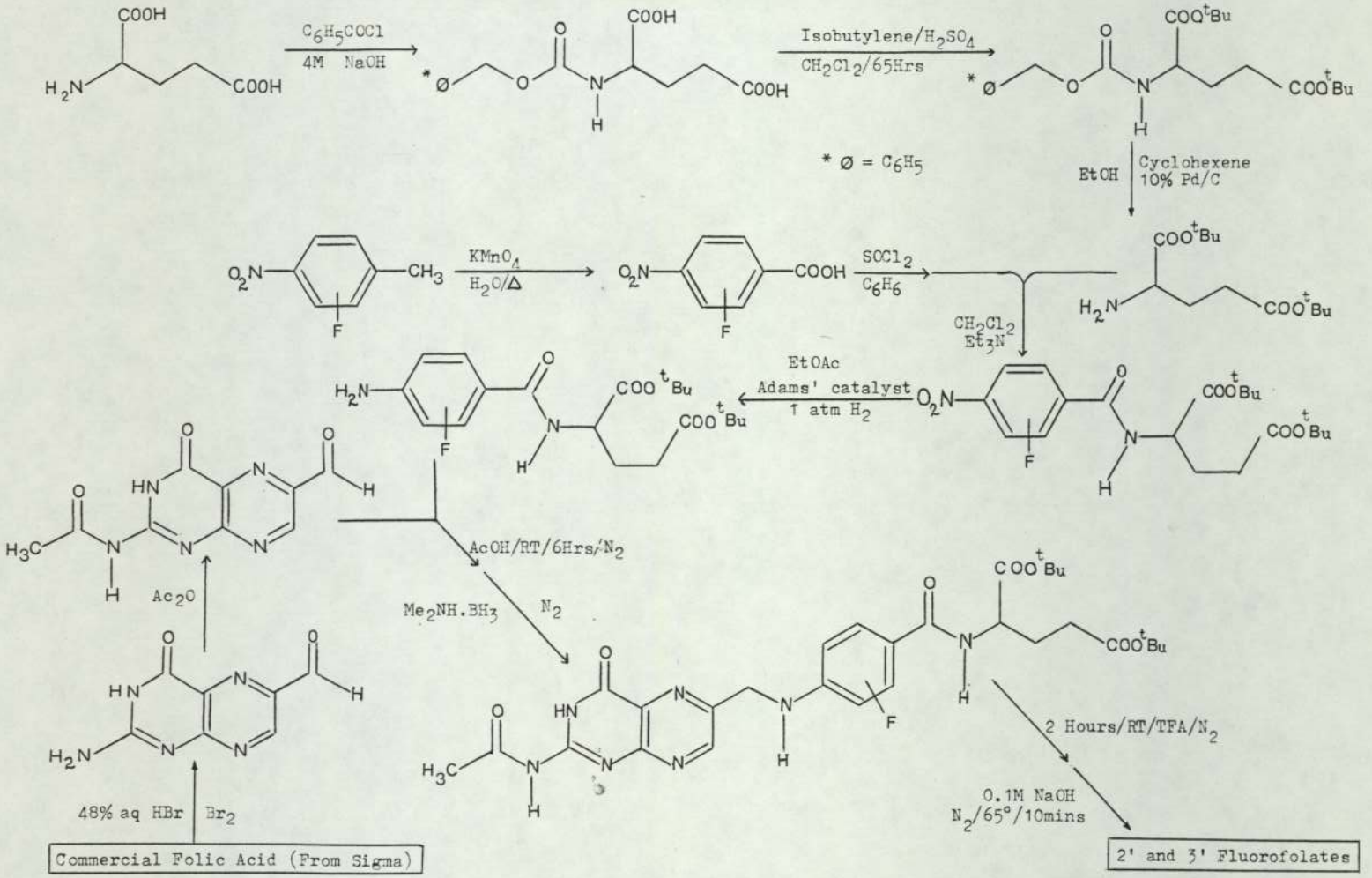


Figure C I Procedure used in the total synthesis of 2' and 3'-fluorofolates.

the dark solution was gradually heated (in the dark, with continuous stirring) to 100°C in 2 hours.

The yellow precipitate which formed was collected by vacuum filtration and thoroughly washed with sufficient ice-cold distilled water until washings became colourless. The residue was suspended in acetone (30 ml) and boiled for 10 minutes before decanting away the acetone. This procedure was repeated 5 times. Residue left after the final washing was dried overnight in vacuo over phosphorus pentoxide and silica gel. Yield of crude pterin-6-carboxaldehyde 1.38 g (73%).

TLC on cellulose-coated plastic sheets using 3% aq. NH_4Cl, 5% aq. AcOH and 0.05M pH 7.0 sodium phosphate buffer as eluting solvents revealed the presence of an impurity. It is yellow when observed in white light and did not fluoresce or absorb when viewed under ultraviolet light at 254 and 366 nm. Attempts to isolate pure material were unsuccessful. However, since the impurity did not significantly affect the acetylation of the 2-amino group in the following reaction, we decided not to proceed any further with the purification. Pterin-6-carboxaldehyde was too insoluble in $^{2}H_6$-dimethylsulphoxide for either a proton or ^{13}C NMR spectrum to be recorded. Ultraviolet spectroscopy (0.05M pH 8.0 sodium phosphate buffer) λ_{max} 361, 320 sh, 278 and 217 nm (cf. Viscontini *et al.*, 1958; λ_{max} 361, 322 and 278).

b Preparation of 2-acetyl-6-formylpterin (2-Ac-6-CHO-pterin)

2-Acetyl-6-formylpterin was prepared by acetylation of pterin-6-carboxaldehyde in acetic anhydride as described by Bieri and Viscontini (1973).

Dry, finely powdered 6-CHO-pterin (1 g) was suspended in 1.35 L of dry, freshly redistilled acetic anhydride (Analar grade, BDH, 98%). This mixture was kept in the dark at about 130^{o}C for four hours under a slow stream of dry nitrogen.

The orange coloured reaction mixture was cooled to room temperature. Acetic anhydride was removed under reduced pressure. The residue, containing traces of solvent, was dissolved in hot water (about 400 ml) using a boiling water bath. Suspended solids were removed by filtration. This solution was left to cool to room temperature. 2-Ac-6-CHO-pterin was allowed to crystallise out overnight at 4^{o}C. The product was harvested by vacuum filtration and washed thoroughly with ice-cold water. Filtrates were concentrated to yield a second crop of crystals. These two crops of 2-acetyl-6-formylpterin were allowed to dry overnight in vacuo over silica gel and phophorus pentoxide. Total yield of dry products 0.79 g (65%).

TLC on cellulose sheets using 3% aq. NH_4Cl. 5% aq. AcOH and 0.05M pH 7.0 sodium phosphate buffer all revealed the presence of impurities. As in the case of 6-CHO-pterin, these proved too difficult to remove, even with anion-exchange chromatography. Again, as they did not seem to interfere significantly with the reaction between 2-Ac-6-CHO-pterin and the para-aminobenzoylglutamate derivatives, we decided to use the material without further purification. Proton and ^{13}C NMR spectra of impure product yielded many resonances, making complete assignment of peaks impossible. Ultraviolet spectroscopy (0.05M pH 7.0 sodium phosphate buffer) λ_{max}

352, 274 and 205 nm.

c Preparation of di-*tert*-butyl-L-glutamate (Glu-$^{t}Bu_2$)

Di-*tert*-butyl-L-glutamate was prepared by deprotection of the corresponding amino-protected product according to the procedure of Anantharamaiah and Sivanandaiah (1977). It involved catalytic hydrogen transfer using ethanol and cyclohexene.

1.2 g of di-*tert*-butylbenzyloxycarboxyl-L-glutamate[1] was dissolved in a solution of absolute alcohol (95%, 48 ml) and cyclohexene (24 ml, 99%, SLR grade, BDH). To this was added 1.5 g of used[2] 10% palladium on activated carbon (Aldrich). The resultant mixture was gently refluxed under stirring for 90 minutes.

After allowing the reactants to cool to room temperature, the catalyst was removed by vacuum filtration and thoroughly washed with more ethanol (50 ml). Care was exercised during this washing to ensure that the catalyst never completely dried out. Solvent from the combined folates was removed under reduced pressure to leave a pale buff-coloured syrup. This was left to dry in vacuo for 72 hours at room temperature over silica gel and phosphorus pentoxide. This long process was necessary in order to

1 This tri-protected glutamic acid was synthesized in our laboratory by Dr. Rasool Naygir-Mazhir. L-glutamic acid was initially reacted with benzyloxycarbonylchloride in 4M sodium hydroxide according to the method of Bergman and Zervas (1932). The product was dissolved in dichloromethane containing a catalytic amount of sulphuric acid and treated with isobutylene gas for 65 hours to convert the two carboxyl groups into the corresponding tertiary butyl esters. This was based on the recipe of Kovacs *et al.* (1968).

2 This catalyst was previously used for the same purpose and had been carefully washed with a large volume of ethanol prior to use. Extreme care was exercised whilst handling this catalyst as it showed a tremendous ability to self-ignite, especially on warm days.

remove traces of ethanol which would interfere with the reaction between this ester and the acid chlorides. Yield of dry product 0.74 g (94%).

The dried glutamate gave a positive ninhydrin test. Proton and ^{13}C NMR spectra confirmed that the amino group had been deprotected whilst the two tertiary butyl functionalities were still intact.

di Preparation of 3-fluoro-4-nitrobenzoic acid

3-Fluoro-4-nitrobenzoic acid was prepared from the corresponding toluene by oxidation in aqueous medium using potassium permanganate. This procedure was based on the method of Schmelkes and Rubin (1944).

A mixture of 4.5 g of 3-fluoro-4-nitrotoluene (99%, Koch-Light Laboratories, Suffolk) and 14.3 g potassium permanganate (Analar grade, 99.5%, Hopkin and Williams) in distilled water (500 ml) was allowed to reflux gently for 4.5 hours. As a result of sublimation, large amounts of starting material were deposited on the condenser walls. These were regularly scraped away and allowed to fall back into the refluxing mixture.

At the end of the reaction, the brown solution was cooled under a tap. Insoluble manganese dioxide was removed by vacuum filtration and thoroughly washed with diethyl ether to remove unreacted starting material. The clear, brownish-orange filtrate was extracted with diethyl ether (5x300 ml). Organic extracts were pooled and ether removed under reduced pressure. Unreacted 3-fluoro-4-nitrotoluene was recrystallised once from water and reused.

The aqueous layer was allowed to stand overnight at 4°C. To this cooled solution was gradually added concentrated bydrochloric acid (10M). Its colour changed from orange to bright yellow and finally to pale yellow. Further acidification precipitated crude 3-fluoro-4-nitrobenzoic acid. It initially appeared as a fine suspension which aggregated after standing for 90 minutes in ice. Product was collected by vacuum filtration and recrystallised from water to give fine, yellow needles. These were dried overnight in vacuo over silica gel and phosphorus pentoxide. Yield of dry crystals 1.34g (25%), melting point 168-9°C. Infrared spectroscopy (KBr Disc) Broad band around 2900 (COOH), 1690 and 1600 (Carboxyl C=0), 1540 and 1350 (NO_2), 1230 (C-F) cm^{-1}. TLC was performed on silica gel coated plastic sheets using 1:1 ethanol/petroleum ether (40-60) as eluting solvent. The product was unmoved and showed no impurities but starting material and the ether extract gave coincident spots. Proton and ^{13}C NMR spectroscopy confirmed that the product was uncontaminated with starting material.

dii Preparation of 2-fluoro-4-nitrobenzoic acid

This preparation was based on the method for synthesizing the 3-fluoro isomer. 6.75 g of 2-fluoro-4-nitrotoluene (98%, Aldrich) was oxidised by 17.2 g of potassium permanganate in 250 ml of water. Removal of manganese dioxide gave a pale yellow filtrate which became more greenish coloured as ether extraction of unreacted starting material progressed. Acidification with concentrated hydrochloric acid gave a pale white precipitate. Recrystallisation from hot water yielded 2.43 g (30%) of buff-coloured irrisdescent plates. Melting point 166-8°C. Infrared spectroscopy (Nujol) Broad band

around 3000 (COOH), 1700 and 1600 (Carboxyl C=0), 1530 and 1350 (NO_2), 1240 (C-F) cm^{-1}. Proton and ^{13}C NMR spectra confirmed the absence of unreacted 2-fluoro-4-nitrotoluene.

ei Preparation of di-*tert*-butyl-N-(3-fluoro-4-nitrobenzoyl)-L-glutamate

The preparation initially involved the conversion of 3-fluoro-4-nitrobenzoic acid to the acid chloride by treatment with thionyl chloride. The acid chloride was not isolated but treated directly with the protected glutamate.

0.4 g (4 mmol) of redistilled thionyl chloride (Aldrich) and 10 mg of redistilled dimethylformamide (Aldrich) was dissolved in 20 ml of sodium-dried benzene. To this mixture was added dry, recrystallised 3-fluoro-4-nitrobenzoic acid (0.37 g, 2 mmol) and the resultant yellow mixture stirred under dry nitrogen for 4 hours at 70-75°C. Solvent and unreacted thionyl chloride were removed under reduced pressure with careful exclusion of moisture.

A solution of dichloromethane (20 ml) containing 0.5 g (5 mmol) triethylamine (99%, Aldrich) and 0.74 g (2.3 mmol) di-*tert*-butyl-L-glutamate was added to the residue. This brown mixture was stirred for two hours at room temperature under a stream of dry nitrogen. Solvent was removed under reduced pressure and residue taken up in 50 ml sodium-dried ether. Salts were removed by vacuum filtration and washed with more dry ether (20 ml). Filtrates were pooled, concentrated to 10 ml and applied immediately to a 2x30 cm column of silica gel in 1:1 redistilled n-hexane/sodium-dried diethyl ether. The column was eluted with the same solvent system. A bright yellow

band was collected. Removal of solvents yielded 0.526 g (64%) of a yellow oil. Infrared spectroscopy (Liquid Film) 2990 (aromatic C-H), 1740 (ester C=0), 1680 (amide C=0), 1600 (amide II), 1550 and 1330 (NO_2), 1240 (intense C-F) cm^{-1}. With the exception of signals arising from the glutamate moiety, proton, ^{13}C and ^{19}F NMR spectra were all similar to the spectra of the starting material. TLC on silica gel gave one major and two minor dark spots when viewed under ultraviolet light at 254 nm. Eluting solvents used included 3:2 methanol/water (R_f of major spot 0.48) and 1:1 methanol/water (R_f 0.36).

eii **Preparation of**

di-*tert*-butyl-N-(2-fluoro-4-nitrobenzoyl)-L-glutamate

The procedure for coupling 2-fluoro-4-nitrobenzoic acid to di-*tert*-butyl-L-glutamate was the same as that used in preparing 3-fluoro isomer. Instead of using protected glutamate prepared as described in section 3B VIIIc above, this synthesis used the corresponding hydrochloride salt purchased from Sigma.

2.73 g of the benzoic acid derivative yielded 2.98 g (51%) of pinkish-white oil. Infrared spectroscopy (Liquid Film) 2970 (C-H), 1720 (ester C=0), 1670 (amide C=0), 1600 (amide II), 1530 and 1350 (NO_2), 1220 (C-F) cm^{-1}. Proton, ^{13}C and ^{19}F NMR spectra contained signals arising from both glutamate and benzene ring moieties. Compared to the corresponding nuclei of 2-fluoro-4-nitrobenzoic acid, the chemical shifts of benzene ring nuclei showed changes consistent with the formation of an amide linkage to the glutamate. TLC on silica gel using methanol/water (1:1 and 3:2) revealed

the presence of at least one minor contaminant.

fi Preparation of di-*tert*-butyl-N-(3-fluoro-4-aminobenzoyl)-L-glutamate

Di-*tert*-butyl-N-(3-fluoro-4-aminobenzoyl)-L-glutamate was prepared from the corresponding nitro compound by catalytic hydrogenation over 1 atmosphere of hydrogen using Adams' catalyst.

The hydrogenation apparatus was constructed as described in Practical Organic Chemistry by Vogel (4th edition, 1978). The nitro compound (0.339 g, 0.788 mmol) was dissolved in ethyl acetate (24.5 ml). To this mixture was added 24 mg of Adams' catalyst (amorphous, 79% platinum, Aldrich) and the resultant mixture hydrogenated at room temperature under 1 atmosphere hydrogen for 8 hours. The solution changed from brown to black, then to greenish black and, finally, dark green. Taps were turned off overnight and opened again for another 5 hours the following day.

At the end of the reaction, ethyl acetate was gently removed from the pale green solution by a rotary evaporator. The dark green residue was dissolved in dry diethyl ether (about 40 ml). Catalyst was removed by bench centrifugation. The supernatant was divided into two portions and applied to separate 2x30 cm columns of silica gel (35-70 mesh) in 1:1 dry ether/redistilled n-hexane. Columns were initially eluted with approximately a column volume of the same solvent system followed by dry diethyl ether. Evaporation of ether gave a thick pink oil which was dried overnight at room temperature in a vacuum dessicator over phosphorus pentoxide and silica gel. Yield of oil 0.28 g (90.6%). TLC on silica gel plates using 4:1 chloroform/acetone, 2:1 methanol/water, 1:1 methanol/water and 2:1 ethanol/water all gave one major ninhydrin

positive spot and three minor ones. Infrared spectroscopy (chloroform), 3680 and 3620 (amine N-H), 3020 (aromatic C-H), 2400 (probably C-F overtone, 1720 (ester C=0), 1630 (amide (C=0), 1220 (intense C-F) cm^{-1}. The ^{19}F NMR resonance was shifted upfield substantially when compared to the signal from the nitro compound. The proton NMR spectrum showed significant changes in the aromatic region and the appearance of a signal from the amino group. Changes were observed in the ^{13}C spectrum as well.

fii Preparation of

di-*tert*-butyl-N-(2-fluoro-4-aminobenzoyl)-L-glutamate

The same method as described above for hydrogenating the 3-fluoro isomer was also used in the reduction of the 2-fluoro isomer. 3.92 g of di-*tert*-butyl-N-(2-fluoro-4-nitrobenzoyl)-L-glutamate was dissolved in ethyl acetate (280 ml) containing a suspension of amorphous Adams' catalyst (0.28 g). During hydrogenation, the solution changed from brown to dark green and then to greenish black. Products were chromatographed on silica gel and eluted with 1:1 dry ether/redistilled n-hexane followed by dry ether. On standing at room temperature for 30 minutes, the ether fraction deposited a white precipitate. The suspension was cooled in an ice-freezing salt bath (30 minutes) and collected by vacuum filtration. Filtrate was concentrated and a second crop of crystals recovered. After washing with ice-cold diethyl ether, the products were allowed to dry in vacuo over phosphorus pentoxide and silica gel. Yield of white solid 3.894 g (80%). Melting point 97.5-98.5°C. TLC on silica gel gave one ninhydrin-positive spot and a minor impurity spot. Eluting solvents used were 2:1 methanol/water (R_f 0.9 for the major spot) and 1:1 methanol/water (R_f

0.76). Infrared spectroscopy (KBr disc) 3470 and 3420 (amine N-H), 3380 (amide N-H), 3000 (aromatic C-H), 1730 (ester C=O), 1625 (amide C=O) and 1230 (C-F) cm^{-1}. The ^{19}F signal was not shifted quite as substantially as that of the 3-fluoro compound when compared to the starting material. However, aromatic regions of the proton and ^{13}C spectra showed some quite significant changes.

gi Preparation of 2-acetyl-3'-fluorofolic acid (2-Ac-3'-F-FA)

This preparation was based on the recipe of Taylor *et al* .(1983) for the synthesis of 5-deazapteridines.

A mixture of di-*tert*-butyl-N-(3-fluoro-4-aminobenzoyl)-L-glutamate (71 mg, 0.18 mmol) and 2-acetyl-6-formylpterin (42 mg, 0.15 mmol) in 5 ml dry, glacial acetic acid (redistilled, GPR grade) was stirred under dry nitrogen[1] at room temperature in the dark for 6 hours. 3 mg (0.056 mmol) dimethylamine borane complex (Aldrich) was added to this reaction mixture. Stirring under nitrogen at room temperature was continued for an additional 40 minutes before warming to 60°C. After 10 minutes at this temperature, the reactants were cooled. Solvent was removed under reduced pressure to leave a red-brown oil. This was dissolved in chloroform (10 ml) and applied to a 2x30 cm column of silica gel (35-70 mesh) in chloroform. After initial washing with 50 ml chloroform, the column was developed with 1:4 (v/v) 95% ethanol/redistilled chloroform. A bright yellow band was collected. The solution containing this partially-purified product was concentrated to 5 ml and reapplied to another column

1 White spot (oxygen-free) nitrogen, dried by passing over concentrated sulphuric acid and self-indicating silica gel.

(2x30 cm) of silica gel in chloroform. Elution was effected with a stepwise gradient of 95% ethanol in chloroform, from 0-25% v/v. A lemon yellow coloured band was isolated. Evaporation of solvent left an orange solid which was allowed to dry in vacuo over phosphorus pentoxide and silica gel. Yield of dry product 72.6 mg (66%). TLC on silica gel using 2:1 methanol/water, 2:1 ethanol/water and 5:3 methanol/water gave one major fluorescent spot and at least one smaller spot.

53 mg of crude di-*tert*-butyl-2-acetyl-3'-fluorofolic acid was dissolved in a slight excess of trifluoroacetic acid (about 4 ml, 99% purity, Aldrich) and stirred in the dark at room temperature under a slow stream of dry nitrogen. Solvent was gently removed under reduced pressure to leave a dark brown oil. This was taken up in ice-cold dilute sodium hydroxide (pH 13) to give a brown solution. Insoluble material was removed by filtration. Slow acidification of the filtrate with dilute hydrochloric acid to pH 3.5 precipitated a brown solid. This suspension was allowed to stand in ice for 30 minutes. Product was collected by bench centrifugation (top speed, room temperature, 5 minutes) and washed with ice-cold water (2x20 ml). Crude 2-acetyl-3'-fluorofolate was dried overnight in vacuo over phosphorus pentoxide and silica gel. Yield of dry folate 48 mg (93%). TLC on cellulose-coated plastic sheets gave one major blue fluorescent spot and at least two minor impurity spots. Eluting solvents used were 3% aq. NH_4Cl (R_f of major spot 0.73), 0.05M pH 7.0 sodium phosphate buffer (R_f 0.95) and 5% aq. AcOH (Rf =0). Attempts to purify 2-Ac-3'-F-FA on anion-exchange chromatography were unsuccessful.

The proton NMR spectra confirmed that coupling had indeed occured between the pteridine and para-aminobenzoylglutamate moieties - a signal belonging to the protons of

the methylene bridge at position 9 had appeared at δ 4.62 ppm. Changes were observed in the aromatic region. Signals from methyl protons of the tertiary butyl groups have also disappeared. Corresponding changes were also noted in the ^{13}C spectrum. The ^{19}F signal was not shifted much from that of the starting material. Ultraviolet spectroscopy (in pH 7.0 0.05M sodium phosphate buffer) λ_{max} 204, 215 (shoulder), 260 (shoulder), 285 and 334 nm.

gii Preparation of 2-acetyl-2'-fluorofolic acid (2-Ac-2'-F-FA)

The procedure used in preparing this compound was essentially the same as that used to synthesize the 3'-fluoro isomer.

95.2 mg (0.24 mmol) of di-*tert*-butyl-N-(2-fluoro-4-aminobenzoyl)-L-glutamate was reacted under dry nitrogen, in glacial acetic acid (5 ml) for 6 hours with 51 mg (0.18 mmol) of 2-acetyl-6-formylpterin. After addition of dimethylamine borane complex (4.5 mg, 0.084 mmol), stirring at room temperature was allowed to proceed for an hour followed by 20 minutes of reaction at 60°C. The product was worked-up as described previously. Di-*tert*-butyl-2-acetyl-2'-fluorofolate was a brown solid. Yield of dry product 104.1 mg (69%). TLC on silica gel using 1:1 methanol/water (R_f of major spot 0.83) and 4:3 water/methanol (R_f 0.52) indicated the presence of at least two minor contaminants.

Saponification of the ester using trifluoroacetic acid at room temperature gave a greenish-brown solid in 91% yield. TLC on cellulose gave results very similar to those obtained for the 2-acetyl-3'-fluoro compound - one major fluorescent spot and at least

two minor impurities. The product did not migrate when 5% aq. AcOH was used but 3% aq. NH_4Cl and 0.05M pH 7.0 sodium phosphate buffer gave R_f values of 0.75 and 0.91 respectively for the fluorescent spot. Proton, ^{13}C and ^{19}F NMR spectra were consistent with that expected from a pteroylglutamate. Ultraviolet spectroscopy (0.05M pH 7.0 sodium phosphate buffer) λ_{max} 202, 214 (shoulder), 261 (shoulder), 284 and 337 nm.

hi Preparation of 3'-fluorofolic acid (3'-F-FA)

2-Acetyl-3'-fluorofolic acid was deprotected by stirring in 0.1M sodium hydroxide at 65°C. This was based on the methods of Khalifa *et al.* (1973 and 1976) and Sengupta *et al.* (1975). We have experimented with various concentrations of sodium hydroxide (0.01 to 1.0M), reaction temperatures (60° to refluxing temperature) and durations of deprotection (1-20 minutes) and have found the method described below the most suited to our compound.

0.1M sodium hydroxide was degassed at room temperature for an hour. Nitrogen gas was bubbled through this solution for 5 minutes and the degassing repeated for another hour. After resaturating the degassed solution with nitrogen, 2-acetyl-3'-fluorofolate (10 mg) was dissolved, under a stream of nitrogen, in a small volume (about 3 ml) of this solution. Deprotection was allowed to proceed under nitrogen for 15 minutes at 65°C.

The brownish coloured reaction mixture was cooled to room temperature. Slow acidification with hydrochloric acid (1M) to pH 3.0 precipitated crude 3'-fluorofolate.

This suspension was left for 30 minutes in an ice bath. Product was collected by bench centrifugation (5 minutes, maximum speed, room temperature), washed with ice-cold water (2x15 ml) and dried overnight in vacuo over phosphorus pentoxide and silica gel. Yield of dry 3'-F-FA 9 mg(98%).

This impure yellow-brown solid (9 mg) was shaken for 30 minutes in degassed 0.05M pH 7.0 sodium phosphate buffer and applied to a DE 52 anion-exchange column. Purification conditions were the same as those utilized for the other folates. Pure 3'-fluorofolate appeared between fractions 60 and 71. These were collected and cooled in ice. Slow acidification with dilute hydrochloric acid (1M) to pH 3 reprecipitated a yellow, gelatinous product. After allowing the suspension to stand for 30 minutes in ice, aggregated product was collected by bench centrifugation (maximum speed, 5 minutes). Residue was thoroughly washed with ice-cold distilled water (2x15 ml) and dried overnight in vacuo over phosphorus pentoxide and silica gel to leave a yellow solid. Yield of pure 3'-F-FA 8 mg (89%). TLC on cellulose using 3% aq. NH_4CL (R_f 0.83), 5% aq. AcOH (R_f 0.83) and 0.05M pH 7.0 sodium phosphate buffer (R_f 0.87) all gave a single fluorescent spot. Proton and ^{13}C NMR spectra confirmed the removal of the 2-acetyl group to leave the free primary amine. The ^{19}F spectrum was also recorded. Ultraviolet spectroscopy (0.05M pH 7.0 sodium phosphate buffer) λ_{max} 279 and 364 nm. Elemental analysis gave C 40.2, H 3.7 and N 17.6%. Calculated for $C_{19}H_{18}N_7O_6F.6H_2O$ - C 40.2, H5.3 and N 17.3%.

hii Preparation of 2'-fluorofolic acid (2'-F-FA)

2-Acetyl-2'-fluorofolic acid was deprotected under the same conditions used for deprotecting the 3'-fluoro analogue. 15 mg of acetylated product yielded 12.7 mg (92%) of crude 2'-fluorofolate. Following anion-exchange chromatography on DEAE cellulose (2'-F-FA appearing between fractions 80 and 98), the amount of pure folate obtained was 11.4 mg (90% yield based on crude 2'-fluorofolate). TLC of this yellow solid on cellulose gave one fluorescent spot with essentially the same R_f values as the 3'-fluoro isomer. Eluting solvents used were 3% aq. NH_4Cl (R_f 0.81), 5% aq. AcOH (R_f 0.81) and 0.05M pH 7.0 sodium phosphate buffer (R_f 0.87). The ultraviolet spectrum was very similar as well. λmax (0.05M pH 7.0 sodium phosphate buffer) 279 and 346 nm. Proton and ^{13}C NMR results were consistent with a free 2-amino group. The ^{19}F chemical shift was very similar to that of the starting material. Elemental analysis yielded C 31.3, H 2.9 and N 13.5%. Calculated for $C_{19}H_{18}N_7O_6F.15H_2O$ - C 31.2, H 6.6 and N 13.4%.

IX SYNTHESIS OF N(10)-FORMYL 2'- AND 3'-FLUOROFOLATES

a Preparation of N(10)-formyl-3'-fluorofolic acid (N(10)-CHO-3'-F-FA)

We initially attempted to synthesize N(10)-formyl-3'-fluorofolic acid by direct formylation of di-*tert*-butyl-2-acetyl-3'-fluorofolate and 2-acetyl-3'-fluorofolate. We

had expected formic acid to remove the protecting groups in addition to introducing a formyl functionality at N(10). In the event, only tertiary butyl groups were removed, leaving 2-acetyl-N(10)-3'-fluorofolic acid. Numerous attempts to selectively deprotect the 2-amino group failed. Finally, we decided to formylate 3'-fluorofolate directly.

20 mg of 3'-fluorofolate were dissolved in a minimum amount of redistilled formic acid (about 5 ml, BDH) and left to stand in the dark at room temperature for 65 hours. These reactants were then added at a slow, dropwise rate to an excess of sodium-dried diethyl ether (about 25 ml), precipitating a creamy-white solid. This was collected by bench centrifugation (5 minutes, room temperature, top speed) and thoroughly washed with more dry ether (4x30 ml). The product was dried overnight in vacuo over phosphorus pentoxide and silica gel. Yield of dry product 19 mg (89%).

TLC on cellulose gave one major fluorescent spot and at least three minor spots from contaminants. R_f values of 0.92, 0.90 and 0.89, respectively, were obtained when 0.05M pH 7.0 sodium phosphate buffer, 3% aq. NH_4Cl and 5% aq. AcOH were used as eluting solvents. The product could not be purified by anion-exchange chromatography on DE 52 because it adhered strongly to the gel, defying even repeated washing in 0.1M sodium hydroxide. Gel filtration chromatography on Sephadex G15 in 0.05M pH 7.0 sodium phosphate buffer or distilled water (slightly better resolution) only succeeded in removing a small amount of contaminants. Ultraviolet spectroscopy (0.05M pH 7.0 sodium phosphate buffer) λ_{max} 252, 276(shoulder) and 338 nm. Proton and ^{13}C NMR spectra confirm the introduction of a formyl group at N(10). The ^{19}F chemical shift showed that formylation had greatly deshielded the fluorine nucleus on this compound with respect to 3'-fluorofolate.

b **Preparation of N(10)-formyl-2'-fluorofolate (N(10)-CHO-2'-F-FA)**

Attempts to prepare this compound by direct formylation of the corresponding 2-acetyl and di-*tert*-butyl-2-acetyl analogues were also unsuccessful, yielding the 2-acetyl-N(10)-formyl compound. Selective deprotection of the 2-amino group in this compound also failed. Consequently, 2'-fluorofolic acid was formylated in the same manner as described for the formylation of the 3' isomer. The yield was slightly lower here. 24 mg of starting material gave only 21 mg (82%) of a brownish-white solid. TLC results were similar to those of the 3'-fluorofolate. 3% aq. NH_4Cl (R_f of major spot 0.89), 5% aq. AcOH (R_f 0.89) and 0.05M pH 7.0 sodium phosphate buffer (R_f 0.91) all demonstrated the presence of one major fluorescent spot and at least three contaminants. Attempts to purify the compound by anion-exchange or gel-filtration chromatography failed as well. Ultraviolet spectroscopy (0.05M pH 7.0 sodium phosphate buffer) λ_{max} 255, 276 (shoulder) and 338 nm. Proton and ^{13}C NMR spectra confirmed that 2'-F-FA had been formylated only at N(10). Unlike the 3'-fluoro isomer, the ^{19}F resonance was very close to that of the starting material.

X SYNTHESIS OF 7,8-DIHYDROFOLIC ACID (7,8-DHF)

7,8-Dihydrofolate was prepared by dithionite reduction of folic acid using a modification of the method of Blakley (1960).

5 g of the sodium salt of ascorbic acid (Sigma) was dissolved in water (25 ml) in a 100 ml beaker. The pH was adjusted from approximately 8 to 6 by slow addition of hydrochloric acid (1M) before making the solution up to 50 ml with more water. To this 10% w/v ascorbate solution was added a 0.1M sodium hydroxide solution (about 10 ml) containing 190 mg of folic acid. pH was adjusted from about 6.5 to 6.0 with 1M hydrochloric acid. Sodium dithionite (2 g, GPR grade, BDH) was slowly added to this solution under stirring. After all the solid had dissolved, stirring was continued for another 5 minutes. Another 1 g of dithionite was added, followed by another 10 minutes stirring.

An ice bath was placed around the beaker to reduce the temperature to 4°C. Under rapid stirring, 3M hydrochloric acid was slowly added over a 15 minute period to reduce the pH to 2.8. The product was precipitated. After stirring for another 5 minutes, this suspension was poured into two pre-cooled centrifuge tubes (50 ml) and spun at 4°C for 5 minutes at 10,000 rpm. Supernatant was discarded and precipitate resuspended in freshly prepared cold sodium ascorbate (2°C, 10%, 50 ml). pH was adjusted from approximately 5 to 6.0 using sodium hydroxide (3M). After stirring for 5 minutes, hydrochloric acid (3M) was added slowly and under rapid stirring over a period of 15 minutes. The pH was reduced to 2.8 and stirring continued for another 10 minutes before centrifuging this suspension at 10,000 rpm for 5 minutes at 4°C. The residue was suspended in cold hydrochloric acid (30 ml, 2°C, 1mM) containing 50 mM mercaptoethanol. This suspension was recentrifuged under the same conditions as

above. Residue was collected and washing repeated once more. Residue from the two centrifuge tubes were combined and made up to 30 ml with the dilute hydrochloric acid solution containing 50 mM mercaptoethanol. This suspension was divided into 10x3 ml aliquots and stored in the dark at -20^{o}C for periods not exceeding 5 weeks. Ultraviolet spectroscopy had shown that 7,8-DHF was stable for this duration under these conditions. The product was used in DHFR assays.

XI MISCELLANEOUS

In addition to the syntheses described above, we have also attempted a number of other reactions. We attempted to nitrate folic acid with different molar equivalents of nitrating agent (mixtures of nitric acid and sulphuric acids). Reaction times were also changed in an effort to synthesize 3'-nitrofolic acid. These reactions either failed to proceed at all or gave highly impure mixtures of products from which it was impossible to isolate the desired material. Anion-exchange and gel-filtration chromatography failed to isolate 3'-nitrofolate. TLC, proton and ^{13}C NMR results seemed to indicate that these mixtures, inexplicably, comprised mainly 3',5'-dinitrofolic acid, unreacted folic acid and small amounts of some other products.

Next, we attempted to nitrate N(10)-acetylfolic acid. We reasoned that the deactivating effect of the acetyl group and its steric bulk should make it easier to form the mono-nitro compound. If the N(10)-acetyl group was not removed in the acid medium, it could be removed at the end of the reaction by treatment with dilute sodium hydroxide. Variation of the molar equivalent of nitric acid and reaction times again failed to yield a reasonably pure product. The mixture seemed to be dominated by unreacted

N(10)-Ac-FA. We were unsuccessful in isolating the minor components of the reaction mixture, either by anion-exchange or gel-filtration chromatography.

Attempts were also made to introduce a fluorine atom directly onto the benzene ring in folic acid. We were interested in this reaction because it provided a route whereby an ^{18}F nucleus could be quickly incorporated into a folate molecule. The resultant compound could then be used to study *in vivo* distribution of fluorinated antifolates and folates by means of positron emission tomography (Ter-Pogossian *et al.*, 1980). Indeed, one of the reasons for attempting to synthesize 3'-nitrofolate was the hope that a fluorine nucleophile could be used to displace the nitro group from the benzene ring. These fluorination experiments, however, involved the reaction of 3'-iodofolic acid and sodium fluoride in neutral and acid media. Different molar equivalents of sodium fluoride, reaction times and temperatures were tried. In addition, we also attempted fluorination by *in situ* generation of hydrogen fluoride by treatment of 3'-iodofolaate with sodium fluoride in concentrated sulphuric acid. None of these reactions were successful, as shown by an absence of ^{19}F NMR signals from the products.

Methotrexate was also treated for extended periods (up to 2 weeks) at room temperature with concentrated deuteriated sulphuric acid. We wanted to investigate if this could result in the incorporation of more than one deuterium atom into the molecule. Results showed that extended treatment resulted mainly in breakdown of the pteroylglutamate molecule. Proton NMR spectra of intact MTX showed only mono deuteriation, at the α-position. Ratios of "non-acidic" protons to one another had the usual values, indicating the absence of partial deuteriation. This observation is supported by results from ^{2}H NMR spectra which showed only a single peak.

C ENZYME STUDIES

Assays of antifolates against bacterial and mammalian dihydrofolate reductases were conducted in this laboratory by Drs. A. Sahota, C. Hamon and J. Brown.

7,8-Dihydrofolate was prepared from folic acid by dithionite reduction as described in section 3B X. They were stored as 3 ml aliquots (about 8 mM) at -20^{o}C for periods not exceeding 5 weeks. Ultraviolet spectra of substrates and inhibitors were examined prior to beginning the assays to ensure that they had not decomposed. The tetrasodium salt of reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Sigma.

I PREPARATION OF RAT LIVER DIHYDROFOLATE REDUCTASE

Rats were sacrificed by ether anaesthesia followed by cervical dislocation. Livers were removed and rinsed three times with an excess of ice-cold pH 7.4 0.05M potassium phosphate buffer. After drying these livers by blotting, they were cut into small pieces, weighed and divided into two portions.

Four volumes of phosphate buffer was added to one of these portions and the mixture homogenised for a minute at full speed in a 50 ml Potter homogeniser. This procedure was repeated with the second portion. Care was taken to ensure that both samples were kept cold during homogenisation. Homogenates were poured into two 50ml polycarbonate centrifuge tubes and spun at 21,000 rpm for about 15 minutes at 4^{o}C using the 8x50 ml rotor head of the MSE Superspeed 50. Supernatants were strained through double layers of cheese cloth.

To the ice-cold filtrate was added 0.2 volumes of 2% protamine sulphate (Grade X, Sigma) in phosphate buffer over a period of about 5 minutes. This mixture was stirred for 15 minutes, centrifuged for 5 minutes under the conditions described above and the residue discarded. Over a period of about 15 minutes, ammonium sulphate (Analar grade, BDH) was added to the supernatant to 70% saturation (44.2 g per 100 ml of supernatant). After 30 minutes stirring in an ice bath, this solution was centrifuged for 15 minutes as above and the precipitate discarded. Ammonium sulphate was added to this supernatant over a period of about 10 minutes to 95% saturation (17.3 g per 100 ml). This supernatant was stirred for about 30 minutes, centrifuged for 15 minutes and the supernatant discarded.

Products from the ammonium sulphate precipitation were dissolved in about 10 ml of buffer and dialysed overnight at 4^{o}C against about 500 ml of phosphate buffer. Products from dialysis were applied to a G50 gel filtration column and eluted with phosphate buffer. Fractions were checked for activity in the usual manner. Tubes containing the enzyme were pooled and divided into 1 ml aliquots. These were stored at -20^{o}C and found to be stable for several weeks. It is possible that some enzymic activity was lost during the purification steps. Recent work by Webber and Whiteley (1985) has shown that purified rat liver DHFR was unstable to dialysis and freeze/thawing.

II BOVINE LIVER AND *LACTOBACILLUS CASEI* DHFRs

Bovine liver DHFR was purchased from Sigma as a suspension in 3.8 mM ammonium sulphate. Freeze-dried *Lactobacillus casei* dihydrofolate reductase was a kind gift from Dr. Gordon Roberts (National Institute of Medical Research, Mill Hill,

London). The specific activity of the mammalian enzyme was 10.0 units/mg protein whilst that of bacterial DHFR was 22.3 units/mg. One unit is defined as the amount of enzyme which reduces 7,8-DHF to tetrahydrofolate at the rate of 1 μmol/min under the conditions described below.

III DIHYDROFOLATE REDUCTASE ASSAY

An aliquot of DHF was thawed out, centrifuged and the supernatant discarded. The precipitate was dissolved in pH 7.4 0.05M potassium phosphate buffer (10 ml). This solution was diluted 1 in 2 (5 ml to 10 ml) with the same buffer and stored as 2 ml aliquots at -20^{o}C. Aliquots were thawed out as required. The concentration of 7,8-DHF was about 1 mM. NADPH was made up in buffer to give 2 ml of approximately 5mM solution. Antifolates were dissolved in buffer to give the required concentrations. 1 mg of bacterial enzyme was dissolved in buffer (2 ml) to give 0.5 mg/ml and the bovine enzyme was diluted 1 in 10. All solutions were kept on ice.

Exact concentrations of DHF, antifolate and NADPH were calculated from the absorbances at λmax in pH 7.4 0.05M potassium phosphate buffer. Mercaptoethanol was omitted from these solutions as it absorbed strongly in the lower uv region (200-260 nm).

The assay mixture (1.25 ml) contained pH 7.4 0.05M potassium phosphate buffer, 7.2 mM mercaptoethanol, 80 μM NADPH and appropriate concentrations of 7,8-DHF, enzyme and antifolate. The standard assay mixture contained 40 μM 7,8-DHF, 80 μM NADPH and 20 μL of an appropriate dilution of DHFR. Bovine liver enzyme was

further diluted 1 in 20 and *L. casei* enzyme 1 in 50, giving approximately 0.14 μg protein in the assay. The rat liver DHFR assay contained 0.11 μg protein. This gave rates of reduction of dihydrofolate of 1.5 nmol/min for the bovine enzyme, 2.4 nmol/min for the bacterial enzyme and 0.26 nmol.min for the rat enzyme. Blank rates in the absence of enzyme (or 7,8-DHF) were 0.1 nmol/min. Results were corrected for this. Previous experiments (Sahota, 1983) have shown that the K_ms for dihydrofolate were 1.5 μM and 2.0 μM for the bovine and bacterial DHFRs respectively. The DHF K_m for rat liver DHFR was 14 μM. In the assays to determine potencies of the antifolates (K_i values), the assay mixture contained 0.14 μg enzyme, 80 μM NADPH and three different concentrations of dihydrofolate (16,40 and 80 μM). At least six different concentrations of antifolate were tested at each substrate concentration. Concentration ranges for the bovine enzyme were 0.05 to 2.50 μM and 2.50 to 62.5 μM for the *L. casei* enzyme. For rat liver DHFR, the concentration range used was 25 to 160 μM.

Reactions were started with NADPH following a two minute pre-incubation at 37^{o}C. Blanks lacked either DHF or enzyme. Assays were carried out in duplicate. DHFRs were assayed by following the decrease in absorbance at 340 nm using the Phillips PU8800 ultraviolet spectrophotometer with an incorporated four cell programmer. Samples were thermostatted at 37^{o}C. Each cell was read for 2 seconds against a reference which contained only buffer. Cycle times were 34 seconds and eleven cycles (initial absorbance followed by 10 absorbance changes) were recorded for each cell. Analysis times were just over six minutes. Absorbance spans were set to 0.5A with automatic offset corrections.

Decreases in absorbance at 340 nm were due to the oxidation of NADPH and the simultaneous reduction of dihydrofolate or antifolate as these compounds also absorbed at this wavelength. By using the combined extinction coefficients of DHF, NADPH and antifolate, the percentage absorbance change due to substrate and inhibitor reduction can be obtained. From these, the concentrations of antifolate needed to give a 50% inhibition can be calculated.

Inhibition data were analysed by plotting the following parameters against antifolate concentration at three different substrate concentrations: -

(a) percentage inhibition,

(b) substrate concentration divided by the rate (s/v), and

(c) the ratio of uninhibited and inhibited rates (v/v_i).

From these plots, the type of inhibition, the dissociation constant (K_i) and the concentration of inhibitor required to give 50% inhibition were calculated. K_m values can also be obtained from these graphs.

CHAPTER 4 RESULTS

A ULTRAVIOLET SPECTROSCOPY

0.05M sodium phosphate buffer was used to record ultraviolet spectra at pH 7.0. For spectra recorded at pH 1.0 and 13.0, dilute hydrochloric acid and dilute sodium hydroxide respectively were used. Spectra of some folates were also recorded at pH 7.4. These spectra were recorded because DHFR assays were conducted at this pH and we were interested to see if they differed significantly from spectra recorded at pH 7.0. 0.05M sodium phosphate buffer was used for these spectra.

The extinction coefficients, ε_{max}, of some novel antifolates were determined. About 0.3±0.01 mg of compound was weighed into a volumetric flask and made up to 10 cm^3. ε_{max} was calculated using the Beer-Lambert equation

$$A = \varepsilon cl$$

where A is the absorbance, c is the concentration of folate in mol dm^{-3} and l is the path length of the ultraviolet cells (1 cm in all experiments). Since A is measured at the absorbance maxima, ε_{max} is the extinction coefficient at the absorbance maxima. The errors involved in weighing out samples led to an error of around $2x10^3$ in the calculated ε_{max}.

The results show that, at a fixed pH, closely related analogues can have different numbers of absorption maxima. When chromophores were electronically modified by

COMPOUNDS \ pHs	pH 1.0				pH 7.0				pH 7.4				pH 13.0			
Folic Acid		245	296			282		346			282	345		256	284	365
N(10)-Me	216sh	248		308		282sh	302	337						254	302	363
N(10)-CHO		252		320	247	267		350						255		365
N(10)-NO		250	283		232	276		342						258		364
N(10)-CH_3CO	204	245		322	239	270		347	203	239	269	348	219	254		365
3'-I	222		299		223	279		346		224	279	346	225sh	254	281	364
3'-F	211	249	294			279		346	214		278	347	226	255	282	364
2'-F	213	249	294			279		346	215		279	347		256	281	363
N(10)-CHO-2'-F	204	241	277	332	255	276sh		338	202	255	275sh	340		257	280	352
N(10)-CHO-3'-F	206	237	278	328	252	276sh		336	203	253	275sh	338		255	276	357

TABLE III - Absorption Maxima Of Folates At Four Different pHs. λ_{max} are given in nm. sh stands for shoulder.

COMPOUNDS \ pHs	pH 1.0				pH 7.0				pH 7.4				pH 13.0			
Folic Acid		12.8	20.4[1]			27.6		7.2[1]						24.6	24.5	8.6[1]
N(10)-Me		10.7		17.5		20.0	18.2	18.1						17.6	17.0	12.8
N(10)-CHO		21.8		7.6[2]	20.0	20.9		5.2[2]						31.7		6.8[1]
N(10)-NO		13.3		19.7	6.7	24.7		20.0					5.5	22.7		12.9
N(10)-CH_3CO	9.1	7.1		2.6	11.3	8.8		2.9	17.3	13.0	10.2	3.6		11.9	21.0	5.0
3'-I	13.9		8.6		24.2	20.3		7.4	25.5		21.1	4.7	26.6	23.0	22.1	9.0
3'-F	21.4	11.2	17.6			23.1		8.8	20.2		22.8	5.9		11.0	26.3	9.5
2'-F	6.5	4.4	6.9			19.2		4.7	15.1		18.2	4.6		14.8	14.8	4.8
N(10)-CHO-2'-F	4.8	3.9	4.2		5.1	3.6		1.3	5.0	3.8	2.5	0.8		54.8	54.8	15.8
N(10)-CHO-3'-F	18.1	15.1	11.6	6.5	15.5	11.1		4.9	19.5	14.9	10.9	4.4		20.7	12.1	5.3

TABLE IV - Extinction Coefficients, $e_{max} \times 10^3$, Of Folic Acid And Its Analogues At Four Different pHs. Values for the N(10)-formylated 2'- and 3'-fluorofolates are unreliable due to the prescence of impurities.

1. Data obtained from JC Rabinowitz (1960).
2. Data obtained from Blakley (1969).

substituents (eg. by conjugation), this could result in shoulders being resolved into new peaks or the merging of two peaks. The new peak may, of course, be due directly to the substituent only.

Bulky groups, with their large steric requirements, could bring about conformational changes when introduced at N(10) or the benzene ring. Both λ_{max} and ε_{max} would be affected by the changes in relative position of the benzene and pteridine rings. Studies by Lam and Kotowycz (1972), Poe (1973) and Pastore (1971) have shown that folates can self-associate in aqueous solutions. Conformational changes in the folate molecule may affect the manner in which these molecules self-associate. Consequently, there may be both inter and intramolecular contributions to changes in ε_{max} and λ_{max}.

Comparison of spectra recorded at pH 7.0 and 7.4 did not reveal any differences. This indicated that the pK_a of the ionizable parts of chromophores did not lie close to this region. Whitely and Huennekens (1967) have shown that, for spectra recorded at pH 7.0, the 282 nm peak in folic acid was due to the para-aminobenzoylglutamate moiety and the pteridine peak was located at 346 nm. Gready (1980) has shown that pK_as of ionizable groups in both chromophores were far away from neutral pH. The closest was N(3), which had a pK_a of 8.4 (Poe, 1973).

Changing the pH from 7.0 to 1.0 resulted in hypsochromic shifts of both peaks in folic acid and its analogues. This could be attributed to protonation at N(1) (pK_a in folic acid 2.35) shifting the λ_{max} of the pteridine peak from 346 to 296 nm. The movement of the benzene ring λ_{max} from 282 to 245 nm was probably due to protonation of the

amide at C(11) as N(10) has a pK_a of around 0.2.

λ_{max} for the benzene ring absorption at pH 1.0 was quite constant in the folate analogues, all occuring at around 245 nm except the 3'-iodo compound whose peak was at 222 nm. The pteridine absorption, however, showed more variation. The blue shifts varied from 20 to 50 nm, with the N(10)-nitroso analogue showing a shift of 57 nm. The N(10)-formylated 2'- and 3'-fluorinated folates showed an additional absorption shifted only about 10 nm from its position at pH 7.0. We cannot be sure of its authenticity because of the presence of impurities in the sample.

At pH 13.0, the pteridine peak in folic acid underwent a bathochromic shift from 346 to 365 nm. This could be attributed to deprotonation at N(3) whose pK_a is 8.4. The benzene ring absorption did not undergo much change but a new peak appeared at 256 nm. The spectra of the other compounds were very similar to that of folic acid except for the N(10)-formyl, nitroso and acetyl analogues which lacked the benzene ring peak at 280 nm. It is possible that this peak was buried under the 255 nm peak.

Folates with halogens attached to the benzene ring gave spectra at pH 7.0 that were very similar to that of folic acid. The λ_{max} at 282 was not shifted much although substitution occurred at the chromophore. ε_{max} seemed to be reduced slightly. However, compounds with electron-withdrawing substituents at N(10) had blue shifted peaks here whilst the pteridine ring absorptions remained largely unperturbed. This was expected because groups like nitroso, formyl and acetyl contain double bonds that could be conjugated with the benzene ring via the lone pair of the nitrogen at position 10. As this chromophore is separated from the pteridine moiety by the saturated methylene

bridge at C(9), it would have had little effect on the latter's absorption. With the exception of 2' and 3'-fluorofolates, all these compounds showed an additional absorption maxima between 223 and 255 nm.

Unlike the compounds above, the λ_{max} of the benzene ring in N(10)-methylfolic acid was at the same position as that of folic acid. This may be due to the inability of the alkyl group to conjugate appreciably with the chromophore. Moreover, the pteridine ring peak was blue-shifted, possibly due to steric requirements of the methyl group. A new absorption has also appeared between the pteridine and benzene ring absorptions.

At pH 13.0, the benzene and pteridine absorption maxima were very similar for the compounds examined with the exception of the N(10)-formyl, nitroso and acetyl compounds which lacked a peak at around 280 nm.

The benzene ring absorptions of the folates at pH 1.0 were also very similar. However, more variations were seen in the pteridine ring absorptions. Most compounds had maxima between 280 and 300 nm although N(10)-formyl and acetyl folates had peaks at 320 and 322 nm respectively.

ε_{max} values of the folate analogues showed a much greater variation than the λ_{max} values. As mentioned previously, the errors involved here were rather large and too much reliance could not be put on them. ε_{max} values were included here only to serve as a rough guide. They can be used to compare relative intensities of peaks in the same spectrum.

B NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

The conditions under which these spectra were recorded have been detailed in the NMR chapter (Chapter 2). These spectra were required for our attempts to determine how minor modifications to the folate molecule affected chemical shifts of nuclei in the vicinity of the modification. Besides the direct effects of steric and electronic perturbation from the substituents, chemical shifts could be affected by conformational changes as well.

I TEMPERATURE AND CONCENTRATION STUDIES OF FOLIC ACID IN $^{2}H_{6}$-DIMETHYLSULPHOXIDE

Lam and Kotowycz (1972), Pastore (1971) and Poe (1973) have demonstrated that proton and chemical shifts of folic acid in aqueous solution were dependent on pH, temperature and solute concentration. These changes were attributed to self-association. We have examined the effects of temperature and concentration on the ^{1}H chemical shifts of folic acid in $^{2}H_{6}$-dimethylsulphoxide ($^{2}H_{6}$-DMSO). Results are summarised in tables V andVII and graphs VII and VIII.

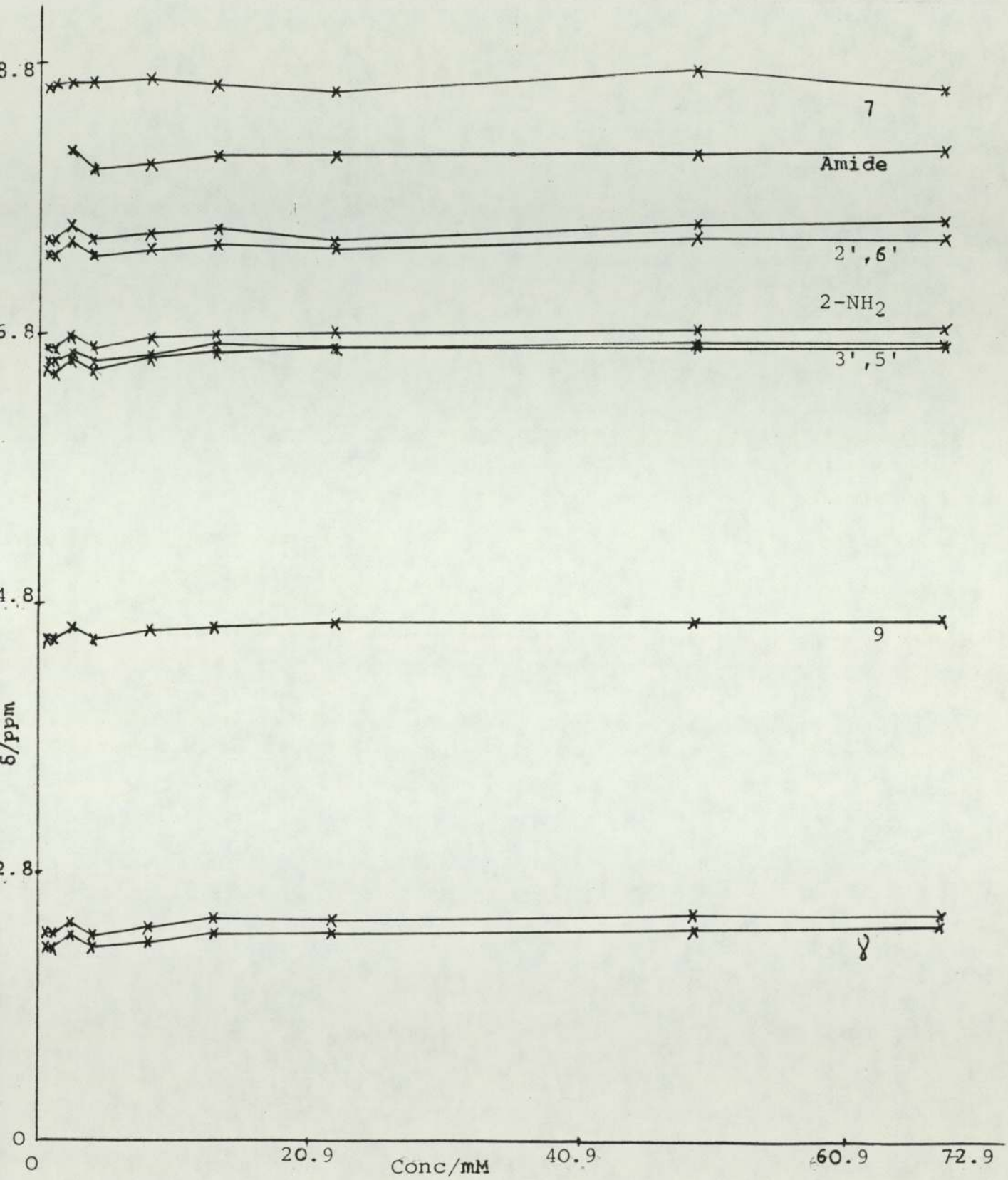
From the two graphs of chemical shifts versus temperature (graph VIII) and versus concentration (graph VII), it can be seen that chemical shifts were not uniformly affected by increasing concentration or temperature in the ranges studied. These results suggest that folic acid did not form dimers (or higher polymers) in $^{2}H_{6}$-DMSO. We do not anticipate minor modifications to the folate structure to radically affect the behaviour of the other analogues in solution. If they did, large changes in proton and ^{13}C chemical shifts would appear in nuclei located far away from the points of modification.

Protons / Conc/mM	2	7	9	2',6'	3',5'	12	13	14	15
0.95	6.54	8.64	4.53	7.50 7.39	6.69 6.57	-	-	-	2.34 2.24
1.90	6.51	8.64	4.53	7.51 7.38	6.69 6.57	-	-	-	2.34 2.25
3.04	6.61	8.65	4.61	7.60 7.49	6.78 6.66	8.16	-	1.99	2.43 2.34
4.60	6.52	8.65	4.52	7.50 7.38	6.69 6.57	8.01	-	1.92	2.33 2.23
9.10	6.62	8.68	4.59	7.54 7.44	6.74 6.63	8.06	-	2.02	2.39 2.28
13.70	6.66	8.65	4.61	7.58 7.47	6.77 6.66	8.11	4.46	2.04	2.44 2.34
22.70	6.71	8.65	4.64	7.44 7.49	6.80 6.68	8.11	4.47	2.07	2.44 2.35
45.30	6.73	8.79	4.66	7.63 7.54	6.83 6.74	8.15	4.50	2.08	2.49 2.39
67.90	6.75	8.64	4.69	7.67 7.55	6.85 6.74	8.17	4.34	2.08	2.50 2.43

TABLE V - The Effect Of Concentration On ^{1}H Chemical Shifts (In ppm) Of Folic Acid In $^{2}H_{6}$- Dimethylsulphoxide. Samples Run At Fixed Temperature, 27.5 °C.

Protons / Temp. °C	7	9	2',6'	3',5'	13	14	15
27.5	8.64	4.48	7.69 7.60	6.68 6.59	4.34	2.02	2.38 2.30
40.0	8.65	4.49	7.70 7.61	6.70 6.60	4.37	1.99	2.36 2.25
50.0	8.63	4.46	7.68 7.58	6.68 6.58	4.33	1.96	2.30 2.23
58.0	8.65	4.50	7.70 7.61	6.71 6.61	4.39	1.99	2.36 2.25
70.0	8.69	4.54	7.74 7.64	6.76 6.66	4.44	2.06	2.41 2.30
80.0	8.63	4.48	7.68 7.60	6.69 6.61	4.37	2.08	2.31 2.24
89.0	8.63	4.48	7.76 7.69	6.70 6.61	4.39	2.03	2.35 2.24

TABLE VI - The Effect Of Temperature On ^{1}H Chemical Shifts (In ppm) Of Folic Acid In $^{2}H_{6}$-Dimethylsulphoxide. Concentration Of Folic Acid Fixed At 68mM.

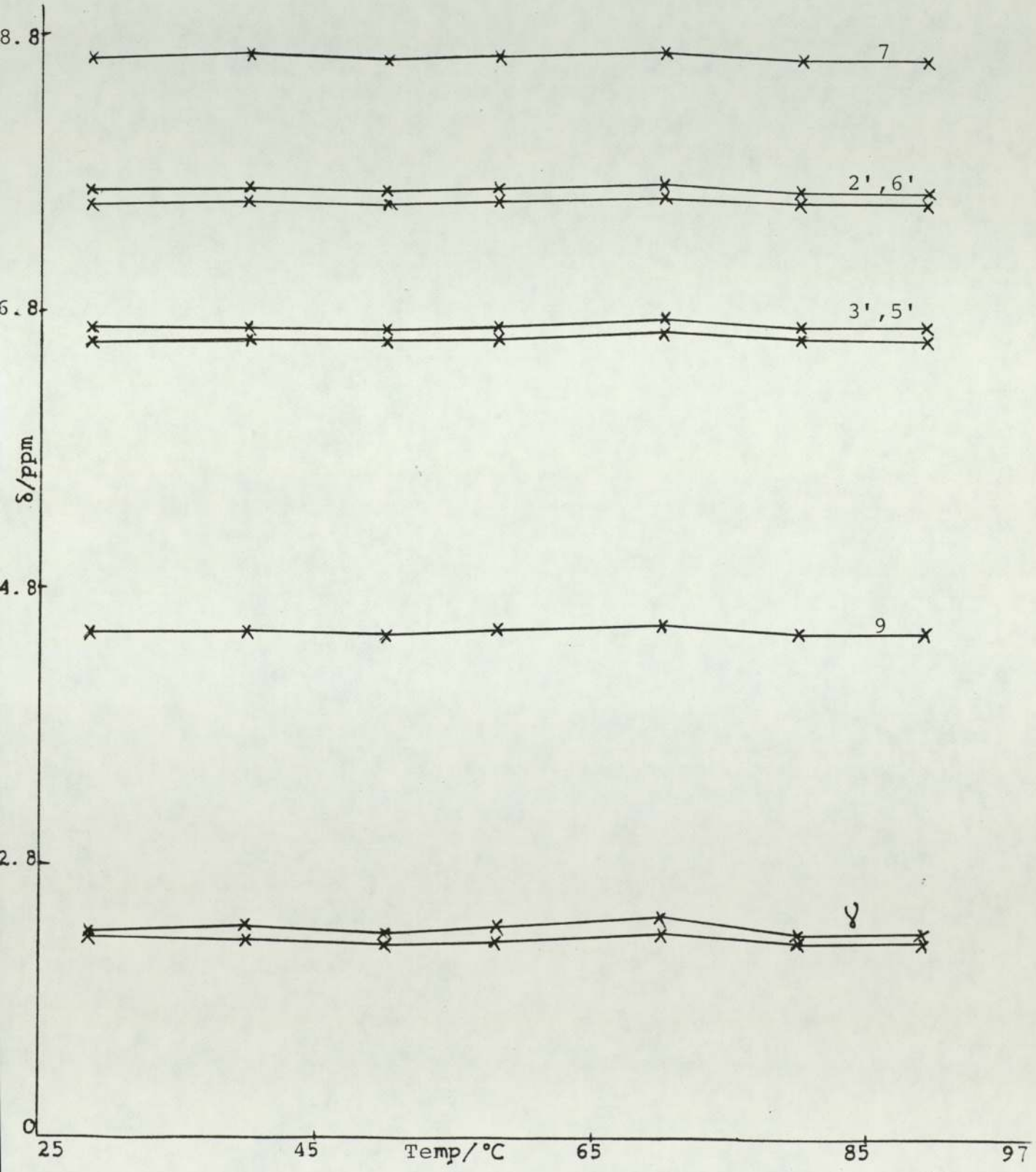


GRAPH VII - Graph Of Folic Acid Chemical Shifts Versus Concentration.

Scale: x axis 1cm = 4mM
y axis 1cm = 0.4ppm

GRAPH VIII - Graph Of Folic Acid Chemical Shifts Versus Temperature.

Scale: x axis 1cm = 0.4ppm
y axis 1cm = 4mM



Protons / Folates	7	9	2'	6'	3', 5'
FA	8.65	4.51&4.46	7.70&7.61		6.69&6.59
N(10)-NO	8.70	5.48	8.10&8.00		7.93&7.83
N(10)-Me	8.47	4.77	7.75&7.66		6.82&6.74
N(10)-CHO	8.62	5.20	7.85&7.94		7.52&7.62
N(10)-CH_3CO	8.62	5.03	7.93&7.84		7.57&7.48
3'-I	8.62	4.60&4.65	8.26	7.76&7.66	6.66&6.56
3'-F	8.65	4.58&4.52	7.66&7.50	7.52	6.80,6.73,6.63&6.53
2'-F	8.66	4.51&4.45	7.59,7.50&7.40		6.56 - 6.36
N(10)-CHO-2'-F	8.83	5.18	7.53,7.45&7.26		8.27,7.77&7.67
N(10)-CHO-3'-F	8.63	5.10	All These Protons Located Between δ 7.91&7.65		
MTX	8.57	4.78	7.78&7.68		6.87&6.78
α-^{2}H-MTX	8.58	4.80	7.78&7.69		6.88&6.78
AMT	8.72	4.50	7.76&7.66		6.80&6.70

Protons / Folates	2-NH_2	12	13	14	15	MISCELLANEOUS
FA	6.89	8.19&8.10	4.33	2.00	2.25&2.32	
N(10)-NO	7.33	8.19&8.08	4.54	2.03	2.31&2.38	
N(10)-Me	6.94	8.18&8.10	4.37	2.14	2.31&2.36	Me - 3.18
N(10)-CHO	6.90	8.55&8.62	4.54	1.99	2.27&2.35	CHO - 8.79
N(10)-CH_3CO	7.11	8.68&8.59	4.36	2.07	2.34	CH_3CO - 1.91
3'-I	6.90	8.35&8.26	4.32	1.95	2.27&2.32	10-H - 6.29
3'-F	6.93	8.29&8.19	4.34	1.93	2.23&2.32	
2'-F	7.11	7.86,7.76&7.69	4.32	1.99	2.21&2.28	10-H - 7.18,7.26& 7.32
N(10)-CHO-2'-F	7.19	8.05,8.12&8.17	4.18	1.98	2.20&2.28	CHO - 8.62
N(10)-CHO-3'-F	7.43	8.75&8.84	4.37	2.05	2.29&2.37	CHO - 8.46&8.49
MTX	6.61	8.13&8.22	4.23	2.01	2.26&2.32	Me - 3.21 4-AMINO -7.58
α-^{2}H-MTX	6.71	8.20	-	2.00	2.25&2.33	Me - 3.21 4-AMINO - 7.90
AMT	6.80	8.11&8.20	4.35	1.96	2.26&2.34	4-AMINO - 7.95

TABLE IX - ^{1}H Chemical Shifts Of Folates in $^2H_6$-Dimethylsulphoxide. Chemical Shifts Given In ppm Downfield From Internal Tetramethylsilane(TMS).

Our experiments were conducted in $^{2}H_{6}$-DMSO. Since this solvent is more hydrophobic than water, it is possible that self-association of folates in $^{2}H_{6}$-DMSO is less energetically favourable than in water. The amount of stabilization energy derived from the solvation by DMSO molecules or the adoption of new conformations may have been greater than the energy derived from vertical coplanar stacking of the two aromatic rings.

Poe (1973) proposed a fully stretched-out conformation for the folic acid molecules which dimerized in aqueous solution. A head-to-tail arrangement was suggested, with the pteridine ring of one molecule vertically stacked in a coplanar manner with the benzene ring of the other molecule and the two glutamate side chains as far away from one another as possible. As a result of their size and the flexibility of the unsaturated bonds, folates could exist in a large number of conformations. In the absence of dimerization, the individual molecules could adopt a completely-folded conformation where the benzene ring and pteridine rings are almost coplanar. This and the fully stretched-out conformation represent two extremes. The exact conformation would be governed by a large number of factors including temperature, concentration, pH, solvent, steric and electronic properties of the substituents.

II PROTON CHEMICAL SHIFTS OF FOLATES

The proton chemical shifts of folic acid and its analogues have been tabulated in table 4 IX. These spectra can be conveniently divided into three regions. The low field region - between 6 and 8 ppm - contain the resonances of 7(H) (usually a sharp singlet),

the benzene ring protons, the two amino groups and the amide H(12). In the high field area - from 1.9 to 3.5 ppm - we find the glutamate protons (γ and β) and the methyl groups of substituents. Located between these two areas (from around 4 to 6 ppm) are the methylene bridge protons and the α multiplet from the glutamate side chains.

The amide protons, H(12), were usually split into doublets as a result of coupling with the adjacent α protons. Coupling constants varied between 2 and 2.5 Hz. In the 2' substituted folates, H(12) was split into a triplet, most likely the result of coupling with the 2'-fluorine. Since these nuclei are separated by four bonds, ^{1}H-^{19}F coupling must have occurred via a "through-space" mechanism. Model building experiments have demonstrated that these nuclei could be made to approach one another to within 3Å. Long range spin-spin coupling between protons and fluorine by "through-space" mechanisms has been well-documented (Hulton and Sutcliffe, 1975; Gribble and Kelly, 1985). Yamamoto and Ōki (1985) have recently demonstrated "through-space" ^{19}F-^{1}H coupling over a distance of 2.7Å.

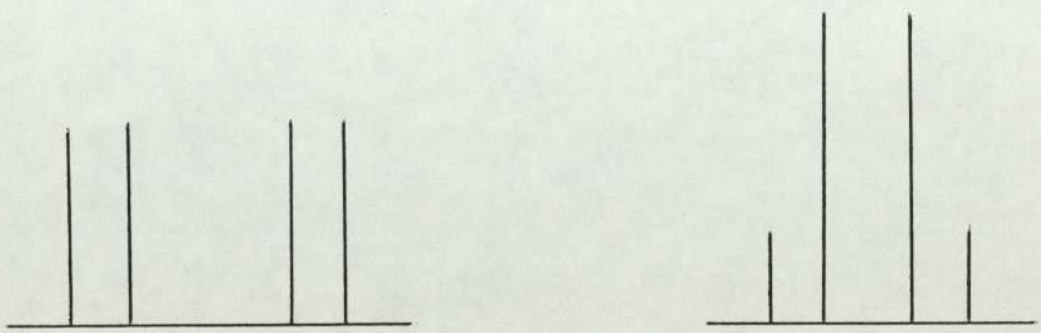


Figure D I The appearences of two doublets in the absence (left) and presence (right) of AB interaction.

RESULTS

In the N(10) modified folates, benzene ring protons appeared as two doublets with coupling constants of around 9 Hz. Since δ_A-δ_B (the chemical shift difference between the 2',6' and 3',5' protons) were of the same magnitude as J_{AB} (the coupling constants between the 2',6' and 3',5' protons), these resonances formed an AB pair. The intensities of the 'inside' peaks have been enhanced at the expense of the intensities of the 'outside' peaks (figure D 1).

Splitting patterns in the benzene ring regions of some of the 2' and 3'-fluorinated folates were extremely complicated. In addition to proton-proton coupling, the spectra also showed significant coupling between fluorine and all the protons of the benzene ring. Assignments were made on the basis of comparison with spectra of intermediates in the synthesis of these folates and by selective irradiation experiments.

Addition of a drop of ^2H_2O to the dimethylsulphoxide solution of 3'-iodofolic acid resolved the peaks formed from the coupling between the 2' and 6' protons of the benzene ring. We measured a coupling constant of 2 Hz.

Resonances belonging to 9(2H) in the benzene ring substituted folates showed partially resolved doublets with coupling constants of about 4.5 Hz. These resonances collapsed into singlets when ^2H_2O was introduced into the solution. Singlets were also observed for 9(2H) in N(10)-substituted folates. These observations rule out the possibility of the 9(2H) doublet being due to magnetically inequivalent protons but suggest that these doublets were the result of 9(2H)-N(10)H coupling. Failure to detect N(10)H in the NMR spectra of these folates could be due to various factors, eg. extremely broadened signals, signals located at very low field or buried under other

resonances.

The α and β protons of the glutamate side chains appeared as complex multiplets. The γ proton, however, were doublets in all the spectra recorded.

III ^{13}C CHEMICAL SHIFTS OF FOLATES

The carbon-13 chemical shifts of folic acid and its analogues have been tabulated in table X.

The ^{13}C spectra of folic acid and its N(10)-substituted analogues gave up to twenty resonances, depending of the types of N(10) groups. In the benzene ring halogenated folates, equivalence of the 2',6' and 3',5' protons have been destroyed. In addition, coupling also occured between the fluorine and the ipso, ortho and meta carbon-13 nuclei. Consequently, these folates contained more ^{13}C resonances than the N(10)-substituted compounds.

^{13}C spectra could conveniently be divided into low and high field regions. No signals were found between 45 and 80 ppm. Resonances from carbons 9, α, β, γ and the septet formed from ^{13}C-^{2}H coupling in the methyl groups of the solvent were found in the high field region. Carbons 9 and α were low field with respect to the septet whilst carbons β and γ were to high field. Assignments were made on the basis of off-resonance decoupling and by comparison with spectra of other compounds.

FOLATES \ ^{13}C	2	4	4a	6	7	8a	9	1'
FA	153.65	160.91	127.81	148.40	148.40	156.28	45.78	121.15
N(10)-NO	153.60	159.99	129.22	143.79	148.83	154.36	46.70	132.42
N(10)-Me	153.71	160.86	128.14	147.81	147.81	156.09	54.78	121.04
N(10)-CHO	153.76	160.64	127.92	145.47	148.56	156.29	46.60	130.90
N(10)-CH_3CO	153.93	161.08	-	146.23	145.26	-	51.90	132.74
3'-I	153.66	160.75	127.65	147.48	148.33	156.14	46.16	123.26
3'-F	153.71	160.80	127.70	147.86	148.45	155.06	45.46	121.20&120.93
2'-F	148.51	163.30	127.81	147.81	148.51	153.60	45.57	109.17&108.58
N(10)-CHO-2'-F	148.07	162.38	127.92	145.15	148.56	153.76	46.38	119.17&119.34
N(10)-CHO-3'-F	153.70	160.17	127.81	145.09	148.56	154.40	47.57	134.26&133.99
MTX	162.59	145.91	122.07	162.60	149.04	154.03	54.78	120.98
α-^2H-MTX	162.21	146.18	121.31	162.54	148.99	154.41	54.72	121.04
AMT	162.70	161.83	120.82	146.18	149.43	153.81	45.57	121.31

FOLATES \ ^{13}C	2'	6'	3'	5'	4'	11
FA	128.84		111.07		150.62	166.23
N(10)-NO	128.68		119.47		143.47	165.52
N(10)-Me	128.73		110.91		150.73	166.06
N(10)-CHO	128.57		121.63		143.25	165.58
N(10)-CH_3CO	128.52		127.76		150.68	165.58
3'-I	138.27	129.14	83.33	109.50	149.21	164.81
3'-F	113.01&113.89	110.74	144.50&153.68	124.67	138.54&139.08	165.20
2'-F	155.93&166.88	131.39	97.25&98.64	108.30	152.03&153.63	160.75
N(10)-CHO-2'-F	156.20&167.62	130.95	108.90&110.02	117.41	144.12&144.60	160.74
N(10)-CHO-3'-F	115.07&116.00	128.24	150.54&161.56	124.13	130.36&130.90	164.33
MTX	128.79		110.91		150.84	166.17
α-^2H-MTX	128.79		110.96		150.84	166.17
AMT	128.84		111.23		150.62	166.28

TABLE X - ^{13}C Chemical Shifts Of Folates In $^2H_6$-Dimethylsulphoxide. Chemical Shifts Given In ppm Downfield From Internal TMS. Table X Continued On Following Page.

FOLATES \ ^{13}C	13	14	15	16	17	MISCELLANEOUS
FA	51.63	25.60	30.34	173.75	173.60	
N(10)-NO	51.90	25.79	30.29	173.65	173.10	
N(10)-Me	51.80	26.11	30.50	173.86	173.70	N(10)-Me 38.96
N(10)-CHO	51.85	25.79	30.29	173.70	173.16	N(10)-CHO 162.43
N(10)-CH_3CO	51.43	25.92	30.40	173.70	173.16	Acetyl Me 22.32, Acetyl CO 169.73
3'-I	51.69	25.84	30.29	173.65	173.43	
3'-F	51.74	25.84	30.29	173.70	173.43	
2'-F	51.63	26.06	30.02	173.65	173.05	
N(10)-CHO-2'-F	52.28	26.44	30.04	173.18	173.28	N(10)-CHO 165.36
N(10)-CHO-3'-F	51.80	25.57	30.12	173.48	173.72	N(10)-CHO 162.81
MTX	51.63	25.95	30.34	173.70	173.54	N(10)-Me 40.15
α-^{2}H-MTX	50.93	25.84	30.29	173.75	173.53	N(10)-Me 39.01
AMT	51.63	25.95	30.34	173.75	173.60	

TABLE X(Continued) - ^{13}C Chemical Shifts Of Folates In $^{2}H_6$-Dimethylsulphoxide.

^{13}C / FOLATES	2	4	4a	6	7	8a	9	1'	4'
FA	153.65	160.91	127.81	148.40	148.40	156.20	45.78	121.51	150.62
N(10)-NO	0.06	0.92	-1.41	4.60	-0.43	1.92	-0.92	-11.27	7.15
N(10)-Me	-0.05	0.05	-0.33	0.59	0.59	0.19	-9.00	0.11	-0.10
N(10)-CHO	-0.10	0.27	-0.11	2.92	-0.16	0.14	-0.81	-9.75	7.37
N(10)-CH_3CO	-0.11	-0.17	-	2.17	3.14	-	-6.12	-11.59	-0.06
3'-I	-0.01	0.16	0.16	0.92	0.07	0.14	-0.38	-2.11	1.41
3'-F	-0.06	0.11	0.11	0.54	-0.05	1.22	0.32	0.08	11.81
2'-F	5.14	-2.39	0.00	0.59	-0.11	2.68	0.21	12.28	-1.71
N(10)-CHO-2'-F	5.58	-1.47	-0.11	3.25	-0.16	2.52	-0.16	1.50	6.26
N(10)-CHO-3'-F	-0.05	0.74	0.00	3.31	-0.16	1.88	-1.79	-12.98	19.99
MTX	-8.94	15.00	5.74	-14.20	-0.64	2.25	-9.00	0.17	-0.22
α-^{2}H-MTX	-8.56	14.73	6.50	-14.14	-0.59	1.87	-8.94	0.11	-0.22
AMT	-9.05	-0.92	6.99	2.22	-1.03	2.47	0.21	-0.16	0.00

^{13}C / FOLATES	2'	6'	3'	5'	11	13	14	15	16	17
FA	128.84		111.07		166.23	51.63	25.90	30.34	173.15	173.60
N(10)-NO	0.16		-8.40		0.71	-0.27	0.11	0.05	0.10	0.49
N(10)-Me	0.11		0.16		0.16	-0.16	-0.22	-0.16	-0.11	-0.10
N(10)-CHO	0.27		-10.59		0.65	-0.22	0.11	0.05	0.05	0.43
N(10)-CH_3CO	0.32		-16.69		0.65	0.20	-0.02	-0.06	0.05	0.44
3'-I	-9.43	-0.30	27.74	1.57	1.42	-0.06	0.06	0.05	0.10	0.17
3'-F	15.39	18.10	-38.02	-13.60	1.03	-0.11	0.06	0.05	0.05	0.17
2'-F	-32.56	-2.55	13.12	2.77	5.48	0.00	-0.16	0.32	0.10	0.55
N(10)-CHO-2'-F	-33.07	-2.11	1.61	-6.34	5.59	-0.65	-0.54	-0.06	-0.06	-0.18
N(10)-CHO-3'-F	13.30	0.60	-44.98	-13.06	1.90	-0.17	0.33	0.22	0.27	-0.12
MTX	0.05		0.16		0.06	0.00	-0.05	0.00	0.05	0.06
α-^{2}H-MTX	0.05		0.11		0.06	0.70	0.06	0.05	0.00	0.07
AMT	0.00		-0.16		-0.15	0.00	-0.05	0.00	0.00	0.00

TABLE XI - Changes In ^{13}C Chemical Shifts Of Folates With Respect To Folic Acid. The sign convention used in designating chemical shift changes are based on an absolute scale of nuclear screening where increases in shielding are given as positive values. This convention is opposite to that commonly found in the literature.

^1H / FOLATES	7	9	2'	6'	3'	5'	2-NH_2
FA	8.65	4.46&4.51	7.61&7.70		6.59&6.69		6.89
N(10)-NO	-0.05	-1.00	-0.40&-0.40		-1.24&-1.24		-0.35
N(10)-Me	0.18	-0.29	-0.05&-0.06		-0.14&-0.15		-0.05
N(10)-CHO	0.03	-0.72	-0.25&-0.24		-0.93&-0.93		-0.11
N(10)- CH_3CO	0.02	-0.54	-0.23&-0.23		-0.88&-0.88		-0.22
3'-I	0.03	-0.14 & -0.14	-0.06	-0.05		0.03	-0.01
3'-F	0.00	-0.07& -0.06	0.11	0.14	-	-0.03	-0.04
2'-F	-0.01	0.00 & 0.01	-	0.16	0.18		-0.22
N(10)-CHO-2'-F	-0.18	-0.70	-	0.24	-1.26		-0.30
N(10)-CHO-3'-F	0.02	-0.62	0.13		-	-1.14	-0.54
MTX	0.08	-0.30	-0.08&-0.08		-0.18&-0.19		0.28
α-^2H-MTX	0.06	-0.31	-0.08&-0.08		-0.19&-0.19		0.18
AMT	-0.08	-0.02	-0.06&-0.06		-0.11&-0.11		0.09

^1H / FOLATES	12	13	14	15
FA	8.10&8.19	4.33	2.00	2.25&2.32
N(10)-NO	0.00&0.01	-0.21	-0.03	-0.06&-0.06
N(10)-Me	0.01&-0.01	-0.04	-0.14	-0.04&-0.06
N(10)-CHO	-0.43&-0.45	-0.21	0.01	-0.02&-0.03
N(10)-CH_3CO	-0.49&-0.49	-0.03	-0.07	-0.06
3'-I	-0.16&-0.16	0.01	0.05	-0.01&-0.02
3'-F	-0.10&-0.09	-0.01	0.07	0.00&0.02
2'-F	0.37	0.01	0.01	0.04&0.04
N(10)-CHO-2'-F	0.03	0.15	0.02	0.04&0.04
N(10)-CHO-3'-F	-0.65&-0.65	-0.04	-0.05	-0.05&-0.04
MTX	-0.03&-0.03	0.10	-0.01	0.00&0.01
α-^2H-MTX	-0.05	-	0.01	-0.01&0.00
AMT	-0.01&-0.01	-0.02	0.04	-0.02&-0.01

TABLE XII - Changes In ^1H Chemical Shifts Of Folates With Respect To Folic Acid. The sign convention used to designate chemical shift changes in this table is the same as that used in Table XI.

The carbonyl carbons from the α and γ carboxylic acid groups formed the low field extreme in ^{13}C NMR of folates. These resonances normally occur close to one another. Signals belonging to other carbonyl carbons were located a little upfield from these resonances. The other benzene and pteridine ring carbons were found further upfield. Carbons 4a and 8a were easily identifiable from their chemical shifts and intensities - these were usually the smallest signals in the spectra. Proton bearing carbons usually gave the most intense signals because of nuclear Overhauser enhancement (NOE) derived from ^{13}C-^{1}H decoupling.

IV INTERPRETATION OF CHANGES IN PROTON AND ^{13}C CHEMICAL SHIFTS OF SUBSTITUTED FOLATES

To facilitate study of the effects of substituents on the proton and ^{13}C chemical shifts, tables of changes in these resonances with respect to the corresponding nuclei in folic acid have been included in tables XI and XII.

One has to be extremely cautious when interpreting chemical shift changes. It is easy to attribute large changes remote from the point of substitution to conformational change or 'through-space' steric and anisotropic effects of the substituents. However, changes in the vicinity of the modification may be due to a combination of the above factors and the electron-donating or withdrawing properties of the substituents. 'Ring-current' effects from the benzene and pteridine rings make interpretation of the data in tables XI and XII extremely difficult. Even minor conformational change as a result of substitution could cause nuclei in the vicinity of the two rings to experience

large chemical shift changes. Further complicating factors in the interpretation of chemical shift changes include the 'through-space' influences of magnetically anisotropic N(10)-acetyl, formyl and nitroso groups.

Table XII showed large changes in protons located far away from the point of substitution in N(10)-formyl, N(10)-acetyl and N(10)-formyl-3'-fluorofolates. H(12) was deshielded by 0.4 to 0.6 ppm in these compounds. However, in N(10)-formyl-2'-fluorofolic acid, this proton was shielded by 0.03 ppm. Examination of the 2'-fluorofolic acid spectrum revealed that this proton was shielded by 0.37 ppm. It is possible that in the corresponding formylated compound, the shielding effects of the 2'-fluorine has overridden the deshielding effects of the N(10)-formyl groups. In these 2'-fluorinated folates, it was easy to account for chemical shift changes at H(12) by citing 'through-space' or possibly even 'through-bond' effects of the 2'-fluorine. These reasons, however, are not applicable in the N(10) substituted folates. It is unlikely that electron-withdrawing acetyl and formyl groups could transmit their effects to H(12) via the σ electrons of the benzene ring as neither the electron-withdrawing nitroso nor the electron-donating methyl groups had any effect on H(12). The possibility of the magnetically anisotropic carbonyl groups directly affecting H(12) by 'through-space' interactions can be ruled out because of the great distances separating them.

These large changes in H(12) can be explained if we assumed that these folates existed in a completely folded conformation in dimethylsulphoxide. The benzene ring would be bent over the pyrazine ring and the amide bond located over the pyrimidine ring. Thus H(12) would be under the influence of the π-cloud from the pyrimidine ring. Assuming that formylation or acetylation at N(10) had altered the conformation of these molecules with respect to folic acid, then movement of H(12) relative to the pyrimidine

ring would affect its chemical shift.

Changes at H(12) could also be explained without invoking a 'bent' folate. Movement of the C(11)-N(12) bond relative to the benzene ring would affect the orientation of H(12) with respect to the electron cloud and alter its chemical shift.

The lack of change observed in H(12) of N(10)-methylfolic acid may be because of the absence of significant conformation change with respect to folic acid. Alternatively, it may also have resulted from the proton being moved into a new environment where, coincidentally, it is experiencing the same amount of shielding as H(12) of folic acid.

Introduction of electron-withdrawing substituents at N(10) caused large chemical shift changes in benzene-ring protons whilst halogenation of the benzene ring did not have large effects on these protons. In the N(10)-substituted compounds, the 3',5' protons were generally affected to a greater extent than the 2',6' protons. These changes are partially due to electronic effects - the 3',5' protons being ortho to the point of substitution whilst the 2',6' protons were in a meta position. However, 'through-bond' effects alone could not have brought about such large changes. Now, assuming a 'bent' folate, the 3',5' protons would be closer to the pyrazine ring. Movement of these two rings relative to each other should affect these protons to a greater extent than the 2',6' protons. Some of the chemical shift changes could also be due to anisotropy of the C=O and N=O functionalities in the substituents. This would also explain the absence of large changes for the benzene ring protons of N(10)-methylfolic acid.

With the exception of H(2') in 3'-iodofolic acid, the aromatic protons of our folates were not significantly perturbed by halogenation of the benzene ring. It seemed unlikely that halogenation had only perturbed these protons to such a small extent. It is possible that the effects of halogenation were nullified by opposing effects arising from

conformational change. The large change observed for H(2') in 3'-iodofolic acid may be evidence of these two factors not cancelling each other. It may also be because of H(2') being sited ortho to the large iodine nucleus.

The methylene bridge protons, 9(2H), experienced larger changes in the N(10)-substituted compounds than in the benzene ring halogenated folates. The distance of 9(2H) from the benzene ring meant that electronic effects could not be significantly transmitted through the σ bonds. Influences from the π cloud of the benzene ring, assuming a completely folded conformation, were not expected to be strong due to its distance from these protons. The large changes experienced by 9(2H) in the N(10) substituted folates were most likely caused by electronic effects transmitted through the σ electrons of the C(9)-N(10) bond, the magnetic anisotropy of the N(10) groups and steric effects arising from their bulk. 'Ring current' contributions from the π cloud of the pteridine ring may also have affected chemical shifts at 9(2H), depending on the amount of rotation about the C(6)-C(9) bond in the substituted folates relative to folic acid. It was interesting to note that both electron donating and withdrawing groups deshielded 9(2H).

Modification to the folate molecule had little effect on 7(H). 'Through-bond' effects should be small because 7(H) was separated from the substituents by at least three bonds. Assuming a 'bent' folate, 'ring-current' effects from the benzene ring should be small because of its distance from 7(H). In this conformation, it would have been possible for N(10) and 3'- substituents to influence chemical shifts at 7(H) because of their proximity in space. Absence of such changes could either mean that a 180^{o} rotation had occured at the C(6)-C(9) bond to place 7(H) on the opposite side of these

substituents or that folates do not exist in a bent conformation in $^{2}H_{6}$-DMSO.

It was difficult to draw conclusions from chemical shifts of the 2-amino group due to their sensitivity to a large number of factors. These include methods of working-up the folates (ie. whether they were washed in distilled water or dilute acids prior to drying), the types of acids used and the presence of impurities.

Chemical shifts of the α, β and γ protons of the glutamate side chain were not greatly affected by modification. Large changes were experienced by the α-protons in the N(10)-nitroso, N(10)-formyl and N(10)-formyl-2'-fluorofolate but these were exceptions as all the other protons were affected by no more than 0.04 ppm.

We have tried to attribute changes in proton chemical shifts to small conformational change in a completely folded folate. By linking these changes to changes in ^{13}C chemical shifts one should be able to get a clearer picture of the effects of modification and the actual folate conformation in $^{2}H_{6}$-dimethylsulphoxide.

In table XI, we see that N(10) substitution had caused large changes in the ^{13}C chemical shifts at C(1') and smaller changes at C(11) in all compounds except N(10)-methylfolic acid. It seem unlikely that changes of this magnitude (0.65 to -11.59 ppm) could be transmitted through the σ bonds of the benzene ring. Movement of these nuclei relative to the π cloud of the pteridine ring in a bent folate could, however, affect chemical shifts to such a large extent.

Changes experienced by ^{13}C nuclei in N(10)-methylfolic acid were generally smaller than those of corresponding nuclei in other folates. One major exception was C(9), which was deshielded by 9 ppm. It appears likely that this was due to

'through-space' and 'through-bond' effects of the methyl group. Chemical shift changes in carbons 4' and 3',5' also differed from corresponding nuclei in the other N(10)-modified folates, ie. these ocurred in opposite directions. These observations were not unexpected as one would expect substituents to exercise profound influence on chemical shifts of nuclei in their vicinity - the other N(10)-modified folates contained electron-withdrawing substituents.

Assuming that chemical shift changes in nuclei remote form N(10) were due to conformational changes in bent folates, the small changes in the ^{13}C nuclei of N(10)-methylfolate would mean that the molecule did not experience large conformational change as a result of modification. This proposition would agree with conclusions drawn from proton NMR data.

Data from table XI also suggest a bent conformation for the halogenated folates. The results show that halogenation had affected C(6). The effects of the halogens could not have been transmitted through the methylene bridge. However, in a 'bent' folate, C(6) could be influenced by the π cloud of the benzene ring. Moreover, 3' substituents would also be close enough to have 'through-space' steric effects on the chemical shifts at C(6).

Changes experienced by C(8a) in the halogenated compounds could be attributed to movement of the benzene and pteridine rings relative to each other. However, as in the case of C(6), these changes could have arisen from 'through-space' effects of the 2'- and 3'- substituents in a folded folate. Thus, although these results suggest a 'bent' folate, they do not provide conclusive evidence that halogenation had caused conformational changes in these molecules.

Carbons 2, 4, 8a and 11 in the formylated 2' and 3'-fluorofolates had chemical shifts which differed from the corresponding nuclei of the unformylated compounds. In a folded molecule, these carbons would be too remote to be directly affected by the formyl groups via 'through-bond' or 'through-space' effects. The observations suggest that formylation had caused relative movement between the benzene and pteridine rings.

The above arguments were based on the supposition that folic acid and its analogues had a 'bent' conformation in $^{2}H_{6}$-DMSO. Modifications at N(10) on the benzene ring did not appear to have caused large scale conformational change for this would have resulted in the majority of the nuclei in the analogues having chemical shifts radically different from those of folic acid. It would be possible to account for some chemcial shift changes by proposing other conformations for the folates (eg. less 'tightly-folded' conformations or N(10)-substituents lying over the pyrazine ring). Our proposal was based on the expectation that stabilization energy derived from vertical, coplanar stacking of pteridine and benzene rings would offset destabilizing factors like steric congestion or possible loss of entropy. Less tightly folded structures were rejected because it would be more difficult to account for chemical shift changes remote from the points of substitution. Moreover, less stabilization energy would be derived from the interactions between the two ring systems of folates having this conformation.

In summary, the NMR results were too incomplete to allow us to draw definite conclusions on folate conformation in $^{2}H_{6}$-dimethylsulphoxide. It was impossible to utilize charts of chemical shift changes induced in protons by π-clouds - the so-called ring-current effects [Johnson & Bovey, 1958; Emsley *et al.*, 1965; Bovey, 1969; Haigh & Mallion, 1972] - because of the presence of two substituted ring systems and the lack

COMPOUNDS / ^1H	3'	5'	6'	Me	α	β	γ	α-^{t}Bu	γ-^{t}Bu	Amide	4-NH_2
2-Fluoro-4-Nitrotoluene	7.95, 8.05		7.51, 7.60, 7.68	2.35, 2.38							
2-Flouro-4-Nitrobenzoic Acid	7.99-8.24										
Di-^{t}Butyl-2-Fluoro-4-Nitrobenzoylglutamate	8.92, 9.01	8.11-8.29			4.42	1.99	2.28, 2.36	1.41	1.44	7.72-7.90	
Di-^{t}Butyl-2-Fluoro-4-Aminobenzoylglutamate	6.23-6.43		7.32, 7.42, 7.52		4.33	1.96	2.21, 2.29	1.38	1.41	7.69, 7.76, 7.96	5.97
Di-^{t}Butyl-2-Acetyl-2'-Fluorofolic Acid	6.40-6.59		7.38, 7.47, 7.57		4.29	1.93	2.21, 2.26	1.36	1.39	7.72, 7.77	

Di-^{t}Butyl-2-Acetyl-2'-Fluorofolic Acid	2.21	8.88	4.62	7.42
	Me of 2-Acetyl	7	9	Amide of 2-Acetyl

TABLE XIII - ^1H Chemical Shifts Of Intermediates In The Synthesis Of 2'-Fluorofolic Acid. Spectra recorded in dimethylsulphoxide. Numbering of nuclei based on the system used for folates eg. in 2-fluoro-4-nitrotoluene, 6' denotes the proton attached to position 6 of the benzene ring.

^1H / COMPOUNDS	2'	5'	6'	Me	α	β	γ	α-^{t}Bu	γ-^{t}Bu	Amide	4-NH_2
3-Fluoro-4-Nitrotoluene	7.50, 7.35	7.96, 8.06, 8.15	7.23, 7.32	2.43							
3-Fluoro-4-Nitrobenzoic Acid	7.42 - 8.35										
Di-^{t}Butyl-3-Fluoro-4-Nitrobenzoylglutamate	7.31, 7.42, 7.53	8.20, 8.29, 8.38	7.85, 7.94, 8.06		4.36	2.03	2.30, 2.38	1.39	1.41	8.96, 9.05	
Di-^{t}Butyl-3-Fluoro-4-Aminobenzoylglutamate	7.46-7.57	6.65, 6.75, 6.85	7.64		4.26	1.92	2.23, 2.32	1.39	1.40	8.15, 8.22	5.72
Di-^{t}Butyl-2-Acetyl-3'-Fluorofolic Acid	7.50-7.53	6.64, 6.74, 6.84	7.58, 7.68		4.18	2.00	2.22, 2.30	1.38		8.20, 8.29	
2-Acetyl-3'-Fluorofolic Acid	7.52, 7.55	6.64, 6.74, 6.84, 6.94	7.59, 7.68		4.36	1.98	2.21, 2.29	-	-	8.22, 8.31	

	Me of 2-Ac	7	9	Amide of 2-Ac
Di-^{t}butyl-2-Acetyl-3'-Fluorofolic Acid	2.21	8.82	4.67	6.95
2-Acetyl-3'-Fluorofolic Acid	2.21	8.88	4.64	6.88

TABLE XIV - Proton Chemical Shifts Of Intermediates In The Synthesis Of 3'-Fluorofolic Acid. For explanation of numbering system, refer to Table XIII.

^{13}C / COMPOUNDS	1'	2'	3'	4'	5'	6'	Me
2-Fluoro-4-Nitrotoluene	146.56, 146.18	154.20, 165.20	109.66, 110.91	133.39	119.09, 119.20	132.14, 132.36	14.14, 14.30
2-Fluoro-4-Nitrobenzoic Acid	125.05, 125.54	154.46, 166.01	111.99, 113.24	150.02; 150.46	119.14, 119.31	132.96	
Di-^{t}Butyl-2-Fluoro-4-Nitrobenzoylglutamate	129.71, 130.41	152.79, 164.06	111.34, 112.53	148.89, 149.26	119.58	130.90, 131.06	
Di-^{t}Butyl-2-Fluoro-4-Aminobenzoylglutamate	108.52, 107.83	155.95, 166.88	98.44, 99.64	153.22, 153.81	109.28	131.50, 131.66	
Di-^{t}Butyl-2-Acetyl-2'-Fluorofolic Acid	108.86, 109.68	155.67, 166.77	96.04, 97.36	151.92, 152.46	108.36	131.49	

	Amide	α	β	γ	Me of α-t_{Bu}	Me of γ-t_{Bu}	4° C of α-t_{Bu}	4° C of γ-t_{Bu}
Di-^{t}Butyl-2-Fluoro-4-Nitrobenzoylglutamate	162.60	52.39	25.84	30.99	27.52	27.63	79.75	80.84
Di-^{t}Butyl-2-Fluoro-4-Aminobenzoylglutamate	163.46, 163.57	52.18	26.11	31.04	27.58		79.65	80.56
Di-^{t}Butyl-2-Acetyl-2'-Fluorofolic Acid	160.01	52.12	26.01	31.05	27.58		79.64	80.62

^{13}C / COMPOUNDS	COOH	CO of α-COOH	CO of γ-COOH	Me of 2-Ac	CO of 2-Ac
2-Fluoro-4-Nitrobenzoic Acid	163.51, 163.63				
Di-^{t}Butyl-2-Fluoro-4-Nitrobenzoylglutamate		170.18	171.26		
Di-^{t}Butyl-2-Fluoro-4-Aminobenzoylglutamate		170.83	171.43		
Di-^{t}Butyl-2-Acetyl-2'-Fluorofolic Acid		170.72	171.43	23.89	173.64

	2	4	4a	6	7	8a	9
Di-^{t}Butyl-2-Acetyl-2'-Fluorofolic Acid	149.81	163.41	130.36	151.32	148.94	154.52	45.67

TABLE XV - ^{13}C Chemical Shifts Of Intermediates In The Synthesis Of 2'-Fluorofolic Acid. Spectra recorded in d_6-dimethylsulphoxide. For explanation of numbering system, refer to Table XIII.

^{13}C / COMPOUNDS	1'	2'	3'	4'	5'	6'	Me
3-Fluoro-4-Nitrotoluene	148.56	118.82, 117.90	148.18, 160.21	134.13	125.64&125.81		20.86
Di-^{t}Butyl-3-Fluoro-4-Nitrobenzoylglutamate	125.10	116.76, 117.73	148.34, 160.11	139.41, 140.60	123.97, 124.13	126.40	
Di-^{t}Butyl-3-Fluoro-4-Aminobenzoylglutamate	120.61 120.82	113.62 114.43	143.96 154.46	139.35 139.95	124.45	114.43	
Di-^{t}Butyl-2-Acetyl-3'-Fluorofolic Acid	120.98 121.26	113.04 114.00	144.44 155.06	138.49 139.02	124.72	110.80	
2-Acetyl-3'-Fluoro-Folic Acid.	121.15 121.42	113.13 113.94	144.49 155.06	138.43 138.97	124.67	110.85	

	2	4	4a	6	7	8a	9
Di-^{t}Butyl-2-Acetyl-3'-Fluorofolic Acid	151.11	161.15	130.14	150.89	148.67	154.74	45.62
2-Acetyl-3'-Fluoro-Folic Acid	151.11	159.13	130.20	149.16	154.80	45.62	

^{13}C / COMPOUNDS	Amide	CO of α-COOH	CO of γ-COOH	α	β	γ
Di-^{t}Butyl-3-Fluoro-4-Nitrobenzoylglutamate	165.14	170.40	171.26	52.72	25.74	31.15
Di-^{t}Butyl-3-Fluoro-4-Aminobenzoylglutamate	165.36	171.15	171.43	52.28	25.90	31.32
Di-^{t}Butyl-2-Acetyl-3'-Fluorofolic Acid	165.25	171.10	171.37	52.28	25.90	31.26
2-Acetyl-3'-Fluoro Folic Acid	165.14	173.38	173.65	51.74	25.84	30.29

^{13}C / COMPOUNDS	Me of α-^{t}Bu	Me of γ-^{t}Bu	4°C of α-^{t}Bu	4°C of γ-^{t}Bu	Me of 2-Ac	CO of 2-Ac
Di-^{t}Butyl-3'-Fluoro-4-Nitrobenzoylglutamate	27.52	27.63	79.75	80.84		
Di-^{t}Butyl-3-Fluoro-4-Aminobenzoylglutamate	27.58		79.65	80.30		
Di-^{t}Butyl-2-Acetyl-3'-Fluorofolic Acid	27.58		79.64	80.35	24.06	173.32
2-Acetyl-3'-Fluoro Folic Acid					23.84	173.97

TABLE XVI - ^{13}C Chemical Shifts Of Intermediates In The Synthesis Of 3'-Fluoro-Folic Acid. Spectra recorded in d_6-dimethylsulphoxide. For explanation of numbering system, refer to Table XIII. Peak assingments for 3-fluoro-4-nitrobenzoic acid not attempted due to complexity of spectrum.

	X = 2'	X = 3'
X-Fluoro-4-Nitrotoluene	5.84	0.20
X-Fluoro-4-Nitrobenzoic Acid	12.41	1.16
Di-^{t}Butyl-X-Fluoro-4-Nitrobenzoylglutamate	8.70	1.13
Di-^{t}Butyl-X-Fluoro-4-Aminobenzoylglutamate	7.01	-16.49
Di-^{t}Butyl-2-Acetyl-X-Fluorofolic Acid	7.80	-15.33
2-Acetyl-X-Fluorofolic Acid	7.89	-15.27
X-Fluorofolic Acid	7.91	-15.37
N(10)-Formyl-X-Fluorofolic Acid	8.19	-3.40

TABLE XVII - ^{19}F Chemical Shifts Of 2'- And 3'-Fluorofolates, Intermediates In Their Preparation And The N(10)-Formyl Analogues. Spectra recorded in d_6-dimethylsulphoxide. Chemical shifts given in ppm relative to the middle of the spectrum, negative values indicatig peaks upfield from the middle.

of suitable protons. However, conformational studies of folates may be possible by using ^{13}C relaxation times, ^{13}C-proton coupling constants and nuclear Overhauser effects. One conformational utilizing these parameters has been demonstrated by Niccolai *et al.* (1985) on the natural product Rifamycin S.

V MISCELLANEOUS

Proton, ^{13}C and ^{19}F chemical shifts of the intermediates in the preparation of 2' and 3'-fluorofolates have been tabulated in tables XIII to XVII. These data were of immense use in characterization of the compounds and assignments of resonances.

C DIHYDROFOLATE REDUCTASE (DHFR) ASSAYS

I BEHAVIOUR OF A SINGLE ENZYME TOWARDS A RANGE OF FOLATES

a Bovine liver DHFR

Of all the compounds studied, N(10)-nitrosofolic acid was the most potent inhibitor of this reductase. The N(10)-formyl analogue was slightly less potent whilst the N(10)-methyl, N(10)-acetyl and folic acid were all an order of magnitude less potent. Benzene ring halogenated folates were significantly weaker inhibitors of the bovine enzyme.

Relative reduction rates were all quite small. Folic acid and its N(10)-methyl analogue gave rates of 0.7 and 0.2% respectively, whilst the N(10)-nitroso and formyl

	L. casei [1]		Bovine Liver [1]		Rat Liver [4]		Mouse Plasma Cytoma (PC 6) [4]		Mouse Sarcoma (M 5) [4]
FOLATES	K_i	Rate	K_i	Rate	K_i	Rate	K_i	Rate	K_i
FA	ND[2]	0.7	0.9	0.7		26		100	
N(10)-Me	No Inhibition	0.1	0.3	0.2					
N(10)-NO	6	0.3	0.012	UD[3]					
N(10)-CHO	ND	2.2	0.075	UD	3.77	18	13.94	81	0.63
N(10)-CH_3CO			0.53 [4]						4.56
3'-I			10.41[4]		12.0	69			8.00
3'-F			3.44 [4]	UD	0.056[4]				
MTX [6]	10.8×10^{-6}	NR	0.0063	NR[3]	1.5×10^{-5}	NR			

TABLE XVIII - K_is And Reduction Rates Of Folates For DHFRs From Various Sources.

(K_is are given in μM and reduction rates in % relative to 7,8-DHF.

1. All assays conducted in this lab by Dr. A Sahota except (4) and (6).
2. ND means not determined (where K_is are very high).
3. UD represent undetectable reduction rates, NR means not reduced.
4. Assays performed by Jayne Brown.
5. Result obtained by Dr. C Hamon.
6. MTX data obtained from the literature - L. casei results from Antonjuk et al. (1984), bovine liver from Baker and Ashton (1970), rat liver from Lovesey (1971).

analogues together with 3'-fluorofolate gave undetectable rates. The differences observed between the K_is and reduction rates suggest that the undetectable rates of the fluoro and the nitroso and formyl analogues were due to different causes.

b Mouse sarcoma (M5) DHFR

Mouse sarcoma (M5) DHFR was inhibited by N(10)-formylfolate to an order of magnitude more strongly than the corresponding acetyl derivative. Unlike the bovine reductase, differences in K_i between the latter and 3'-iodofolate appeared less marked. All three compounds were poor inhibitors with K_is varying over a rather small range. This was in contrast with the bovine reductase where small modifications to the folate molecule produced inhibitors with quite different K_is.

c Rat liver DHFR

Relative magnitudes of K_is were very different in rat liver DHFR when compared to the previous two reductases. Here 3'-fluorofolic acid was the most potent inhibitor by about two orders of magnitude over the formylfolate. 3'-iodofolate was the weakest inhibitor with a K_i of 12 μM, about 3 times less potent than N(10)-formylfolate. These differences suggest changes in the folate binding cleft of the enzyme, and this seems to be borne out by the relative reduction rates.

The rat liver enzyme is more efficient at reducing folates than the previous two

reductases. Reduction rates varied considerably, from 18% for the formyl analogue to 69% for 3'-iodofolic acid. It would be interesting to examine the amino acid sequence of rat liver DHFR to discover how the changes in primary structure, if any at all, could result in such significant alterations to reduction rates and K_is.

d *LACTOBACILLUS CASEI* DHFR

The N(10)-formyl analogue of folic acid, like the parent molecule, had very high K_is for *L. casei* DHFR. The actual figures were not determined. N(10)-methylfolate did not inhibit this enzyme at all whilst the nitroso analogue had a K_i of 6 μM.

L. casei DHFR was only able to reduce folic acid and its three N(10)-substituted analogues at a small relative rate (compared to the natural substrate 7,8-dihydrofolate). Values ranged from 0.1% for the methyl analogue to 2.2% for N(10)-formylfolic acid.

All four compounds seemed to have K_is and reduction rates quite different to those of the bovine enzyme. The relative values of these folates against the bacterial enzyme were different as well. Comparison of amino acid sequences has failed to reveal the source of these differences. They may be due to subtle changes in tertiary structure which could be uncovered by X-ray crystallography of bovine liver DHFR or the assay of a wider range of antifolates against both reductases.

e MOUSE PLASMA CYTOMA (PC6) DIHYDROFOLATE REDUCTASE

Only two assays were conducted on this enzyme. The results showed that reduction rates and K_i were extremely high. Assays covering a wider range of antifolates would reveal if these values were exceptional or that antifolates were indeed loosely bound and rapidly reduced by this reductase.

II BEHAVIOUR OF A SINGLE FOLATE TOWARDS A RANGE OF ENZYMES

a N(10)-formylfolic acid

The lowest K_is of this antifolate were against bovine liver and mouse sarcoma (M5) reductases. A very high K_i was recorded for the bacterial enzyme whilst those for rat liver and mouse plasma cytoma reductases were 3.77 and 13.94 μM respectively.

Reduction rates varied from 81% for mouse plasma cytoma DHFR to undetectable for the bovine enzyme. Rat liver DHFR had a value of 18% and *L. casei* DHFR reduced N(10)-formylfolic acid at 2.2% the rate of dihydrofolate.

There does not appear to be any obvious relationship between reduction rates and K_is. It was interesting to note that there is a large difference between the K_is of the bovine liver and *L. casei* reductases. A large difference was also observed for folic acid against these two enzymes.

b Folic acid

The K_is were determined against only two DHFRs. Bovine liver reductase gave a value of 0.9 μM whilst *L. casei* DHFR gave a very large K_i.

Both reductases reduced folic acid at a rate of 0.7% relative to DHF. Rat liver DHFR gave a rate of 26% and mouse plasma cytoma reduced folic acid just as efficiently as it reduced the natural substrate!

The assay of N(10)-formylfolic acid against this PC6 enzyme has already suggested that alterations had occured in the folate binding pocket. The ability to utilize folic acid or other oxidised folates would greatly enhance the survival of the cytoma. It would be interesting to uncover the changes which have improved the ability of the enzyme to utilize these folates.

c N(10)-methylfolic acid

This folate did not inhibit *L. casei* DHFR at all. Its K_i for the bovine liver enzyme was 0.3 μM and both DHFRs reduced this compound at approximately equal rates.

It was noticed that these folates, with electron donating moieties at N(10), both had very similar reduction rates with the two enzymes. Both were extremely poor inhibitors of the bacterial reductase and weak inhibitors of the bovine liver enzyme.

d N(10)-nitrosofolic acid

This compound was an extremely potent inhibitor of the reductases studied. It had the lowest K_i of all the folates tested against bovine liver DHFR. The same was true for *L. casei* DHFR. It was imperceptibly reduced by the vertebrate enzyme whilst the reduction rate by *L. casei* DHFR was 0.3%.

For the bovine liver enzyme, the low K_i seemed to have decreased the reducibility of this folate but the same was not true for the bacterial reductase. Like all the other N(10)-substituted folates, this compound was a better inhibitor of bovine liver enzyme than bacterial reductase, it was bound 500 times more strongly to the vertebrate DHFR.

e N(10)-acetylfolic acid

The K_i against mouse sarcoma (M5) DHFR was about 9 times that of bovine liver DHFR. Reduction rates were not determined. M5 DHFR seemed to bind N(10)-substituted folates in a similar manner but appear to bind the benzene ring substituted compounds differently.

f 3'-iodofolic acid

K_i values against the three enzymes tested were very similar, ranging from 8 μM for M5 DHFR to 12.0 μM for rat liver reductase. This enzyme reduced the folate at 69% the rate of DHF.

The lack of selectivity of 3'-iodofolic acid may be due to the steric bulk of the halogen causing the compound to be weakly bound by the reductases studied.

g 3'-fluorofolic acid

This was extremely strongly bound by rat liver DHFR. Its K_i against bovine liver reductase was 3.44 μM. Its behaviour is opposite to that of N(10)-formylfolate which was very strongly bound by bovine liver DHFR and weakly bound by the rat liver enzyme. It was interesting to observe that such small modifications had produced two folates which were able to discriminate between these two vertebrate enzymes.

The low K_i of 3'-fluorofolic acid against rat liver DHFR is not likely to be caused by reduced electron density at the benzene ring. The main effects of the electron withdrawing properties of the halogen would be to rearrange the distribution of charges at the ring, possibly increasing the charge at the ipso carbon. The strong binding of this folate would, most likely, be caused by other factors - like the electronegative fluorine participating in hydrogen-bonding with hydrophilic side chains or other hydrogen bond donors in the benzene ring binding cleft. ^{19}F NMR spectroscopy on the enzyme-folate complex here could yield some interesting results.

CHAPTER 5 CONCLUSION

This project involved the preparation of a large number and variety of conservatively modified folic acid analogues and the study of changes in carbon-13 and proton NMR chemical shifts with respect to the corresponding atoms of the parent molecule, folic acid. We aimed to investigate if these changes could be correlated in a simple manner to the electronic properties of the substituents at N(10) or at the benzene ring. The purpose was to ascertain if sterically-undemanding, electron donating or withdrawing functionalities at these positions yielded compounds with significantly different binding properties to dihydrofolate reductases from a variety of sources. Comparison of the reduction rates of these compounds with one another and with the natural substrate - 7,8-dihydrofolate - was hoped to provide information concerning the mechanism by which DHFR catalyses the conversion of DHF to THF.

In addition to studies to determine the effects of small, electron withdrawing substituents at the benzene ring, fluorinated folates were also synthesized for three other purposes.

Firstly, due to the small size of the fluorine atom, it would be interesting to compare the binding strengths of the two isomers. We did not expect the differences, if any at all, to be due to electron withdrawing properties of the halogens because their main effect would be to redistribute electron density within the benzene ring, possibly resulting in small differences in negative charges at the respective ipso carbons. Binding strength differences were more likely to be due to hydrogen bonding with hydrophilic side chains

at the binding pocket.

Secondly, we had also planned studies on the benzene ring binding pockets of DHFRs by conducting NMR experiments on solutions containing DHFR, flurofolate and NADPH. The aim was to study how the environmentally-sensitive ^{19}F signal was affected by changing concentrations of antifolate, substrate or stronger inhibitors (like methotrexate), the presence of absence of NADPH, $NADP^+$, NADH or NAD^+ and changing temperatures. During our synthesis of the 2' and 3'-fluorofolates, a paper describing some of these experiments but using 3',5'-difluoromethotrexate (3',5'-F_2-MTX) instead was published by Clore *et al.* (1984). As a result of this and the time-consuming difficulties encountered whilst preparing our compound, we decided to leave these studies to those following up this work.

Clore *et al.* (1984) have demonstrated that the benzene ring of bound 3',5'-F_2-MTX flipped about its symmetry axis at a rate of 7.3×10^3 s^{-1} at 298K. Following from the studies by Ozaki *et al.* (1981) which revealed similarities in the manner in which the benzene rings of MTX and folic acid were bound by DHFR, it would be interesting to investigate, using variable temperature NMR experiments, whether the benzene rings in our fluorofolates behaved in an identical manner when bound to the enzyme. NMR experiments on solutions containing a greater molar equivalent of antifolate than enzyme would reveal the effects of binding on the ^{19}F chemical shifts of the isomers. Studies of solutions containing enzyme, cofactor and mixtures of substrate, fluorofolate or methotrexate could yield kinetic constants and other information on the manner in which these pteroylglutamates bind to DHFR.

Finally, we had also planned to use these folates for metabolic studies. We were interested in the manner in which reduced folates were utilized, distributed and used in the biosynthesis of other biomolecules, the importance of polyglutamylation and any other roles of these compounds. A conservatively modified folate with the small yet distinctive fluorine labelling would be ideal for our purposes. However, neither isomer was reduced by bovine liver DHFR and, consequently, they would not be incorporated into the reduced folate pool of the animal. Studies of other enzymes were still incomplete at the time of writing. Should any reductase show an appreciable ability to reduce these fluorofolates then the analysis of urine, and possibly even tissue samples, using mass spectrometry, NMR, UV, HPLC and other spectroscopic techniques could yield much useful information. Radiolabelling methods using the positron-emitting ^{18}F isotope may be used to study folate distribution in the living animal. *In vivo* biological NMR studies on cancerous tissue could also be used to examine the interactions between fluorofolates and tumour DHFRs.

Analysis of amino acid sequences of DHFRs from bacterial and vertebrate sources has revealed that homology between these enzymes and even amongst bacterial reductases were as low as 25% when they were aligned according to structural equivalence (Volz *et al.*, 1982). However, if we concentrated on residues directly involved in binding cofactor and inhibitor (or substrate), we immediately notice the high degree to which residues were strictly conserved. In places where changes had occurred, amino acids were often replaced by analogous residues, eg. Asp for Glu or Leu for Val.

Our interest in amino acid sequences stemmed from the fact that these provide a potential route to the exploitation of differences between bacterial and vertebrate

reductases. We were especially interested in cases where alkyl side chains were replaced by aromatic or other hydrophobic groups capable of acting as potential nucleophiles (eg. Ser, Thr or Met). Modification at N(10) of the folate molecule was expected to perturb electron density at the benzene ring and, to a smaller extent, at the pteridine ring as well. Reduction in electron density at the benzene ring could result in these compounds binding more strongly to those enzymes possessing nucleophilic side chains close to the benzene ring of bound folates. Much interest was centered on residues 31 and 60 (C1 numbering; Volz *et al.*, 1982). These residues were, however, acting 'in opposition' to each other. Residue 31 had aromatic side chains in all vertebrate reductases studied and alkyl side chains in bacterial enzymes (Met in *S. faecium* II). The situation was reversed for amino acid 60, -Ile in all vertebrate DHFRs (and *E. coli* MB1428 as well) but *S. faecium II* had Met and *L. casei* had Phe at this position. Nevertheless, contributions from these two potential nucleophiles to the overall binding energy may not be equal and provided that the difference was significant, one may still be able to design inhibitors capable of discriminating between bacterial and vertebrate dihydrofolate reductases.

A BEHAVIOUR OF A RANGE OF ANTIFOLATES TOWARDS A SINGLE ENZYME

I BOVINE LIVER DHFR

Examination of the data in table XVIII has revealed that the antifolates could conveniently be classed into three groups. N(10) nitroso and formylfolates were the best inhibitors by an order of magnitude followed by folic acid, its N(10) acetyl and methyl derivatives. The benzene ring halogenated compounds were the poorest inhibitors.

CONCLUSION

X-ray crystallographic studies of DHFRs containing bound methotrexate and cofactor (Bolin *et al.*, 1982; Matthews *et al.*, 1985) have revealed that residue 31 occurs close to the benzene ring of bound antifolate. These studies were not conducted on bovine liver reductase. However, crystallographic studies of enzymes from bacterial and vertebrate sources have demonstrated that, in spite of large differences in amino acid sequences, the overall architecture of these enzymes, especially at the binding site, were very alike (Matthews *et al.*, 1978; Volz *et al.*, 1982). Alignment of the bovine liver amino acid sequence with sequences from *L. casei* and chicken liver reductases according to structural equivalence have shown that residue 31 in the bovine enzyme is phenylalanine. The aromatic side chain is thought to be close enough to interact with the benzene rings of bound folates.

The main reason behind lower K_i values for the nitroso and formyl folates was probably the electron-withdrawing abilities of the N(10) substituents. Folic acid and its methyl analogue had electron donating functionalities at the corresponding position. N(10) acetylfolic acid did not fall into the same category of the former compounds probably because relative to the formyl and nitroso groups, the acetyl substituent was less electron withdrawing and more bulky. Consequently, the positive contribution to binding from the electron withdrawing N(10) functionality might have been overcome by negative effects resulting from steric interactions between it and the enzyme.

Inductive effects could also be used to account for the weak binding of 3'-iodofolic acid. This electron donating property and especially the steric bulk of iodine may have combined to make the compound a particularly poor inhibitor. Interpretation of the K_i value for 3'-fluorofolic acid was more difficult. Whilst it was easy to explain the lower

K_i relative to the iodo analogue, the reasons behind its weaker binding compared to folic acid were not obvious. The presence of an electron withdrawing group on the benzene ring was expected to make the compound a slightly better inhibitor than the parent molecule. Steric effects due to the halogen were not expected to be significant.

Our results suggest that compounds with small electron withdrawing groups at N(10) were bound more strongly to bovine liver DHFR than compounds with electron donating groups at the same position or with either electron withdrawing or donating groups at the benzene ring. Studies by Clore *et al.* (1984) on 3',5'-F_2-MTX binding to *L. casei* DHFR and Rosowsky and Chen (1974) on 3',5'-Cl_2-MTX binding to both *L. casei* and L1210 reductases both support our observations that electron-withdrawing halogens at the benzene ring did not significantly alter the binding strengths of folic acid analogues. This was probably because by virtue of their structures and position, N(10) substituents in withdrawing electron density from the benzene ring also allowed electron delocalization over a larger area. Whilst electonegative fluorine atoms also withdrew electron density from the benzene ring, their main effect was to rearrange electron density within the aromatic system. This is not likely to make fluorofolates appreciably better inhibitors in the presence of nucleophiles at the benzene ring binding pocket. Had these fluorofolates been better inhibitors than folic acid, the most likely cause would have been other reasons, like hydrogen bonding between the fluorine atom and the enzyme.

All five compounds were reduced very slowly relative to 7,8-dihydrofolate. The reduction rates of the fluoro, nitroso and formyl folates were undetectable whilst those of folic acid and the N(10) methyl analogue were only 0.7 and 0.2%, respectively. The

source of this difference for the nitroso and formyl analogues may have been the electron withdrawing properties of the N(10) substituents. These moieties were expected to reduce electron density at the pyrazine ring to a smaller extent than the reduction experienced by the benzene ring as N(10) is separated by a methylene bridge from the pyrazine ring. Reduction in electron density here would slow down protonation at N(5), the first step in the reduction of folates (Charlton & Young, 1985). If this was the rate determining step, then compounds with electron withdrawing N(10) substituents or reduced electron density at their pyrazine rings would be reduced at slower rates relative to folates with electron rich N(10) substituents or pyrazine rings. The undetectable reduction rate of the fluorofolate was more difficult to explain. The 3'-substituent was not expected to significantly reduce electron density at the pyrazine ring. The poor reducibility of this analogue probably arose from other causes.

II *LACTOBACILLUS CASEI* DHFR

K_i values here were more difficult to interpret due to the lack of exact figures. Like bovine enzyme, the best inhibitor was the nitroso analogue but unlike the vertebrate enzyme, the poorest antifolate was the methyl compound. Amino acid sequence analysis (Bitar *et al.*, 1977) has shown that residue 31 is Leu. The absence of a potential nucleophile here would raise difficulties in explaining the relative order of binding strengths of the four compounds. Fortunately, crystallographic studies by Matthews *et al.* (1978) have revealed that the Phe-60 in *L. casei* also interacts with the benzene ring. The positioning of a potentially nucleophilic aromatic side-chain close to the benzene

rings of bound folates may be one factor responsible for the small differences in relative K_i values.

Reduction rates were also slightly different to those of the bovine enzyme. *L. casei* DHFR reduced folic acid and the methyl analogue at almost the same rate (relative to DHF) as bovine liver reductase. It seemed to be slightly better at reducing the nitrosated compound but as for the formyl analogue, the increase was much bigger. This increase and the fact that N(10)-formylfolate was reduced by the bacterial enzyme at the greatest relative rate compared to the other three compounds were both difficult to explain.

III RAT LIVER DHFR

K_i values for the various antifolates were quite different for this enzyme compared to the previous two reductases. There was a small increase for 3'-iodofolate, a large increase for N(10) formylfolate and a very large decrease for 3'-fluorofolate.

The amino acid sequence for rat liver DHFR was unavailable at the time of writing and we could only speculate on probable changes in the binding cleft. The small increase in the K_i of 3'-iodofolate relative to bovine liver DHFR would seem to suggest that overall architecture was largely unchanged. However, large changes for the other two compounds indicate otherwise. As explained previously, we do not feel that the enhanced binding of 3'-fluorofolic acid was caused by electron withdrawing effects of the halogen but rather by other causes like hydrogen-bonding with side chains in the binding pocket. The change in K_i for N(10)-formylfolate relative to the other reductases could either mean that alterations had also occured in the N(10) binding region as well or

that changes had involved an area intersecting both N(10) and the benzene ring binding regions. X-ray crystallographic studies by Volz *et al.* (1982) and Matthews *et al.* (1985a) have demonstrated that residue 31 interacts with both pteridine and benzene rings of bound folates. This residue was not strictly conserved in vertebrate reductases. It was Phe in bovine liver (Lai *et al.*, 1979) and L1210 murine lymphoma (Stone *et al.*, 1979) and Tyr in chicken liver (Kumar *et al.*, 1980) and porcine liver (Smith *et al.*, 1979). Changes here would have significant effects on the binding of N(10)-formyl and 3'-fluorofolates.

Rat liver DHFR was also able to reduce folates at a greater relative rate compared to the other enzymes. The N(10)-formylfolate rate was still slower than that of folic acid and the iodo analogue. These results, together with K_i changes, both suggest substantial changes in the pteroylglutamate binding pocket of rat liver enzyme.

Comparison of the K_is for N(10)-formyl and 3'-fluorofolates against rat liver and bovine liver reductases showed that small modifications to the folate molecule had produced compounds with widely different binding strengths to two vertebrate enzymes. Whilst these differences were not of the same magnitude as those shown by trimethoprim for bacterial and vertebrate enzymes (Baccanari *et al.*, 1982), nonetheless they provide a small step towards the better understanding of both enzymes. Publication of the rat liver DHFR amino acid sequence and crystallographic data of bovine and rat liver reductases containing bound folates would contribute much to our understanding of the reasons behind this selectivity.

IV MOUSE SARCOMA (M5) AND MOUSE PLASMA CYTOMA (PC6) DHFRs

Only a few results were obtained for these two enzymes. The order of K_i values for mouse sarcoma (M5) DHFR was the same as that for bovine liver enzyme but the range was smaller - an order of magnitude compared to two orders for the bovine enzyme. This apparent lack of selectivity relative to the bovine liver enzyme seemed to be caused by weaker binding of the N(10)-substituted folates and a slightly stronger binding of the halogenated compound. The authenticity of this reduced selectivity could be confirmed when more assays are completed. An amino acid sequence is eagerly awaited as well.

N(10)-formylfolate was bound extremely weakly to PC6 DHFR and reduced at 81% the rate of dihydrofolate. This enzyme was also able to reduce folic acid at the same rate as the natural substrate. It is very likely that major changes have occured here. The determination of the amino acid sequence and the assay of a wide range of antifolates would advance our understanding of this enzyme.

B BEHAVIOUR OF A SINGLE ANTIFOLATE TOWARDS A VARIETY OF ENZYMES

This comparison should reveal if a compound was bound differently by DHFRs from different sources.

N(10)-formylfolic acid was a particularly good inhibitor of bovine liver DHFR. It was slightly weaker towards mouse sarcoma enzyme and a poor inhibitor of the bacterial and mouse plasma cytoma reductases. The difference between the strongest and weakest

K_is was at least 800 times. A large range was also observed for the relative reduction rates. There seems to be a simple, linear relationship between relative reduction rates and K_i values amongst vertebrate enzymes, strongly bound folates being reduced at slower rates than weakly bound ones. This might have resulted from the formyl substituent withdrawing electron density from both the pyrazine and benzene rings.

N(10) nitrosofolic acid was more strongly bound to bovine liver DHFR than the formyl analogue. It was also bound 500 times more strongly to this enzyme than to *L. casei* DHFR. Until we obtain more K_i values for the bacterial reductase, we cannot be sure if small electron-withdrawing substituents at N(10) promoted discrimination between bacterial and bovine liver DHFRs.

The behaviour of N(10) acetylfolic acid towards bovine liver and mouse sarcoma DHFRs closely paralleled that of the formyl analogue, suggesting similarity in the binding of these two compounds. This similarity was not extended to the binding of the iodo analogue, a result which could be interpreted to mean that changes had occured near the benzene ring binding cleft or, alternatively, 3'-iodofolate was bound in a different manner compared to the previous two folates.

3'-iodofolic acid was a poor inhibitor of all the enzymes tested, with the K_i values all very similar. This lack of selectivity may be due, in part, to the steric bulk of the iodine or its electronic properties. The corresponding fluoro compound had quite different K_is against bovine liver and rat liver reductases. Rearrangement of electron density at the benzene ring by the halogens could not be the main cause of differences between the enzymes and the antifolates. Clearly, rat liver DHFR possess factors - like

potential hydrogen bond donors - which enhanced the binding of 3'-fluorofolic acid.

3'-fluorofolic acid, like the N(10) nitroso and formyl analogues, were imperceptibly reduced by bovine liver DHFR. Unlike these folates though, its K_i was quite high. The absence of correlationship between K_i and reduction rate showed that factors that decrease binding strength need not increase reduction rate and vice versa. A low reduction rate could be due either to poor binding (compound not reduced at all) or strong binding (compound bound to enzyme but reduced slowly or reduced compound still strongly attached to the enzyme).

C NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF FOLIC ACID AND ITS ANALOGUES

Interpretation of the results from enzyme assays could have been simplified if we had been able to classify inhibitors into groups based upon the electron densities at their benzene and pteridine rings. We had prepared folates with the intention of discovering whether compounds with electron poor pteridine or benzene rings were better inhibitors of dihydrofolate reductases bearing potential nucleophilic groups at the binding clefts of these aromatic rings. As mentioned earlier, difficulties have been encountered with enzymes for which no amino acid sequences were available. This could have been partially overcome had we known whether the antifolates had electron rich or deficient aromatic systems. If an antifolate with an electron deficient benzene ring was a more potent inhibitor of a particular enzyme than a compound with an electron rich ring, this would suggest the presence of nucleophiles near the benzene ring binding cleft of the

reductase. Conversely, should the compound with an electron rich benzene ring turn out to be the better inhibitor, then this would suggest the presence of electrophiles. In cases where enzymes have been sequenced this lack of knowledge concerning the 'electronic status' of the aromatic systems has only allowed us to speculate that differing binding strengths of a range of compounds may be due to changes in electron density at the pteridine and benzene rings.

This lack of knowledge concerning the electronic status of the two rings has arisen because we were unable to completely interpret results from the NMR spectra of our compounds. We had hoped that comparison of spectra from a large number of conservatively-modified folic acid analogues would allow us to correlate changes in proton and ^{13}C chemical shift with changes in electron density at these nuclei. Folates are large molecules and their many saturated bonds confer upon them great conformational flexibility. Moreover, the presence of two aromatic systems has added more complications to chemical shift interpretation. In retrospect, it seemed extremely optimistic to attempt such a mammoth task within the confines of part of a Ph.D. project.

Analysis of proton and ^{13}C NMR spectra has revealed that modification at N(10) or at the benzene ring produced chemical shift changes in nuclei far removed from the points of modification. Changes occuring in the vicinity of the modification were also too large to be due solely to through-bond effects of the substituent. As discussed in the Results chapter, we have concluded that folates exist in a folded conformation with benzene and pteridine rings interacting closely with each other. Modification produced some small, albeit significant conformational change and because of the anisotropy of the aromatic systems, there were both 'through-bond' and 'through-space' contributions to the changes in the chemical shift. The effects of conformational change has been

potentiated by the fact that folates existed in a folded conformation, small movement of these rings resulting in large chemical shift changes for quite a number of nuclei. These changes made results extremely difficult to interpret as we were unsure of the exact conformation of our compounds. Charts of benzene ring induced ring current effects (Johnson & Bovey, 1958) were thus rendered useless. Moreover, no such charts at all were available for pteridine rings. We had anticipated at the beginning that problems of this type could arise but had hoped that they could be overcome by analysing a large number of folic acid analogues. In the event, due to the numerous time-consuming difficulties encountered, especially in relation to the syntheses of the fluorofolates, we were unable to prepare as many compounds as we would have liked. It would take the analysis of a large number of NMR spectra before one is able to demonstrate that small substituents at N(10) and at the benzene ring did indeed affect electron densities at the benzene and pteridine rings.

Although we have been unable to demonstrate the effects of N(10) and benzene ring substituents on electron densities at the two rings, this study has, nonetheless, yielded some positive results. We were able to show that there apparently was no self-association of folates in dimethylsulphoxide. This is in contrast to the results of Poe (1973) and Pastore (1971) who demonstrated dimerization of folic acid, albeit in aqueous medium. The change to a less protic solvent could have been responsible for the absence of dimerization in DMSO.

In spite of the lack of direct evidence, our results suggest that folates adopt a bent conformation when dissolved in dimethylsulphoxide. This could have resulted from the absence of self-association. Interaction of the two aromatic rings could provide

sufficient stabilization energy to offset opposing effects like steric congestion and reduced entropy.

Finally, if we assume that chemical shift changes were due mainly to conformational change then the results for N(10)-methylfolic acid suggest that the replacement of hydrogen with a methyl group did not produce a large conformational change in the new molecule relative to folic acid. The chemical shifts of its hydrogen and carbon nuclei were little different from those of its parent. We are unsure why there should be so little conformational change in the new molecule.

D SYNTHESIS

Our earlier syntheses were all based on simple one-step methods published in the literature. These included the preparation of the N(10)-formyl, methyl, nitroso and acetyl analogues. 3'-iodofolate was also prepared by a one-step reaction using folic acid as starting material. All products were characterised only by ultraviolet and nuclear magnetic resonance spectroscopy as other spectroscopic techniques like infrared or Raman spectroscopy did not yield much useful information. Attempts have been made by some authors to study these compounds using mass spectrometry (Anbar & St. John, 1976; Hignite & Azarnoff, 1978; Smith *et al.*, 1981) but their techniques were either not widely available or dogged with problems arising from uncertainties about the extent of derivatization. Cheung *et al.* (1985) has recently described the use of ammonia and methane chemical ionization mass spectrometry to study pteroylglutamates. This appeared to have overcome many of the earlier problems and could be of much use in the future. Microanalysis was also performed on our products and results agreed well with theoretical values.

CONCLUSION

α-Deuteriomethotrexate was prepared by the reaction of concentrated deuteriosulphuric acid with MTX. This reaction was investigated with the hope of introducing a deuteron into either the benzene or pteridine rings and using the resultant compound for metabolic and NMR studies. α-^{2}H-MTX has not yet been assayed for its activity towards DHFR. X-ray crystallographic studies by Matthews *et al.* (1978) and Filman *et al.* (1982) have demonstrated the role of the α-carboxylate group in the binding of methotrexate and hence the importance of stereochemistry at the glutamate moiety. However, some enzymic studies (Lee *et al.*, 1978) have failed to uncover any difference in the activities of the (L) and (D) optical isomers of MTX towards L1210 DHFR. It would be interesting to compare the activities of the two optical isomers towards a bigger range of enzymes.

Our results from the studies of optical activity of α-^{2}H-MTX and the other pteroylglutamates were plagued with large errors. Nonetheless, it was still obvious that deuteriation of the (L)-glutamate had not resulted in significant racemization or inversion of stereochemistry. One possible explanation for this observation is that deuteriation involved a relatively long-lived pyramidal carbanion intermediate, probably stabilized by ionic interaction with an adjacent polar group on the methotrexate molecule.

The syntheses of the fluorofolates were the only multi-step preparation that we undertook. The procedure was largely based on the method of Henkin & Washtien (1983) for the preparation of the corresponding aminopterins. Intermediates were carefully characterised to ensure that their spectra and other physical constants were consistent with those reported in the literature. Problems were encountered, particularly

when syntheses were scaled up. The final step - deprotection of the 2-amino group - was especially troublesome.

Formylation of fluorofolates was a relatively straightforward reaction. The 2' isomer gave a slightly lower yield than 3'-fluorofolate. We suspect that the positioning of the 2'-fluorine atom 'meta' to N(10) has reduced its nucleophilicity more significantly than the 'ortho' 3'-fluorine, thus hindering the reaction with formic acid.

Although the syntheses of fluorofolates were trouble-free, their purification was rather more problematical. This, we think, was due to presence of two-electron withdrawing functionalities on the folate molecule, increasing the stability, nucleophilicity and the ease of formation of the folate anion. Consequently, these fluorofolates were bound more strongly to the anion-exchanger DE52 (diethylaminoethyl cellulose) - the gel normally used to purify the other folates. We have attempted, without success, to purify these compounds by gel permeation chromatography. Preparative thin layer chromatography may prove to be the only technique applicable for the purification of these compounds.

CHAPTER 6 FURTHER WORK

This project is concerned with the design of selective inhibitors of dihydrofolate reductase possessing the 2-amino-4-hydroxypteroylglutamate structure. Our rationale was the expectation that enzymes could be distinguished by antifolates solely on the basis of differing electron densities at the benzene ring. This necessarily involved the study of three large areas - the synthesis of new conservatively modified folates with differing electron densities at the benzene ring, the measurement of these electron densities and enzymic assays. Tackling these topics within the confines of a three-year project has meant that we were unable to manage an in-depth investigation into each particular area. Although superficial, our study has uncovered some interesting results and a follow-up study would generate much useful information.

Our understanding of the importance of electron density at the benzene ring to the binding of folates could be advanced by the synthesis of more compounds. Modification should be concentrated at the benzene ring and N(10). It is imperative that substituents are small in order to reduce steric contributions to a minimum. Both electron withdrawing and donating groups are required. The effects of electron withdrawing functionalities capable of promoting delocalization over a large area of the folate skeleton should also be investigated. Study of a large number of compounds would, hopefully, allow us to understand the factors governing binding strengths and reduction rates of folates. This, in turn, could allow us to test a range of compounds against an unsequenced enzyme to probe the nature of its binding pocket.

FURTHER WORK

Synthesis of new folic acid analogues, particularly those with strong electron-withdrawing substituents would necessitate the introduction of new purification techniques. Our problems with the formylfluorofolates clearly demonstrated the need for this. Although preparative thin layer chromatography may yet prove successful in purifying these compounds, we would prefer a more straightforward technique, possibly involving column chromatography. Gel-filtration chromatography has yet to be fully investigated. It might be possible that the use of a new buffer system, a gel with a different mesh size or even a completely different gel could successfully yield impurity-free folates.

A new method for the quick introduction of ^{18}F into the benzene ring of folates is also required. Due to the very short half-life of this isotope, the preparation and purification steps should ideally be completed in less than a day. The increasing use of this isotope in medicine has prompted the publication of a large number of papers describing fast, efficient, selective and mild fluorination reactions and reagents (Lerman *et al.*, 1984; Visser *et al.*, 1984; Idoux *et al.*, 1985). For a large, multifunctional molecule like folic acid, fluorination would most likely involve the reaction of a suitably protected compound. The synthesis would involve the displacement of hydrogen or, preferably, a better leaving group from the benzene ring followed by deprotection and purification.

Preparation of more folic acid analogues would allow us to a more thorough NMR study of folates. It is hoped that this would enable us to separate and quantify the various contributions to chemical shift changes. Identification of these components would improve our understanding of the effects of substituents on electron density at the benzene and pteridine rings. It could also give us a better picture of folate conformation

in dimethylsulphoxide.

A more exhaustive and direct study of folate conformation in DMSO should also be attempted. This would encompass the measurement of relaxation times, coupling constants and nuclear Overhauser enhancements. Selective irradiation of protons and carbon-13 nuclei will be required to determine whether folic acid and its analogues existed in a bent conformation. Several papers describing the use of these methods for the structure determination of larger and more complex molecules have appeared in the recent literature (Niccolai *et al.*, 1985; Smith *et al.*, 1985).

Studies of the binding of fluoro and formylfluorofolates to dihydrofolate reductase was another topic that we have been unable to attempt. This involves the investigation of the interactions between cofactor and antifolate with purified enzyme. Studies conducted on tumour tissue kept alive artificially in a physiological medium could be compared to the previous experiments to determine if any significant differences were evident between *in vivo* and *in vitro* systems. Other experiments involving enzymes have been described in previous chapters. Some of the above experiments may require the use of a more sophisticated spectrometer with a stronger magnet.

Examination of table XVIII immediately revealed the amount of assays that needs to be completed. Completion of this table will give us a better understanding of the factors governing the binding strengths and reducibility of folates. It would also improve our knowledge of the enzymes themselves, particularly the unsequenced ones. Study of the behaviour of a range of folates towards a single enzyme and the response of a single enzyme towards a single compound could go a long way to further our understanding of factors governing selectivity. This table will be especially useful if we are able to increase the number and variety of both enzymes and antifolates.

CHAPTER 7 REFERENCES

1. Anantharamaiah GM and Sivanandaiah KM - J. Chem. Soc. Perkin I (1977), 490-2
2. Anbar M and St. John GA - Anal. Chem. (1976) 48 (1), 199-203
3. Antonjuk DJ, Birdsall B, Cheung HTA, Clore GM, Feeny J, Gronenborn A, Roberts GCK and Tran TQ - Br. J. Pharmac. (1984) 81, 309-15
4. Baccanari DP, Daluge S and King RW -Biochemistry (1982) 21, 5068-75
5. Baker BR and Ashton WT - J. Med. Chem. (1970) 13, 1161-5
6. Baker DJ, Beddell CR, Champness JN, Goodford PJ, Norrington FEA, Roth B and Stammers DK -12th General Assembly, International Union of Crystallography, Ottawa (1981a), C58
7. Baker DJ, Beddell CR, Champness JN, Goodford PJ, Norrington FEA, Smith DR and Stammers DK -FEBS Letts. (1981b) 126 (1), 49-51
8. Beddell CR in X-Ray Crystallography and Drug Action by AS Horn and CJ De Ranter (eds.) Oxford University Press (1984), 169-73
9. Bender RA - Cancer Chemother. Rep. (1975) Part 3, 6, 73
10. Bergman M and Zervas L - Ber. (1932) 65, 1192-1201
11. Bertino JR, Perkins JP and Johns DG -Biochemistry (1965) 4, 839-46
12. Bieri JH and Viscontini M - Helv. Chim. Acta (1973) 56 (8), 2905-11
13. Birdsall B, Feeny J, Pascual C, Roberts GCK, Kompis I, Then RL, Muller K and Kroehn A - J. Med. Chem. (1984) 27, 1672-6

14. Bitar KG, Blankenship DT, Walsh KA, Dunlap RB, Reddy AV and Freisheim JH - FEBS Lett. (1977) 80, 119-22

15. Blakley RL -Biochem. J. (1957) 65, 331-42

16. Blakley RL -Biochem. J. (1959) 72, 707-15

17. Blakley RL - Nature (1960) 188, 231-2

18. Blakley RL -The Biochemistry of Folic Acid and Related Pteridines (1969) North-Holland, Amsterdam.

19. Blakely RL in Molecular Actions and Targets for Cancer Chemotherapeutic Agents (Volume 2) by AC Sartorelli, JS Lazlo and JR Bertino (eds.) Academic Press, New York (1981), 303-32

20. Blaney JM, Jorgensen EC, Connolly ML, Ferrin TE, Langridge R, Datley SJ, Burridge JM and Blake CCF - J. Med. Chem. (1982) 25, 785-90

21. Bloch F, Hansen WW and Packard M - Phys. Rev. (1946) 69, 127-8

22. Bolin JT, Filman DJ, Matthews DA, Hamlin RC and Kraut J - J. Biol. Chem. (1982) 257 (22), 13650-62

23. Bovey FA - Nuclear Magnetic Resonance Spectroscopy, Academic Press, London and New York, Appendix (1969)

24. Brown DJ and Jacobsen NW - J. Chem. Soc. (Lond.) (1961) 4413-20

25. Burchall JJ - J. Infect. Dis. (1973) 128, 5437-441

26. Cayley PJ, Dunn SMJ and King RW -Biochemistry (1981) 20, 874-9

27. Charlton PA, Young DW, Birdsall B, Feeny J and Roberts GCK - J. Chem. Soc. Chem. Commun. (1979), 922-4

28. Charlton PA and Young DW - J. Chem. Soc. Perkin I (1982) 1363-9

29. Charlton PA, Young DW, Birdsall B, Feeny J and Roberts GCK - J. Chem. Soc. Perkin I (1985) 1349-53

30. Cheung HTA, Tattam BN, Antonjuk DJ and Boadle DK - Biomed. Mass Spectrom. (1985) 12 (1), 11-18

31. Clore GM, Gronenborn AM, Birdsall B, Feeny J and Roberts GCK -Biochem. J. (1984) 217, 659-66

32. Cocco L, Groff JP, Temple C Jr., Montgomery JA, London RE, Matwiyoff NA and Blakely RL - Biochemistry (1981a) 20, 3972-8

33. Cocco L, Temple C Jr., Montgomery JA, London RE and Blakley RL - Biochem. Biophys. Res. Commun. (1981b) 100, 413-9

34. Cohen SS - Ann. N.Y. Acad. Sci (1971) 186, 292-301

35. Cosulich DB and Smith JM Jr. - J. Amer. Chem. Soc. (1949) 71, 3574

36. Cosulich DB, Seeger DR, Fahrenbach MJ, Collins KH, Roth B, Hultquist ME and Smith JM Jr. - J. Amer. Chem. Soc. (1953) 75, 4675-80

37. Cramer SM, Schornagel JH, Kalghatgi KK, Bertino JR and Horvath C - Cancer Res. (1984) 44, 1843-6

38. Crippen GM and Havel TF - Acta Crystallogr. Sect. A (1978) A34, 282-4

39. Dann JG, Ostler G, Bjur RA, King RW, Scudder P, Turner PC, Roberts GCK, Burgen ASV and Harding NGL - Biochem. J. (1976) 157, 559-71

40. Dobson CM in NMR in Biology by Dwek RA, Campbell JD, Richards RE and Williams RJP (eds.) Academic Press, London (1977) 63

41. Emsley JW, Feeny J and Sutcliffe LH - High Resolution Nuclear Magnetic Resonance Spectroscopy, Pergamon Press, Oxford (1965) Volume 1, Appendix B

42. Filman DJ, Bolin JT, Matthews DA and Kraut J - J. Biol. Chem. (1982) 257 (22), 13663-72

43. Fontecilla-Camps JC, Bugg CE, Temple C Jr., Rose JD, Montgomery JA and Kisliuk RL - J. Amer. Chem. Soc. (1979) 101, 6114-5

44. Freisheim JH and Matthews DA in Folate Antagonists as Therapeutic Agents Volume 1 (Biochemistry, Molecular Actions and Synthetic Design) by Sirotnak FM, Burchall JJ, Ensminger WD and Montgomery JA (eds.) Academic Press, London (1984), 69-131

45. Ghose AK and Crippen GM - J. Med. Chem. (1983) 26, 996-1010

46. Goldman ID - Trans. NY Acad. Sci. (1971) 186, 400-22

47. Gready JE - Adv. Pharm. Chemother. (1980) 17, 37-102

48. Greenfield NJ, Williams MN, Poe M and Hogsteen K - Biochemistry (1972) 11, 4706-11

49. Gribble GW and Kelly WJ - Tet. Lett. (1985) 26 (32), 3779-82

50. Gupta VS and Huennekens FM - Arch. Biochem. Biophys. (1967) 120, 712-8

51. Haigh CW and Mallion RB - Org. Magn. Reson. (1972) 4, 203

52. Hansch C - Drug Intell. Clin. Phar, (1982) 16, 391-6

53. Henkin J and Washtien WL - J. Med. Chem. (1983) 26, 1193-6

54. Hignite CE and Azarnoff DL - Biomed. Mass Spectrom. (1978) 5 (2), 161-3

55. Hitchings GH - Clin. Pharmacol. Therap. (1960) 1, 570-89

56. Hitchings GH and Roth B in Enzyme Inhibitors as Drugs by M Sandler (ed.) MacMillan Press Ltd., London (1982), 263-80

57. Hood K and Roberts GCK - Biochem. J. (1978) 171, 357-66

58. Hopfinger AJ - J. Amer. Chem. Soc. (1980) 102, 7196-206

59. Hopfinger AJ - J. Med. Chem. (1983) 26, 990-6

60. Hopkins FG - Nature (1889) 40, 335

61. Hopkins FG - Nature (1891) 45, 197-8

62. Hulton J and Sutcliffe LH - Prog. NMR Spectrosc. (1975) 10, 27-39

63. Idoux JP, Madenwald ML, Gracia BS and Chu D-L - J. Org. Chem. (1985) 50, 1876-8

64. International Union on Pure and Applied Chemistry - International Union of Biochemistry Commision on Biochemical Nomenclature - Biochim. Biophys. Acta (1965) 107, 11-13

65. Johnson CE and Bovey FA - J. Chem. Phys. (1958) 29, 1012-4

66. Karplus M and McCammon A - CRC Crit. Revs. Biochem. (1981) 9, 293

67. Khalifa E, Bieri JH and Viscontini M - Helv. Chim. Acta (1973) 56 (8), 2911-9

68. Khalifa E, Sengupta PK, Bieri JH and Viscontini M - Helv. Chim. Acta (1976) 59 (1), 242-7

69. Kovacs J, Schmit GN and Ghatak UR - Biopolymers (1968) 6, 817-36

70. Kumar AA, Blankenship DT, Kaufman BT and Freisheim JH - Biochemistry (1980) 19, 667-78

71. Kuhn R and Cook AH - Ber. Deut. Chem. Ges. (1937) 70, 761-8

72. Kuyper LF, Roth B, Baccanari DP, Ferone R, Beddell CR, Champness JN, Stammers DK, Dann JG, Norrington FEA, Baker DJ and Goodford PJ - J. Med. Chem. (1982) 25, 1120-2

73. Kuyper LF, Roth B, Baccanari DP, Ferone R, Beddell CR, Champness JN, Stammers DK, Dann JG, Norrington FE, Baker DJ and Goodford PJ - J. Med. Chem. (1985) 28, 303-11

74. Lai PH, Pan YC, Gleisner JM, Peterson DL and Blakley RL in Chemistry and Biology of Pteridines by Kisliuk RL and Brown GM (eds.) Elsevier/North-Holland, New York (1979), 437-40

75. Lam Y-F and Kotowycz G - Can. J. Chem. (1972) 50, 2357-63

76. Lee WW, Martinez AP and Goodman L - J. Med. Chem. (1974) 17, 326-30

77. Lerman O, Tor Y, Hebel D and Rozen S - J. Org. Chem. (1984) 49 (5), 806-13

78. Livingston RB and Carter SK - Single Agents in Cancer Chemotheraphy, IFI Plenum, New York (1970), 130-72

79. Lovesey AC - Biochem. Pharmacol. (1971) 20, 2033-41

80. Martin YC - Quantitative Drug Design, Dekker, New York (1978)

81. Matthews DA, Alden RA, Bolin JT, Freer ST, Hamlin R, Xuong N, Kraut J, Poe M, Williams M and Hogsteen K - Science (1977) 197, 452-5

82. Matthews DA, Alden RA, Bolin JT, Filman DJ, Freer ST, Hamlin R, Hol WGL, Kisliuk RL, Pastore EJ, Plante LT, Xuong N and Kraut J - J. Biol. Chem. (1978) 253, 6946-54

83. Matthews DA and Volz K in Molecular Structure and Biological Activity by Griffin JF and Duax WL (eds.) Elsevier Science Publishing Company (1982), 13-26

84. Matthews DA, Bolin JT, Burridge JM, Filman DJ, Volz KW, Kaufman BT, Beddell CR, Champness JN, Stammers DK and Kraut J - J. Biol. Chem. (1985a) 260 (1), 381-911

85. Matthews DA, Bolin JT, Burridge JM, Filman DJ, Volz KW and Kraut J - J. Biol. Chem. (1985b) 260 (1), 392-9

86. Morales DR and Greenberg DM - Biochim. Biophys. Acta (1964), 85, 360-76

87. Mowat JH, Boothe JH, Hutchings BL, Stokstad ELR, Waller CW, Angier RB, Semb J, Cosulich DB and Subba Row Y - Ann. NY Acad. Sci. (1946) 48 Art. 5, 279-82

88. Mowat JH, Boothe JH, Hutchings BL, Stokstad ELR, Waller CW, Angier RB, Semb J, Cosulich DB and Subba Row - J. Amer. Chem. Soc. (1948) 70, 14-18

89. Nagayama K and Wuthrich K - Eur. J. Biochem. (1981) 115, 653-7

90. Niccolai N, Rossi C, Mascagni P, Gibbons WA and Brizzi V - J. Chem. Soc. Perkin I (1985) 239-43

91. Ozaki Y, King RW and Carey PR - Biochemistry (1981) 20, 3219-25

92. Pastore EJ - Ann. NY Acad. Sci. (1971) 186, 43-54

93. Perault A-M and Pullman B - Biochim. Biophys. Acta (1961) 52, 266-80

94. Pfleiderer W, Liedek E, Lohrmann R and Rukwied M - Chem. Ber. (1960) 93, 2015-24

95. Poe M - J. Biol. Chem. (1973) 248 (20), 7025-32

96. Poulsen FM, Hoch JC and Dobson DM - Biochemistry (1980) 19, 2597-607

97. Purcell CM, Torrey HC and Pound RV - Phys. Revs. (1946) 69, 37-8

98. Purrmann R - Ann. Chem. (1940a) 544, 182-90

99. Purrmann R - Ann. Chem. (1940b) 546, 98-102

100. Rabinowitz JC in The Enzymes by Boyer PD, Lardy H and Myrback K (eds.) 2nd edition, Academic Press, New York (1960) Volume 2, 185-252

101. Reddy AV, Behnke WD and Freisheim JH - Biochim. Biophys. Acta (1978) 533, 415-27

102. Rosowsky A and Chen KKN - J. Med. Chem. (1974) 17 (12), 1308-11

103. Roth B, Bliss E and Beddell CR in Molecular Aspects of Anti-Cancer Drug Action by Neidle S and Waring MJ (eds.) MacMillan, London (1985), 363-93

104. Sahota A - unpublished results (1983)

105. Schmelkes FC and Rubin M - J. Amer. Chem. Soc. (1944) 66, 1631-2

106. Schöpf C and Wieland H - Ber. Deut. Chem. Ges. (1926) 59, 2067-72

107. Schöpf C and Reichert R - Ann. Chem. (1941) 548, 82-94

108. Seeger DR, Cosulich DB, Smith JM Jr. and Hultquist ME - J. Amer. Chem. Soc. (1949) 71, 1753-8

109. Sengupta PK, Bieri JH and Viscontini M - Helv. Chim. Acta (1975) 58 (5), 1374-9

110. Simon Z, Badilescu I and Racovitan T - J. Theor. Biol. (1977) 66, 485-95

111. Smith RG, Pegues JC, Farquhar D, Loo TL and Wang Y-M - Biomed. Mass Spectrom. (1981) 8 (4), 145-8

112. Smith RJ, Williams DH, Barna JCJ, McDermott IR, Haegele KD, Piriou F, Wagner J and Higgins W - J. Amer. Chem. Soc. (1985) 107, 2849-57

113. Smith RN, Hansch C, Kim KH, Omiya B, Fukumura G, Selassie CD, Jow PYC, Blaney JM and Langridge R - Arch. Biochem. Biophys. (1982) 215, 319-28

114. Smith SL, Patrick P, Stone D, Phillips AW and Burchall JJ - J. Biol. Chem. (1979) 254, 11475-84

115. Stephan A, Badilescu I and Simon Z - An. Univ. Timisoara, Ser. Sturte Fiz.-Chem. (1977) 15, 61

116. Stone D, Paterson SJ, Raper JH and Phillips AW - J. Biol. Chem. (1979) 254, 480-8

117. Subramanian S and Kaufman BT - Proc. Natl. Acad. Sci. USA (1978) 75, 3201-5

118. Taylor EC, Palmer DC, George TJ, Fletcher SR, Tseng CP and Harrington PJ - J. Org. Chem. (1983) 48, 4852-60

119. Temple C Jr., Bennet LL Jr., Rose JD, Elliott RD, Montgomery JA and Mangum JH - J. Med. Chem. (1982) 25, 161-6

120. Ter-Pogossian MM, Raichle ME and Sobel BE - Sci. Amer. (1980) 243 (4), 140-55

121. Thijssen HHW - Anal. Biochem. (1973) 54, 609-11

122. Viscontini M, Loeser E and Karrer P - Helv. Chim. Acta (1958) 41, 440-6

123. Visser GWM, van Halteren BW, Herscheid JDM, Brinkman GA and Hoekstra A - J. Chem. Soc. Chem. Commun. (1984) (10), 655-6

124. Vogel AI - A Textbook of Practical Organic Chemistry including Qualitative Organic Analysis, Longman (London), 4th edition (1978), 64-66

125. Volz KM, Matthews DA, Alden RA, Freer ST, Hansch C, Kaufman BT and Kraut J - J. Biol. Chem. (1982) 257 (5), 2528-36

126. Webber S and Whitely JM - Arch. Biochem. Biophys. (1985) 236 (2), 681-90

127. Werkheiser WC - J. Biol. Chem. (1961) 236, 888-93

128. Werkheiser WC - Fedn. Proc. (1969) 28, 362

129. Whitely JM and Huennekens FM - Biochemistry (1967) 6, 2620-5

130. Wieland H and Schopf C - Ber. Deut. Chem. Ges. (1925) 58, 2178-83

131. Wills L - Brit. Med. J. (1931) i, 1059

132. Yamamoto G and Oki M - J. Org. Chem. (1984) 49, 1913-7

133. Yamamoto G and Oki M - Tet. Lett. (1985) 26 (4), 457-60

134. Zakrzewski SF - J. Biol. Chem. (1966) 241, 2957-61