THIS WORK WAS CARRIED OUT from October, 1967 to October, 1970 in the Department of Chemistry at the UNIVERSITY OF ASTON IN BIRMINGHAM. It has been done independently and has not been submitted for any other degree.

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THE PREPARATION AND PROPERTIES OF 5-METHYLTETRAHYDROFOLIC ACID AND ITS ANALOGUES.

BY

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A Thesis presented for the degree of
Doctor of Philosophy
in the
University of Aston in Birmingham
JULY 1971

23NOV71 144815

THESIS 547.466 SAU

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SUMMARY

A simple method of preparation of 5 Methyltetrahydrofolic acid has been devised. This material is known to be the major folate present in natural materials and is involved in many biochemical reactions necessary for maintaining life. The calcium and barium salts were prepared and the chemical and physical properties studied. Nuclear Magnetic Resonance and Ultraviolet Spectrometry confirmed its identity and purity. It was shown by E.S.R. to exist as a free radical in the solid state. Microbiological Assay showed it was free from other folates.

The higher analogues, ethyl, propyl and butyl were also prepared and identified by the above techniques.

The toxicity and tumour inhibitory properties of the calcium salts were investigated and showed that they were non toxic and of possible use as tumour inhibitors. 5 Methyltetrahydrofolic acid was shown to effectively reverse the toxicity of methotrexate in the treatment of solid tumours.

The 5 Methyl and 5 Ethyl derivatives of tetrahydromethotrexate were also prepared, purified and identified. The Ultraviolet and Nuclear Magnetic Resonance Spectra were compared with those of the folate series. Trials on their toxicity and tumour inhibitory properties were conducted.

The overall advantage of this method for the production of folate derivatives is that they can be prepared, quickly and cheaply in large quantities without the isolation of intermediates.

However, unsuccessful attempts were made to prepare the 5 isopropyl, the 5 benzyl and 5 β phenyl ethyl derivatives of tetrahydrofolic acid and also 5 methyltetrahydropteridine.

TABLE OF CONTENTS

		Page
SUMMARY		
INTRODUCTION		1
Fig. I		1
Table I	Response of L. casei, P. cerevisiae and S. faecalis to naturally occurring folate derivatives.	1
Fig. II		1
Fig. III		2
Fig. IV		2
Fig. V		2
Fig. Va		3
EXPERIMENTAL		6
Preparation of 5-Methyltetrahydrofolic Acid.		7
Table II	Rf Values in Various Solvents - Experiment A1.	8
Table III	Rf Values in Various Solvents - Experiment A2.	9
Table IV	Rf Values in Various Solvents, Pteridines.	9
Table V	Rf Values in Various Solvents - Experiment A4.	10
Table VI	Rf Values in Various Solvents - Experiment A9.	15
Table VII	Microbiological Assay - Experiment A9.	15
Table VIII	Rf Values in Various Solvents - Experiment A9, Barium Salt.	15
Table IX	Spectral Data of 5-Methyltetrahydrofolic Acid. Barium Salt.	16
Table X	Microbiological Assay - Experiment A14 and A16.	17
Table XI	Spectral Data of 5-Methyltetrahydrofolic Acid. Calcium Salt.	18

Page

Table XII Rf Values in Various Solvents - Experiments A15 and A16. 18

Table XIII Spectral Data of 5-Methyltetrahydrofolic Acid. 18

Preparation of 5-Ethyltetrahydrofolic Acid. 19

Preparation of 5-n Propyltetrahydrofolic Acid. 19

Table XIV Spectral Data of 5-Ethyltetrahydrofolic Acid. Calcium Salt. 20

Table XV Microbiological Assay - Experiment C. 20

Table XVI Rf Values in Various Solvents - Experiments C, G and I. 20

Preparation of 5-n Butyltetrahydrofolic Acid. 21

Table XVII Spectral Data of 5-n Propyltetrahydrofolic Acid. Calcium Salt. 21

Table XVIII Microbiological Assay - Experiments G and I. 21

Table XIX Spectral Data of 5-n Butyltetrahydrofolic Acid. Calcium Salt. 21

Preparation of 5,10-Methylene Tetrahydrofolic Acid. 22

Preparation of 5-Isopropyltetrahydrofolic Acid. 22

Table XX Spectral Data of 5,10-Methylene tetrahydrofolic Acid. Calcium Salt. 23

Preparation of 5-Benzyltetrahydrofolic Acid. 23

Table XXI Proton Magnetic Resonance Data. 23

Table XXII U.V. Spectral Data - Experiments E1 and F1. 23

Preparation of 5-β Phenylethyltetrahydrofolic Acid. 25

Preparation of 5-Methyltetrahydromethotrexate. 25

Preparation of 5-Ethyltetrahydromethotrexate. 26

Table XXIII Spectral Data of Methotrexate Analogues. 26

Table XXIV Rf Values of Methotrexate Derivatives in Various Solvents. 26

		Page
Preparation of 2-amino, 4 hydroxy-tetrahydropteridine.		27
RESULTS AND DISCUSSION		29
Preparation of 5-Methyltetrahydrofolic Acid.		29
Table XXV	Comparison of pH condensation and % Yield.	29
Table XXVI	Comparison of Male Ratio and % Yield.	29
Table XXVII	Spectral Data of Folate Analogues.	30
Table XXVIII	Rf Values in Various Solvents - Experiment A .	30
Fig. VI		30
Preparation of 5-Ethyl, 5-Propyl and 5-Butyltetrahydrofolic Acids.		31
Preparation of 5,10-Methylenetetrahydrofolic Acid.		32
Preparation of 5-Isopropyltetrahydrofolic Acid.		32
Preparation of 5-Benzyltetrahydrofolic Acid.		32
Fig. VII		32
Preparation of 5-β Phenylethyltetrahydrofolic Acid.		33
Preparation of 5-Methyl and 5-Ethyltetrahydromethotrexate.		33
Table XXIX	Spectral Data of Methotrexate Analogues.	33
Table XXX	Rf Values in Various Solvents - Methotrexate Analogues.	33
Fig. VIII		34
PROTON MAGNETIC RESONANCE SPECTROMETRY		35
Table XXXI	Proton Magnetic Resonance Spectra in Deuterium Oxide.	35
Table XXXII	Proton Magnetic Resonance Spectra in Trifluoro Acetic Acid.	35
Table XXXIII	Chemical Shift of C_7 Proton of Folic Acid with Concentration.	35
Table XXXIV	Chemical Shift of α and β Aromatic Protons of Folic Acid with Concentration.	35
Table XXXV	Chemical Shift of α and β Aromatic Protons of pNH_2 Benzoyl L-Glutamic Acid with Concentration.	35

Page

Table XXXVI Chemical Shift of C_6 and C_7 Protons of $2NH_2 4OH$ Pteridine with Concentration. 35

ELECTRON SPIN RESONANCE SPECTROMETRY 36

Table XXXVII Energies of Molecular Orbitals. 36

Results of E.S.R. Determinations. 38

Table XXXVIII E.S.R. of Folinic Acid. 38

Table XXXIX E.S.R. of Tetrahydrofolic Acid. 38

Table XL E.S.R. of A9 (5-Methyltetrahydrofolic Acid). 38

Table XLI E.S.R. of C1 (5-Ethyltetrahydrofolic Acid). 38

Table XLII E.S.R. of Sample E1 and F1. 38

Table XLIII Values of "g" for the 5-Alkyltetrahydrofolic Acids. 39

BIOLOGICAL TESTING OF FOLATE DERIVATIVES 40

Table XLIV Effect of 5-Methyltetrahydrofolic Acid on the Survival of Methotrexate-treated Mice. 40

Table XLV Effect of 5-Methyltetrahydrofolic Acid and Citrovorum Factor on the Survival of Methotrexate-treated Mice. 40

Table XLVI Effect of Folate Analogues on the Survival of Methotrexate-treated Mice. 43

Table XLVII Effect of 5-Methyltetrahydrofolic Acid and Methotrexate on Survival of Mice with R1 Lymphoma. 44

Acknowledgements. 44

References. 44

INTRODUCTION

Folic acid and its derivatives play an important role in metabolism. This was recognised by workers in the 1930's[1-9] and further investigation led to the elucidation of the structure of folic acid in 1946 by Angier et al.[10] It was given the name pteroylglutamic acid but the alternative name folic acid of Mitchell et al.[8] is most commonly used.

The multiplicity of the derivatives existing in biological materials and the complexity of their chemical structure led to much confusion concerning the natural occurrence of the folate derivatives. Early research demonstrated that naturally occurring folate derivatives differed from each other in regard to the number of glutamate residues present in the molecule (Fig. I) the state of oxidation of the pteridine nucleus and the nature of any one-carbon substituents.

Most of the folates have growth promoting effects on various bacteria. This was the reason why microbiological assay was used as a method of identifying and as a criterion of purity for many of the folate analogues. Three organisms have been commonly used: Lactobacillus casei, Streptococcus faecalis, known now as Streptococcus faecium, and Pediococcus cerivisiae. Care must be taken however to ensure that oxidative degradation does not take place before or during assay; this has led to much of the confusion in the literature prior to 1960.

None of these organisms are capable of responding to those derivatives in which more than two glutamate residues (Fig. I) are linked to the folate (Table I) and it is necessary to remove these residues using incubation with chicken liver conjugase before assay.

The main type of folate present in liver is 5-methyltetrahydrofolic acid (Fig. II) in its peptide form. In the serum the folate exists as "free" 5-methyltetrahydrofolic acid. 5-Methyltetrahydro-

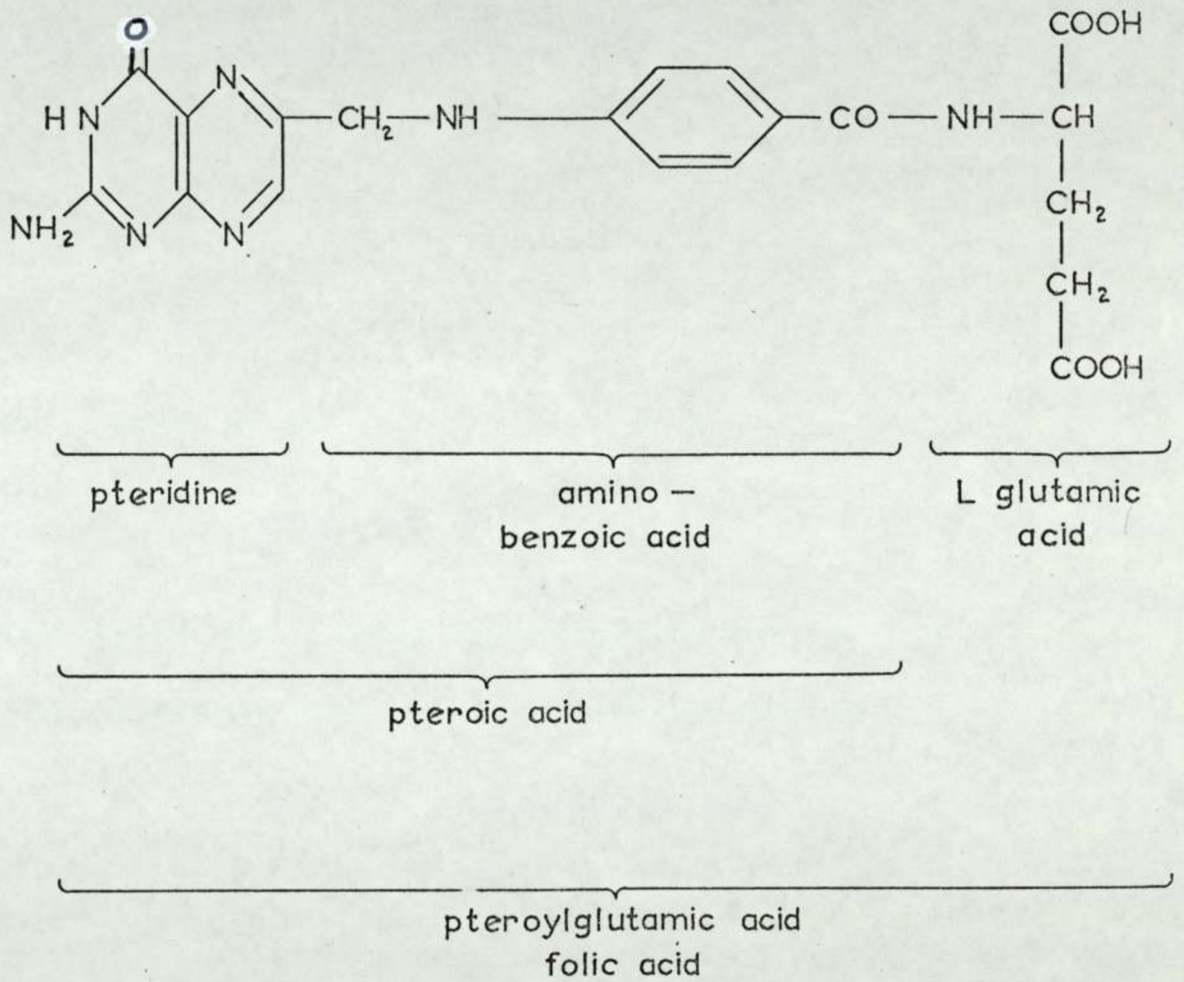


Fig. I

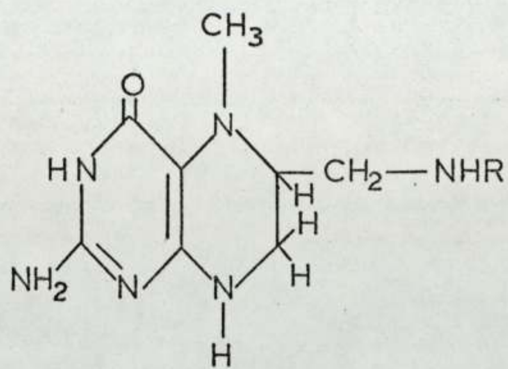
Table I

Response of L. casei, P. cerevisiae and S. faecalis to naturally occurring folate derivatives.

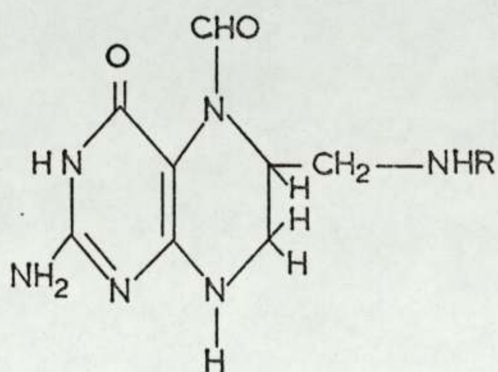
Compound	Growth Activity [a] for		
	L. casei	P. cerevisiae	S. faecalis
F [b]	+	—	+
DHF [c]	+	—	+
THF [d]	+	+	+
5CHOTHF [e]	+	+	+
10CHOF [f]	+	—	+
10CHODHF [g]	+	—	+
10CHOTHF [h]	+	+	+
$5CH_3THF$ [i]	+	—	—
F glutamate [j]	+	—	+
F diglutamate [k]	+	—	—
F hexaglutamate [l]	—	—	—
THF diglutamate [m]	+	+	—

a + indicates a response of at least 50% of a maximum
— indicates a response of less than 5% of the maximum

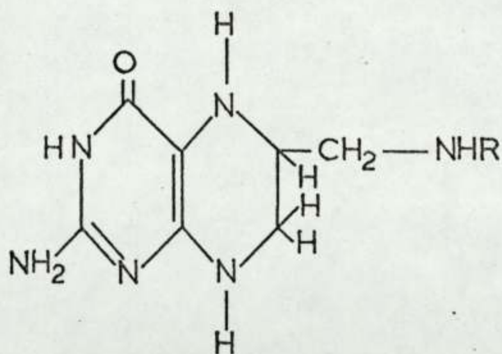
b folic acid
c dihydrofolic acid
d tetrahydrofolic acid
e 5-formyltetrahydrofolic acid
f 10-formylfolic acid
g 10-formyldihydrofolic acid
h 10-formyltetrahydrofolic acid
i 5-methyltetrahydrofolic acid
j folylglutamate
k folyldiglutamate
l folylhexaglutamate
m tetrahydrofolyldiglutamate.



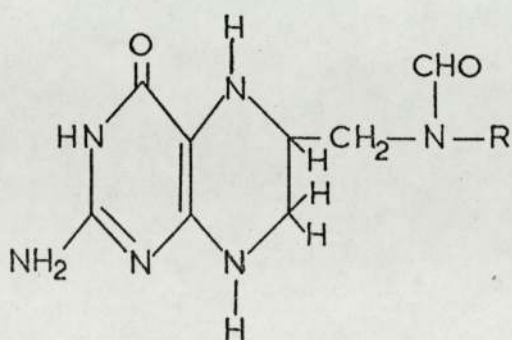
5 Me THF



5 CHO THF



THF



10 CHO THF

Fig. II

folic acid has a different growth response to S. faecalis and L. casei than other folates (Table I). This became a very important criterion with the discovery that 5-methyltetrahydrofolic acid and its derivatives were major constituents of folates in natural materials.

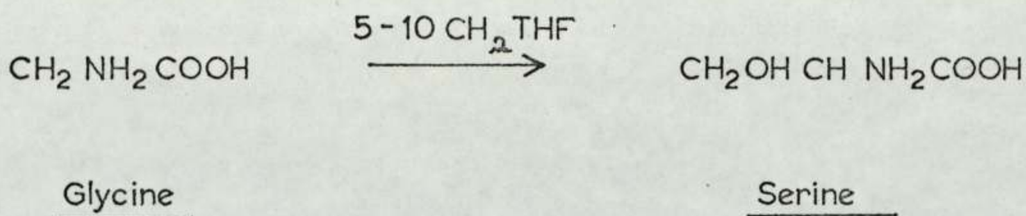
Shortly after the isolation, structure determination, and synthesis of folic acid, work was begun on the physiological changes brought about in folate deficiency. Franklin et al.[11] studied the changes brought about by a folate deficient diet on young female rats. The folate deficient rats were marked by slow growth, anemia, and low white cell count. When the action of folate was inhibited by aminopterin, a folate antimetabolite, death followed. So folate is involved in many biochemical reactions necessary for maintaining life in the rat. This is also true for man.

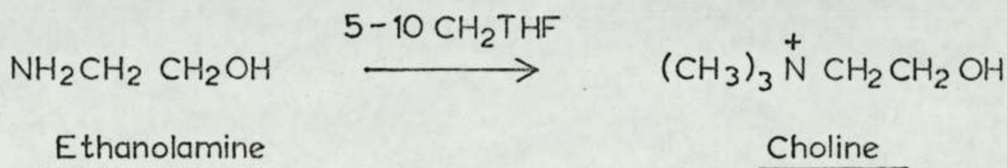
Folic acid coenzymes participate in animal, plant and microbial metabolism by controlling the transfer of single carbon units which are at the oxidation level of formic acid or formaldehyde. Some of the reactions mediated by folic acid coenzymes are:

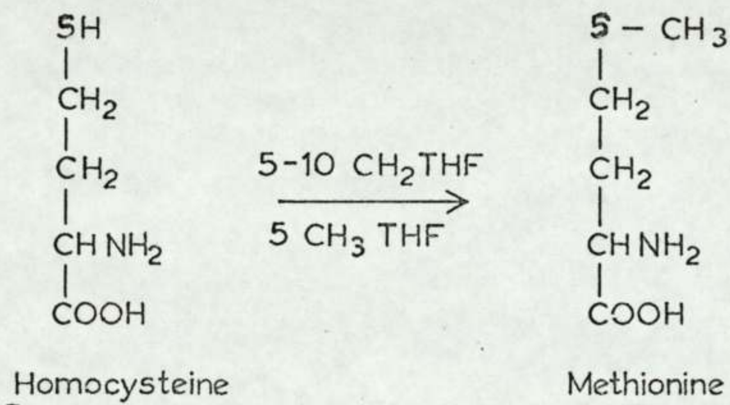
a)	conversion of glycine to serine	Fig. III
b)	methylation of ethanolamine to choline	Fig. III
c)	methylation of homocysteine to methionine	Fig. III
d)	methylation of uracil intermediate to thymine	Fig. III
e)	introduction of C-2 and C-8 in purine biosynthesis	Fig. IV

One or more of the four coenzymes, (Fig. V) all of which are derivatives of 5,6,7,8-tetrahydrofolic acid (II) (Fig. V), are involved in the one carbon transfer reactions listed above.

It has been shown that 10-formyltetrahydrofolic acid (V) is the source of the number two carbon atom in the formation of the purine ring and that 5,10-methenyltetrahydrofolic acid (IV) is the source of the number eight carbon in the same ring[13]. 5,10-Methylenetetra-







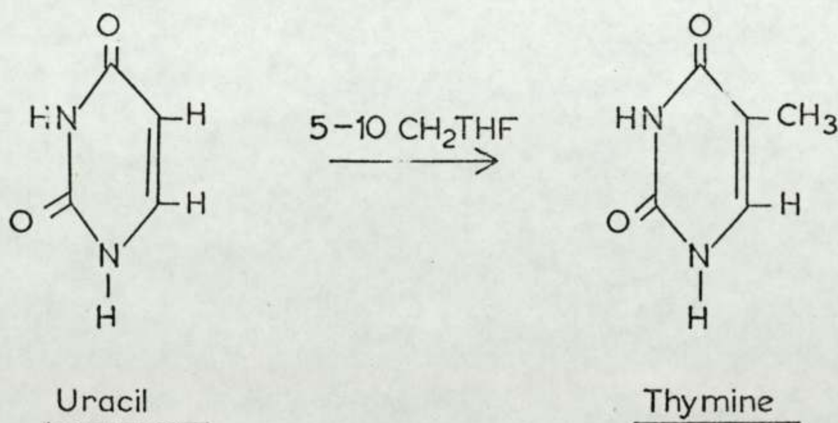
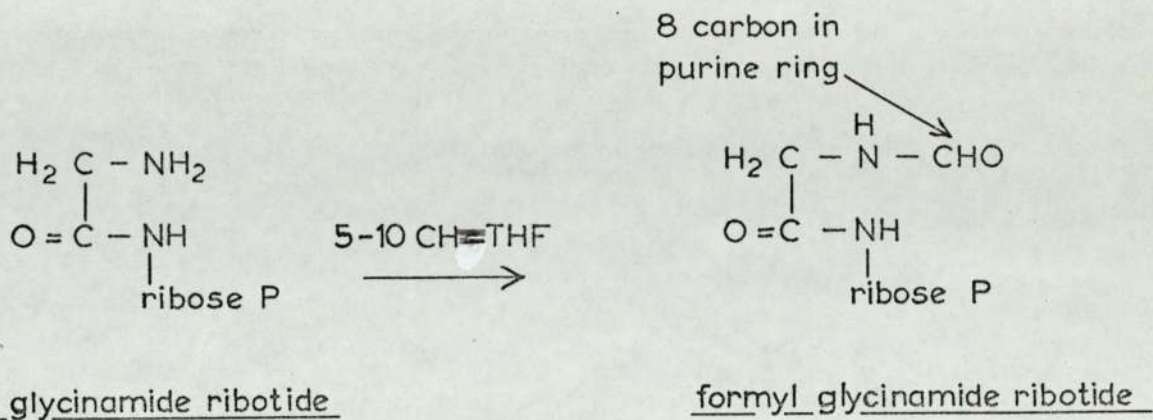


Fig. III



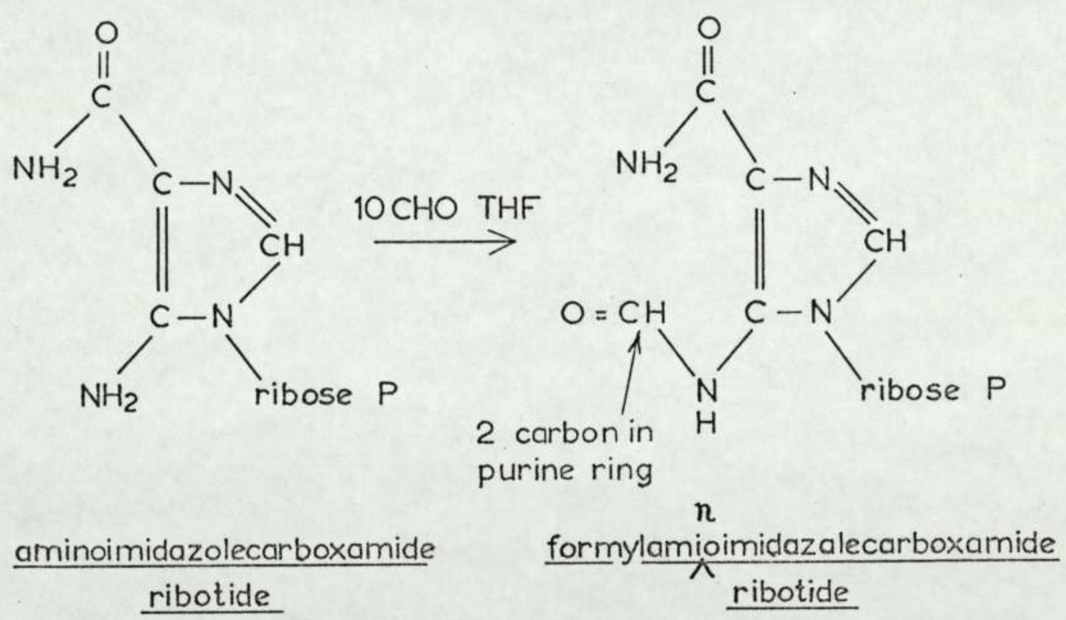


Fig. IV

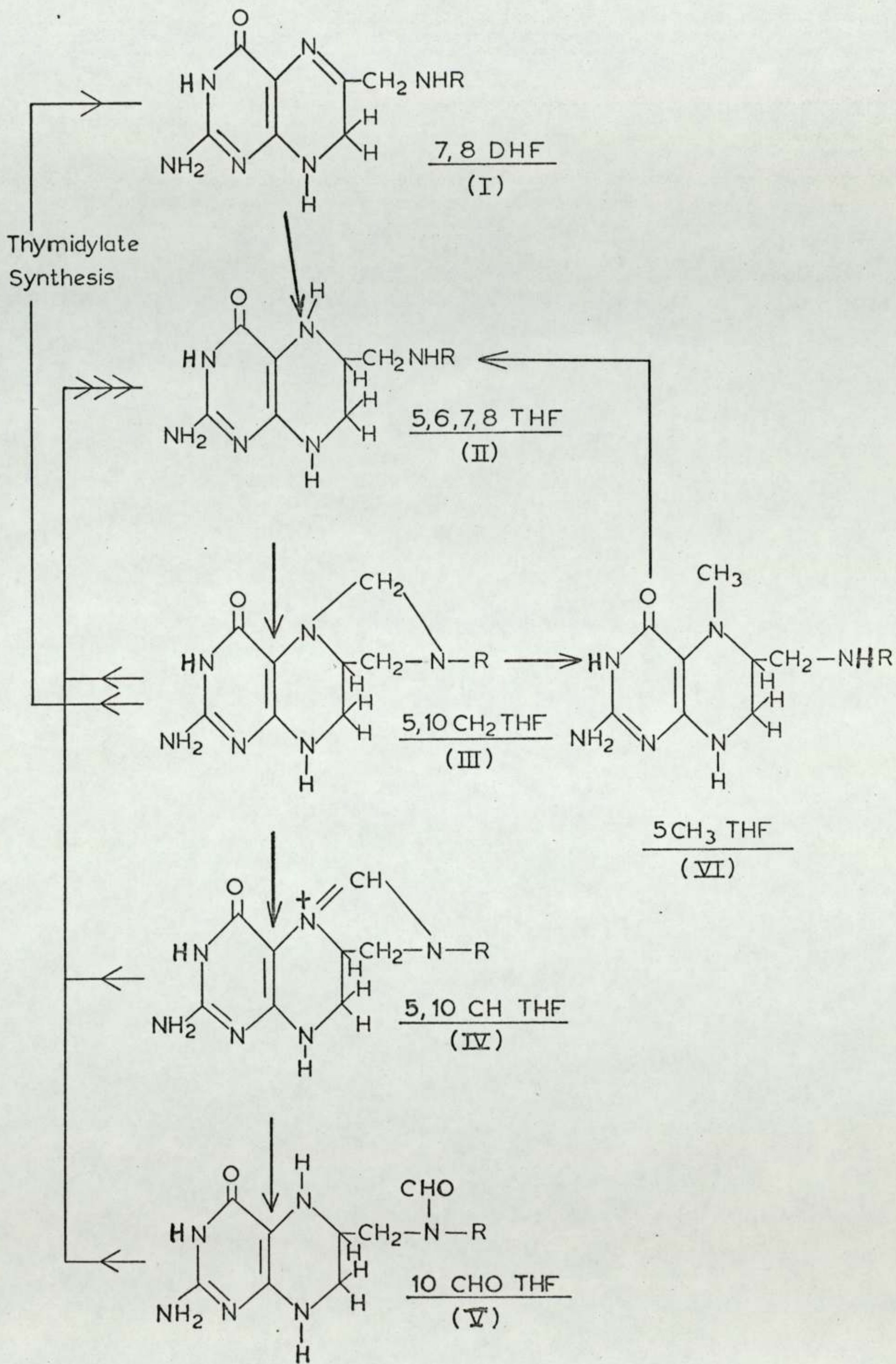


Fig. V

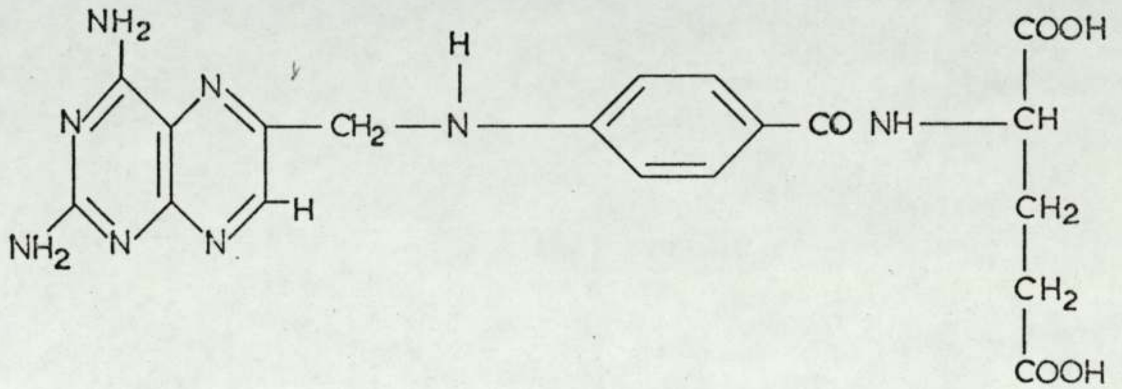
hydrofolic (III) acid is concerned with hydroxymethyl or methyl group transfer to various acceptors. These reactions include the formation of 5-hydroxymethylcytosine from cytosine, methionine from homocysteine, thymine from uracil and serine from glycine. 5-Methyltetrahydrofolic acid (VI) is also involved in the formation of methionine from homocysteine.

The reduction of dihydrofolic acid (I) to tetrahydrofolic acid (II) is performed by the enzyme dihydrofolic reductase. However this reduction can be inhibited by the antimetabolites aminopterin (VII) and methotrexate (VIII) (Fig. Va).

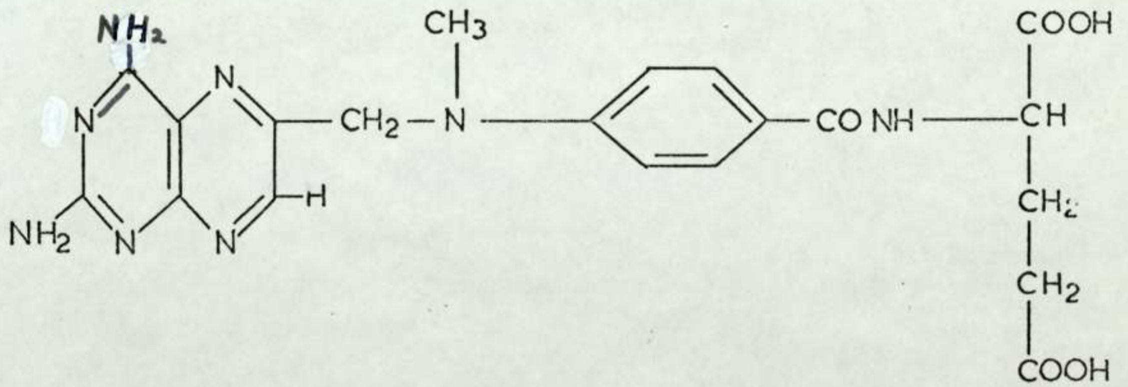
Thus the folic acid enzymes are involved in a wide scope of biochemical transformations which are quite basic to the formation of new cells. Due to this and the relatively high folic acid content and rapid growth of cells that are found in cancers, many workers have connected this disease with a malfunctioning of the folic acid system.

Many antimetabolites have been synthesised with the hope of preferentially inhibiting or preventing this system from functioning. Foremost amongst these are methotrexate (VIII) and aminopterin (VII) as shown in Fig. Va. The inhibition constitutes a block in the conversion of dihydrofolic acid to tetrahydrofolic acid. As a consequence of this block, tissues become deficient in the tetrahydrofolic acid derivatives, and this produces many effects similar to those observed in nutritional folate deficiency.

However a crucial consequence must be the depression of thymidylate synthesis with a consequent failure in DNA synthesis and arrest of cell division. This has lethal results in rapidly proliferating tissues such as intestinal mucosa, bone marrow and the cancer cell. However, these analogues are extremely toxic and although powerful inhibitors of malignant cell growth their use is restricted by their toxicity.



VII Aminopterin



VIII Methotrexcate

Fig. Va

A subsequent search was carried out to produce reversal of the toxic effects of these analogues without effecting their inhibitory properties. Early reports suggested that the toxic effects could be reversed by administration of large doses of folic acid but this is true only to a limited extent.[14-17] However, 5-formyltetrahydrofolic acid has proved much more useful. Combinations of large doses of methotrexate and 5-formyltetrahydrofolic acid give improved survival rates in leukemic mice[18] and appear advantageous in the treatment of human tumours.[19]

It has been already stated that methotrexate would interfere with the production of thymidylate for incorporation into D.N.A. [20,21] This production would utilise 5,10 methylenetetrahydrofolic acid(III) and cause an increase in dihydrofolic acid (I), which is prevented from further reduction by the inhibitory action of methotrexate on dihydrofolic acid reductase. Methotrexate thus irreversibly inhibits D.N.A synthesis and depletes the folate pool.

However administered 5-formyltetrahydrofolic acid can enter the folate pool without reduction by dihydrofolic acid reductase, probably by conversion to the 5,10 methenyl compound (IV) which is then reduced to 5,10-methylenetetrahydrofolic acid (III). This may be further reduced to 5-methyltetrahydrofolic acid (VI) which can be converted by the loss of a methyl group to tetrahydrofolic acid (II), the reaction involving vitamin B_{12}. Any inhibition of the reactions involving vitamin B_{12} would result in inhibition in the growth of tumours.

Since 5-methyltetrahydrofolic acid (VI) features in this cycle and is the main storage form of folates in the body[22] it was anticipated that it would have similar, if not enhanced, protective properties to 5-formyltetrahydrofolic acid as it would require no prior metabolism. Considering the importance of 5-methyltetrahydrofolic acid a convenient method of synthesis was required to produce

the material in quantity to allow its chemical and biological properties to be studied.

5-Methyltetrahydrofolic acid was first isolated from horse liver by Donaldson and Kereztesy[23-25] who named the material "prefolic A". This material was active for L. casei but not for S. faecalis[26]. It has been prepared chemically from tetrahydrofolic acid by formation of 5,10-methylenetetrahydrofolic acid by addition of formaldehyde and further reduction of this compound by sodium borohydride to the 5-methyl derivative[27,28]. The tetrahydrofolic acid is usually obtained by catalytic hydrogenation[29,30] or by chemical reduction of folic acid[31,32]. It has also been prepared from folinic acid[33].

We considered a direct synthesis of 5-methyltetrahydrofolic acid and its analogues from folic acid without the intermediate isolation of tetrahydrofolic acid. A similar application to the production of reduced analogues of methotrexate should also be possible.

All the compounds prepared were submitted for testing for their toxicity and tumour inhibitory properties. 5-Methyltetrahydrofolic acid was submitted for trials on reversal of methotrexate toxicity.

EXPERIMENTAL

Chemicals were obtained from the following sources: folic acid, Koch Light Ltd., Colnbrook, Bucks; methotrexate and folinic acid, Lederle Laboratories, Chadwell Heath, Essex; and DEAE cellulose (DE 22 and DE 52), W. & R. Balston Ltd., Maidstone, Kent.

Ultraviolet spectra were determined in aqueous solution at pH 1.0, 7.0, and 13.0 using a Unicam SP 700 or Hilger & Watts Uvispek.

Thin-layer chromatography was performed with 0.25 mm. depth Cellulose powder MN300 F254 (Macherey, Nagel and Co., Duren, Germany) and developed at room temperature in the dark.

The following solvent systems were used:

(1) 0.1M phosphate buffer, pH 7.0, containing mercaptoethanol (0.5% v/v).

(2) n propanol/water/0.88 s/g aqueous ammonia (200:100:10, v/v) containing mercaptoethanol (0.5% v/v).

(3) The organic phase of 1-butanol/acetic acid/water (4:1:5, v/v) containing mercaptoethanol (0.5% v/v) equilibriated overnight.

Compounds were detected as quenching (folates) or fluorescent spots (unconjugated pteridines) when viewed under ultraviolet light emitting at either 254 or 365 mμ.

Proton magnetic resonance spectra were recorded with a Perkin-Elmer R10 or R14 (60 MHz or 100 MHz) using solvent deuterium oxide (> 99% D_2O) and external reference of tetramethylsilane (TMS) or solvent trifluoroacetic acid (TFA) and internal standard of TMS.

In experiments A1-A17 the yield and purity of freeze dried product was determined in 0.1M phosphate buffer, pH 7.0 $\epsilon = 31.7 \times 10^3$ M^{-1} cm^{-1} at 290 mμ[28].

Yield and purity of calcium and barium salts was determined in 0.1N sodium hydroxide, pH 13.0 $\epsilon = 28.5 \times 10^3$ M^{-1} cm^{-1} at 290 mμ.[28]

Preparation of 5-Methyltetrahydrofolic Acid.

Experiment A1

Sodium borohydride (250 mg., 6.5 mmoles) in water (10 ml.) was added to a stirred solution of folic acid (250 mg., 0.6 mmoles) in 0.66M Tris buffer, pH 7.8 (30 ml.), under nitrogen at room temperature. After 10 minutes, excess sodium borohydride was destroyed with 5N acetic acid and the solution adjusted to pH 7.0. Formaldehyde (0.10 ml., 37% w/v, 4.0 mmoles) was added, immediately followed by sodium borohydride (500 mg., 13.0 mmoles) in water (20 ml.). The mixture was incubated for 1 hour at 37°C under a slow stream of nitrogen and cooled, and mercaptoethanol (1.0 ml.) was added. The pH was adjusted to 7.0 and the mixture diluted to 100 ml. and stored in the fridge.

The reaction mixture was chromatographed on DEAE cellulose (DE 22). Prior to use in column chromatography, the DEAE cellulose was washed with 1.0M Na_2HPO_4, pH 7.0, until the washings were colourless, washed with water until the suspension was approximately pH 7.0 and stored under water. A slurry of the DEAE cellulose was poured into a 3 x 23 cm. glass column to a height of 12 cm. and the column was washed with ammonium acetate (200 ml., 0.13M, pH 6.9) containing mercaptoethanol (0.2M). The reaction mixture was carefully layered at the top of the column and eluted by a linear gradient method in which the mixing chamber contained ammonium acetate (500 ml., 0.13M, pH 6.9) and the reservoir contained ammonium acetate (500 ml., 0.4M, pH 6.9). Both ammonium acetate solutions contained mercaptoethanol (0.2M). The elluent was collected at the rate of 1 ml/min. in 10 ml. fractions using an automatic fraction collector.

The extinction at 290 mμ was determined after dilution with 0.1M phosphate buffer, pH 7.0. A complete spectrum was recorded every fifth fraction. The product, 5-methyltetrahydrofolic acid was found in tubes 70-100 (300 ml.) and was characterized by an extinction maxi-

mum at 290 mμ and a minimum at 250 mμ. Fractions 70-100 were pooled and freeze dried (two days) to give a white slightly oily residue.

The residue was dissolved in ammonium hydroxide (2N) and freeze dried (2 days) to give a yellow solid which was hygroscopic. This material was vacuum dried at 30° overnight. Purity by U.V. 10%. T.L.C. data is given in Table II. Experiment abandoned at this stage and an attempt made to increase the purity.

Table II

Rf Values in Various Solvents [a]

Solvent [b]	F.A. [c]	A1
1	0.5 (0.63) slight blue fluor. [d]	0.80 (0.56) blue fluor. [d]
2	0.23	1.0 (0.57) blue fluor. [d]

a These were detected as quenching spots unless otherwise indicated. Minimum detectable amounts about 5μg in 1μl.

b As in "Experimental".

c Abbreviations as in Table I.

d Fluorescent impurity.

Experiment A2

The preparation was carried out as for Experiment A1. The product was found in tubes 55-105 and these were bulked and freeze dried. The residue after freeze drying consisted of yellow viscous liquid containing long colourless needle like crystals. Purity 20%. These crystals turned to yellow viscous liquid on exposure to the atmosphere.

This liquid was triturated with dry methanol (20 ml.) and the

Table III

R_f values in Various Solvents[a]

Solvent	FA	A2
1	0.53 slight blue fluor.	0.80
	0.39	0.53 blue fluor.
		0.33 green fluor.
2	.39 slight blue fluor.	0.73
	.32	0.53 blue fluor.
		0.32 green fluor.

[a] Abbreviations as in Table I and II.

Table IV

Rf Values in Various Solvents [a]

Solvent	pNH_2BA [b]	pNH_2BGA [c]	$2NH_24OHPt6COOH$ [d]	X [e]	$2NH_24OHPt6CHO$ [f]	$2NH_24OHPt$ [g]
1	0.86 blue fluor.	0.95 blue fluor.	0.46 light blue fluor. 0.56 light blue fluor.	0.35 light green fluor.	0.46 light blue fluor.	0.50 light blue fluor.
2	0.67 blue fluor.	0.60 blue fluor.	0.25 light blue fluor. 0.48 light blue fluor.	0.29 green fluor.	0.48 light blue fluor.	0.51 vivid blue fluor.

[a] Abbreviations as in Table I and II.

[b] pNH_2 benzoic acid.

[c] pNH_2 benzoyl-L-glutamic acid.

[d] $2NH_24OH$ pteridine 6COOH.

[e] xanthopterin.

[f] $2NH_24OH$ pteridine 6CHO.

[g] $2NH_24OH$ pteridine.

residue filtered and dried under nitrogen. Purity as determined by UV 30%. (Extinction maximum at 291 mμ and minimum at 251 mμ). Microbiological assay with Lactobacillus casei was performed and showed a 31% activity. Activity defined as response/ml. to L. casei divided by weight/ml. of sample, using folic acid as a standard. T.L.C. data is shown in Table III and IV. Experiment terminated at this stage.

Experiment A3

The preparation was carried out as for Experiment A1, the solution being stored overnight in the fridge under nitrogen. The reaction mixture was added to the column at 4°C in the dark. The elution was carried out as previously and the first 200 ml. were discarded. The eluate was collected at 1ml./min. in fractions (10 ml). The product was found in tubes 40-80 and these were bulked and freeze dried. The temperature of the cold room rose to 60° overnight. The experiment was terminated at this stage.

Experiment A4

The preparation was carried out as for Experiment A1, the solution being stored overnight in the fridge under nitrogen.

The reaction mixture was added to the column at 4° in the dark. The elution was carried out as previous and the first 200 ml. were discarded. The temperature of the coldroom rose to 20°C. The product was found in tubes 41-81 and these were bulked and freeze dried.

To the freeze dried residue was added ethanol (10 ml.) and the yellow precipitate (A4 I) was filtered using No. 4 sinter. The product was rapidly dried in air but turned slightly brown. Purity as determined by UV 45% (extinction maximum at 291 mμ and minimum at 245mμ).

Microbiological assay with Lactobacillus casei and Streptococcus faecalis showed activities of 16% and 7% respectively. T.L.C. data

is shown in Table V.

Ethanol (10 ml.) was added to another portion of freeze dried residue and the precipitate (A4 II) was filtered and dried under high vacuum at r.t. Purity as determined by UV 35%.

Extinction maximum was at 290 mμ and minimum at 245 mμ. Absorbance ratio $A_{290}/A_{245} = 2.7$. Microbiological assay (sample A4 II) with L. casei and S. faecalis showed activities of 40% and 21% respectively. Microbiological assay (repeat A4I) with L. casei and S. faecalis showed activities of 12% and 8% respectively.

Table V

Rf Values in Various Solvents [a]

Solvent	FA	A4
1	0.29	0.78

[a] Abbreviations as in Table I and II.

Experiment A5

The preparation was carried out as for Experiment A1, the solution being stored overnight in the fridge under nitrogen.

The reaction mixture was added to the column at 4° in the dark. The elution was carried out as previous and the first 200 ml. were discarded. The product was found in tubes 44-94 and these were bulked and freeze dried for 3 days. The freeze dried residue was carefully dissolved in ammonium acetate (20 ml., 0.13M, pH 6.9) containing mercaptoethanol (0.2M) and layered on the top of a DEAE cellulose column. The column material, the preparation and gradient

elution were the same as for the initial purification and the first 200 ml. were discarded. The product was found in tubes 44-96 and these were bulked and freeze dried for 3 days. The freeze dried residue was dissolved in cold deaerated water (40 ml.) containing mercaptoethanol (10^{-3}M) and carefully layered on the top of a cellulose column.

Prior to use the cellulose was washed and equilibrated with cold water (3 litres) containing mercaptoethanol (10^{-3}M). A slurry of the cellulose was poured into a 3 x 23 cm. glass column to the height of 12 cm. and the column washed with cold water (1 litre) containing mercaptoethanol (10^{-3}M). Elution was carried out with water containing mercaptoethanol (10^{-3}M) and 10 ml. fractions were collected at the rate of 1ml./min. The product was found in tubes 6-30 and these were bulked and freeze dried for 3 days.

To the freeze dried residue was added ethanol (20 ml.) and the white precipitate (80 mg.) was filtered using No. 4 sinter. The product was rapidly dried under vacuum. Purity as determined by U.V. 52%. The alcohol filtrate was freeze dried and again treated with alcohol (10 ml.). The off white precipitate was filtered, dried and its purity determined. No UV spectrum was observed.

Preparation of DEAE cellulose column

The DEAE cellulose (DE 22) was washed with 1.0M Na_2HPO_4, pH 9.0 until the washings were colourless (5 litre), washed with water until the washings were pH 7.5. A slurry of the DEAE cellulose was poured into a 3 x 23 cm. glass column to a height of 12 cm. and the column was washed with distilled water (10 litre) until pH 7.1. The cellulose was washed with ammonium acetate (3 litre, 0.13M, pH 6.9) and a sample of washing was freeze dried. This left a small sticky residue. The DEAE cellulose was washed with ammonium acetate (3 litre, 0.5M,

pH 6.9) and a sample was freeze dried. This left a very small residue. The DEAE cellulose column was equilibrated with ammonium acetate (1 litre, 0.13M, pH 6.9) and used for Experiment A6.

Experiment A6

The preparation was carried out as for Experiment A1, the solution being stored overnight in the fridge under nitrogen. The reaction mixture was added to the column at 4° in the dark. The elution was carried out as previous and the first 100 ml. were discarded. The product was found in tubes 1-65 and these were bulked and freeze dried (5 days).

Freeze drying gave white material (290 mg.) which turned grey on exposure to air. Purity 35%. Extinction maximum was at 289 mμ, and minimum at 248 mμ. Absorbance ratio A289/A248 = 2.5.

Preparation of Barium Salt

The freeze dried product (300 mg., 35% pure) was dissolved in deaerated water (10 ml.) containing sodium chloride (100 mg) and the pH was adjusted to 7.0 and the solution filtered. Barium chloride (1 ml. of 22g. $BaCl.2H_2O$/100 ml. water) and mercaptoethanol (1 ml.) were added to the solution. The barium salt was precipitated by addition of absolute ethanol (10 ml.) and the solution cooled overnight. The barium salt was filtered at 5°, washed with 50% aqueous ethanol (10 ml.), followed by absolute ethanol (10 ml.), and dried rapidly under high vacuum at room temperature. Yield 50 mg.

The barium salt was recrystallised by dissolving in minimum amount deaerated water containing mercaptoethanol (10^{-3}M) (10 ml.) and diluting slowly with methanol (10 ml.) to give a crystalline product which was filtered, washed and dried rapidly under vacuum. Yield 10mg. Purity 29%. To the filtrate was added methanol (10 ml.) and the solution cooled in the fridge overnight. Product filtered, washed

and dried rapidly under vacuum. Yield 24 mg. Purity 70%.

Preparation of Column

The DEAE cellulose (DE 22) column was prepared as for Experiment A7 and eluent (3 litre) was freeze dried. This gave a white crystalline solid which was hygroscopic on exposure to air.

Experiment A7

The preparation was carried out as for Experiment A1, the solution being stored overnight in the fridge under nitrogen. The DEAE cellulose column was equilibrated with water containing mercaptoethanol (0.2M) and the reaction mixture was carefully layered at the top of the column and eluted by a linear gradient method in which the mixing chamber contained water (500 ml.) and the reservoir ammonia (500 ml. 1M). Both solutions contained mercaptoethanol (0.2M). The eluent was collected at the rate of 1ml./min. in 10 ml. fractions. The product was found in tubes 10-90 and these were bulked and freeze dried (3 days). This gave a yellow hygroscopic material. Purity 15%. Experiment terminated at this stage.

Experiment A8

The preparation was carried out as for Experiment A1, the solution being stored overnight in the fridge under nitrogen. The reaction mixture was chromatographed on DEAE cellulose (DE 52 preswollen microgranular diethylaminoethyl cellulose).

Prior to use in column chromatography the DEAE cellulose was equilibrated with ammonium acetate (300 ml., 0.13M, pH 6.9) containing mercaptoethanol (0.2M) and the fines removed. The column was packed and eluted as in Experiment A1. The product was found in tubes 17-64 and these were bulked and freeze dried. The temperature of the cold room rose to 60° overnight. The experiment was terminated at this stage.

Recycling of DEAE cellulose (DE 52).

The DEAE cellulose (DE 52) was recycled by washing with hydrochloric acid (15 times volume, 0.5N) and was left standing for 30 minutes. The cellulose was filtered and washed with water until the pH of the eluent was 4.0. It was then washed with sodium hydroxide (15 times volume, 0.5N) and was left standing for 30 minutes. The cellulose was filtered and washed with water until the pH of the eluent was 7.0. The second treatment was repeated.

Experiment A9

Sodium borohydride (6g., 160 mmoles) in water (50 ml.) was added to a stirred solution of folic acid (6g., 15 mmoles) in 0.066M Tris buffer, pH 7.8 (200 ml.), under nitrogen at room temperature. After 15 minutes, excess sodium borohydride was destroyed with 5N acetic acid and the solution adjusted to pH 7.0. Formaldehyde (8 ml., 37% w/v, 100 mmoles) immediately followed by sodium borohydride (6g., 160 mmoles) in water (100 ml). The mixture was incubated for 1 hour at 45° under a slow stream of nitrogen and cooled, and mercaptoethanol (1.5 ml.) added. The pH was adjusted to 7.0 and the mixture diluted to 500 ml. with cold water.

The pale yellow solution was passed through a 3.8 x 45 cm. column of DEAE-cellulose previously equilibrated with ammonium acetate (3 litre, 0.13M, pH 7.0) containing mercaptoethanol (0.2M). Elution was initiated at the rate of 5 ml./min. with ammonium acetate (2 litre, 0.13M, pH 7.0) followed by a gradient of ammonium acetate (0.13-0.4M, pH 7.0, 1 litre each) and completed with ammonium acetate (2 litre, 0.4M, pH 7.0). All solutions contained mercaptoethanol (0.2M). The 0.13M buffer eluate (2 litre) was collected in bulk and discarded. After 200 ml. of the ammonium acetate gradient had passed through the column, the eluate was collected in 15 ml. fractions and the extinction at 290 mμ was determined after dilution with 0.1M phosphate buffer, pH 7.0. A complete spectrum was recorded on every tenth fraction.

The product, 5-methyltetrahydrofolic acid, was found in tubes 31-181 and was characterised by an extinction maximum at 290 mμ and a minimum at 248 mμ; the extinction ratio ($A_{290}:A_{248}$) was greater than 3.0. Tubes 31-181 were combined and freeze-dried. The yield of 5-methyltetrahydrofolic acid was 33%, purity 45%. T.L.C. data is given in Table VI. Microbiological assay with L. casei and S. faecalis is given in Table VII.

Preparation of Barium Salt

To the freeze-dried chromatographically purified 5-methyltetrahydrofolic acid (1.4g) was added deaerated water (25 ml.) containing sodium chloride (300 mg). The pH was adjusted to 7.0 (0.1N NaOH) and the solution filtered. Barium chloride (10 ml. of 22g. $BaCl.2H_2O$/ 100 ml. water) and mercaptoethanol (0.04/35 ml. solution) were added to the filtrate. The barium salt was precipitated by addition of absolute ethanol (140 ml.) and the solution cooled overnight.

The barium salt was filtered at 5°, washed with 50% aqueous ethanol (20 ml.), followed by absolute ethanol (15 ml.), and dried rapidly under high vacuum at room temperature. Yield 1.45g., purity 91%.

The barium salt was recrystallised by warming in deaerated water containing mercaptoethanol (10^{-3}M) and diluting slowly with methanol to give a crystalline product which was filtered, washed, and dried rapidly to yield the barium salt of 5-methyltetrahydrofolic acid. Yield 1.2g. The purity was 100% as shown by UV absorption at pH 13: λ_{max} = 290 mμ, λ_{min} = 245 mμ, extinction ratio ($A_{290}:A_{245}$) was 2.7. T.L.C. data is given in Table VIII. Proton magnetic resonance spectrometry in D_2O gave a characteristic $N-CH_3$ at τ 7.5.

Experiment A10

The preparation was carried out as for Experiment A9. The pro-

Table VI

R_f Values in Various Solvents [a]

Solvent	A9
3	0.58 (0.38 slight blue fluor.)
1	0.85

[a] Abbreviations as in Table I and II.

Table VII

Microbiological Assay

Sample	Concentration [a] mg/ml	Response [b] L. casei mg/ml	S. faecalis
FA [c]	0.98	4.70	0.96
FA [c]	6.65	2.62	---
A9 [d]	2.12	1.30	0.16
A9 [e]	3.71	3.90	0.12
A9 [f]	3.78	0.97	0.14
A9 [e]	2.68	0.53	---
A9 [f]	2.61	0.48	---

[a] As determined by U.V.

[b] Concentration as determined by assay using folic acid as standard.

[c] Abbreviations as in Table I.

[d] Determined as free acid.

[e] Determined as barium salt.

[f] Determined as barium salt plus mercaptoethanol (0.2M).

Table VIII

R_f Values in Various Solvents [a]

Solvent [b]	A9 [c]
1	0.63 (0.45 slight blue fluor.)
3	0.57 (0.34 slight blue fluor.)

[a] Abbreviations as in Table I and II.

[b] T.L.C. run at 4°C.

[c] Determined as Barium salts.

duct was found in tubes 1-150 and these were bulked and freeze dried. Yield 54%. Experiment terminated at this stage.

Experiment A11

The preparation was carried out as for Experiment A9. The product was found in tubes 30-110 and was characterised by an extinction maximum at 290 mμ and minimum at 250 mμ; the extinction ratio (A_{290}: A_{250}) was greater than 3.0. Tubes 30-110 were combined and freeze-dried. Yield 49%. The barium salt was prepared and recrystallised as in Experiment A9. The purity was 100% as shown by UV absorption at pH 13: λ_{max} = 290 mμ, λ_{min} = 245; extinction ratio (A_{290}:A_{245}) was 3.8.

Experiment A12

The preparation was carried out as for Experiment A9 but the pH of the condensation stage was adjusted to pH 7.8. The product was found in tubes 30-120 and was characterised by an extinction maximum at 290 mμ and minimum at 250 mμ; the extinction ratio (A_{290}:A_{250}) was greater than 3.0. Tubes 30-120 were combined and freeze dried. Yield 75%. The barium salt was prepared and recrystallised as in Experiment A9, purity 98%. The recrystallisation was repeated, purity 107%. Calculated for $C_{20}H_{23}N_7O_6Ba.4H_2O$, C 36.02, H, 4.68, N, 14.69, Ba 20.59; found, C, 35.61, H, 4.01, N, 14.45, Ba, 20.32. The UV data is shown in Table IX.

Experiment A13

The preparation was carried out as for Experiment A12. The product was found in tubes 30-120 and these were bulked and freeze dried. Yield 68%. The barium salt was prepared and recrystallised as in Experiment A9. Purity 96%. λ_{max} = 290 mμ; λ_{min} = 245 mμ; extinction ratio (A_{290}:A_{245}) was 3.8.

Experiment A14

The preparation was carried out as for Experiment A9, but the pH

Table IX

Spectral Data of 5-Methyltetrahydrofolic Acid

Barium Salt

pH	λ_{max} mμ	λ_{min} mμ	$\epsilon_{max} \times 10^{-3}$	Absorbance ratio A_{max}/A_{min}
1	270, 294	242, 281	21.0, 20.0	---
7	290	245	31.7	3.8
13	290	245	30.8	3.8

of the condensation stage was adjusted to pH 8.5. The product was found in tubes 30-110 and was characterised by an extinction maximum at 290 mμ and a minimum at 250 mμ; the extinction ratio ($A_{290}:A_{250}$) was greater than 3.0. Tubes 30-110 were combined and freeze dried. Yield 39%.

Preparation of Calcium Salt

To the freeze-dried chromatographically purified 5-methyltetrahydrofolic acid (2.5g.) was added deaerated water (50 ml.) containing sodium chloride (600 mg.) The pH was adjusted to 7.0 (0.1N NaOH) and the solution filtered. Calcium chloride (12 ml. of 10g $CaCl_2 6H_2O$/ 100 ml.) and mercaptoethanol (0.04ml./35ml. solution) was added to the filtrate. The calcium salt was precipitated by addition of absolute ethanol (250 ml.) and the solution cooled overnight.

The calcium salt was filtered at 5°, washed with 75% aqueous ethanol (20 ml.), followed by absolute ethanol (20 ml.), and dried rapidly under high vacuum at room temperature. Yield 1.25 g., purity 93%. Microbiological assay with L. casei and S. faecalis is given in Table X.

Experiment A15

The preparation was carried out as for Experiment A9 but using a reduced amount of formaldehyde (1.2 ml., 37% w/v, 15 mmoles). The product was found in tubes 30-130. These were combined and freeze dried. Yield 53%.

The calcium salt was prepared as in Experiment A14, purity 96%. It was recrystallised by warming in deaerated water containing mercaptoethanol (10^{-3}M) and diluting slowly with methanol to give a crystalline precipitate which was filtered, washed and dried rapidly to yield the calcium salt of 5-methyltetrahydrofolic acid. Purity 123%. At pH 7 in phosphate buffer calculated purity 105%. T.L.C. data is given in

Table X

Microbiological Assay

Sample	Concentration [a] mg/ml	Response [b] L. casei mg/ml	S. faecalis
A14 [c]	2.0	1.0	.03
A16 [d]	1.1	0.36	.02

[a] As determined by U.V.

[b] Concentration as determined by assay using folic acid as standard.

[c] Determined as calcium salt,

[d] Determined as free acid.

Table XII. Calculated for C_{20}, H_{23}, N_7O_6 Ca. $3H_2O$; C, 43.5; H, 5.3; N, 17.8; Ca 7.25; found: C, 43.28, H, 5.05, N, 17.85; Ca 7.0. The U.V. data is shown in Table XI

Experiment A16

The preparation was carried out as for Experiment A9 but using a reduced amount of formaldehyde (2.4 ml., 37% w/v, 30 mmoles). The product was found in tubes 25-130. These were combined and freeze dried. Yield 82%.

Preparation of Free Acid

A. To the freeze-dried chromatographically purified 5-methyltetrahydrofolic acid (2.5 g.) in deaerated water (25 ml.) was added N HCl until the solution was pH 2.0. No precipitation occurred and the solution was cooled overnight. The precipitate was filtered, washed with methanol and dried rapidly under high vacuum. Yield 4.5g. No U.V. spectrum was observed. The filtrate was discarded.

B. To the freeze-dried chromatographically purified 5-methyltetrahydrofolic acid (25 g.) in deaerated water (25 ml.) was added absolute ethanol (300 ml) and mercaptoethanol to give a concentration of 10^{-2}M and the solution cooled overnight. The precipitate was filtered at 5°, washed with absolute ethanol (3 x 50 ml.), and dried rapidly under high vacuum at room temperature.

The product was recrystallised by dissolving in the minimum amount of water containing mercaptoethanol (10^{-2}M) and precipitating with ethanol (100 ml). The white crystalline product was dried over night under high vacuum over P_2O_5 at 25°. Yield 1.2 g. The purity was 95% as shown by U.V. absorption at pH 7: λ_{max} = 290 mμ, λ_{min} = 245 mμ, extinction ratio (A_{290}:A_{245}) was 3.8. Microbiological assay with L. casei and S. faecalis is given in Table X. T.L.C. data is given in Table XII. U.V. data is given in Table XIII. Calculated for C_{20} H_{25} $N_7O_6.2H_2O$, C, 48.7; H, 5.89; N, 19.79; found: C, 47.37; H, 5.80; N, 20.80.

Table XI

Spectral Data of 5-Methyltetrahydrofolic Acid Calcium Salt

pH	λ_{max} (mμ)	λ_{min} (mμ)	$\epsilon_{max} \times 10^{-3}$	Absorbence Ratio A_{max} / A_{min}	
1	270, 294	242, 281		$\frac{A_{270}}{A_{242}}$	2.6
7	290	245	32.4		3.85
13	290	245	35.5		3.4

Table XII

Rf Values in Various Solvents [a]

Solvent [b]	A15 [c]	A16 [d]
3	0.68 (0.45 slight blue fluor.)	0.68 (0.45 slight blue fluor.)

[a] As in Table I and II.

[b] T.L.C. run at room temperature.

[c] Determined as calcium salt.

[d] Determined as free acid.

Table XIII

Spectral Data of 5-Methyltetrahydrofolic Acid

pH	λ_{max} (mμ)	λ_{min} (mμ)	$\epsilon_{max} \times 10^{-3}$	Absorbance Ratio A_{max}/A_{min}
1	270, 294	242, 281	21.1 20.0	3.1, 1.04
7	290	245	30.5	3.7
13	290	245	28.0	3.24

Proton magnetic resonance spectrometry in D_2O gave a characteristic N-CH_3 at $\tau = 7.5$ with a small acetate - CH_3 impurity at $\tau = 8.0$. A triplet at $\tau = 8.8$ and quartet at $\tau = 6.3$ indicated the presence of ethanol which could not be removed by drying.

Experiment A17

The preparation was carried out as for Experiment A9. The product was found in tubes 80-155. These were combined and freeze-dried. Yield 62%. The free acid was prepared and recrystallised as in Experiment A16. Purity 90%.

Preparation of 5-Ethyltetrahydrofolic Acid

Experiment C1

5-Ethyltetrahydrofolic acid was prepared essentially by the method for 5-methyltetrahydrofolic acid (Experiment A9) but replacing formaldehyde with acetaldehyde (5.7 ml. 100 mmoles).

After elution the product, 5-ethyltetrahydrofolic acid was found in tubes 50-130 and was characterised by an extinction maximum at 290mμ and a minimum at 250 mμ; the extinction ratio ($A_{290}:A_{250}$) was greater than 2.5. Tubes 50-130 were bulked and freeze-dried. The yield of 5-ethyltetrahydrofolic acid using an extinction coefficient of 31.7 x 10^{-3} M^{-1} cm^{-1} in phosphate buffer pH 7.0 at 290 mμ (28) was about 70% based on folic acid used.

Preparation of Calcium Salt

To the freeze-dried chromatographically purified 5-ethyltetrahydrofolic acid (2.5 gm.) was added deaerated water (20 ml.) containing sodium chloride (500 mg). The pH was adjusted to 7.0 (0.1N NaOH) and the solution filtered. Calcium chloride solution (12 ml. of 10 gm. $CaCl_2.6H_2O$/100 ml. water) and mercaptoethanol sufficient to give a final concentration of 10^{-2}M was added to the filtrate. The calcium salt was precipitated by the addition of methanol (500 ml.) and the

solution cooled overnight. The calcium salt was centrifuged at 5°, washed with 75% aqueous methanol (20 ml.), followed by methanol (20ml) and dried rapidly under high vacuum over phosphorous pentoxide at room temperature. Yield 1.2 gm., purity 90%.

The calcium salt was recrystallised from deaerated water containing mercaptoethanol (10^{-3}M) and diluting slowly with methanol to give a crystalline precipitate which was filtered, washed, and dried rapidly to yield the calcium salt of 5-ethyltetrahydrofolate. Calculated for $C_{21}H_{25}N_7O_6Ca.4H_2O$, C, 43.22; H, 5.70; N, 16.80; Ca, 6.87; NCH_2CH_3, 4.97; found: C, 43.88; H, 5.22; N, 16.30; Ca, 6.10; NCH_2CH_3, 4.19. U.V. data is given in Table XIV. Microbiological assay with L. casei and S. faecalis is given in Table XV.

Proton magnetic resonance spectrometry in T.F.A. gave a $N\text{-}CH_2CH_3$ splitting pattern. An unresolved triplet at $\tau = 8.5$ and a poorly defined quartet at $\tau = 6.4$. There was no evidence of a methanol CH_3 $\tau = 6.3$ or an acetate CH_3 $\tau = 7.75$. See also Table XXI.

T.L.C. data is given in Table XVI.

Preparation of 5-n Propyltetrahydrofolic Acid

Experiment G1

The preparation was carried out essentially as for the methyl derivative (Experiment A9) but replacing formaldehyde with propionaldehyde (7.3 ml., 100 mmoles). After elution, the product, 5-propyltetrahydrofolic acid was found in tubes 35-135 and was characterised by an extinction maximum at 290 mμ and a minimum at 250 mμ; the extinction ratio ($A_{290}:A_{248}$) was greater than 2.5. The yield of 5-propyltetrahydrofolic acid using an extinction coefficient of $\epsilon = 31.7 \times 10^3\ M^{-1}\ cm^{-1}$ in phosphate buffer pH 7.0 based on folic acid used at 290 mμ was about 80%.

Table XIV

Spectral Data of 5-Ethyltetrahydrofolic Acid Calcium Salt

pH	λ_{max} (mμ)	λ_{min} (mμ)	ϵ_{max} X 10^{-3}	Absorbance ratio A_{max}/A_{min}
1	273 294	235 284	21.5 19.6	$\frac{A273}{A245} = 2.35$
7	292	250	31.4	2.8
13	291	249	29.6	3.0

Table XV

Microbiological Assay

Sample	Concentration [a] mg/ml	Response to L. casei	Response to S. faecalis
		mg/ml	
1	1.0	0.034	—
2	1.0	0.029	—
3	0.5	0.026	0.027
4	0.5	0.022	0.018

Table XVI

Rf Values in Various Solvents [a]

Solvent [b]	5EtTHFA	5PrTHFA	5BuTHFA
a	0.84 (0.52) blue fluor.	0.84 (0.52) blue fluor.	0.84 (0.52) blue fluor.
b	0.55 (0.37) blue fluor.	0.55 (0.37) blue fluor.	0.55 (0.37) blue fluor.
c	0.68 (0.44) blue fluor.	0.68 (0.44) blue fluor.	0.68 (0.44) blue fluor.

[a] Abbreviations as in Table 1.

[b] T.L.C. run at room temperature.

Preparation of Calcium Salt

The calcium salt was prepared and recrystallised as for the ethyl derivative (Experiment C1) Calculated for C_{22} H_{27} N_7O_6 Ca $3H_2O$, C, 45.59; H, 5.74; N, 16.92; Ca, 6.91; $NCH_2CH_2CH_3$, 9.84, found: C, 45.30; H, 5.70; N, 17.86; Ca, 8.1; $NCH_2CH_2CH_3$, 7.08. U.V. data is given in Table XVII. Microbiological assay with L. casei and S. faecalis is given in Table XVIII. Proton magnetic resonance spectrometry in T.F.A. gave a N-CH_2-CH_2-CH_3 splitting pattern. An unresolved triplet at $\tau = 9.0$, and poorly defined peaks at $\tau = 6.4$ and $\tau = 8.1$. There was no evidence of methanol CH_3 at $\tau = 6.3$ or an acetate CH_3 $\tau = 7.75$. See also Table XXI. T.L.C. data is given in Table XVI.

Preparation of 5-n butyltetrahydrofolic acid

Experiment I1

The preparation was carried out essentially as for the methyl derivative (Experiment A9) but replacing formaldehyde with butyraldehyde (9.0 ml., 100 mmoles). After elution, the product, 5-butyltetrahydrofolic acid was found in tubes 45-130 and was characterised by an extinction maximum at 286 mμ and minimum at 250 mμ; the extinction ratio (A_{286}:A_{260}) was greater than 1.5. The yield of 5-butyltetrahydrofolic acid using an extinction coefficient of $\epsilon = 31.7 \times 10^3$ M^{-1} cm^{-1} in phosphate buffer pH 7.0 based on folic acid used was about 60%.

Preparation of Calcium Salt

The calcium salt was prepared and recrystallised as for the ethyl derivative (Experiment C1). Calculated for C_{23} H_{29} N_7O_6 Ca $4H_2O$, C, 45.16; H, 6.10; N, 16.03; N butyl, 11.63; found: C, 44.00; H, 5.95; N, 17.30; N butyl, 4.25. U.V. data is given in Table XIX. Microbiological assay with L. casei and S. faecalis is given in Table XVIII. Proton magnetic resonance spectrometry in T.F.A. gave a N-CH_2-CH_2-CH_2-CH_3 splitting pattern. An unresolved triplet at $\tau = 9.1$ and poorly

Table XVII

Spectral Data of 5-n Propyltetrahydrofolic Acid Calcium Salt

pH	λ_{max} (mμ)	λ_{min} (mμ)	ϵ_{max}	Absorbance ratio Amax/Amin
1	274, 290	244, 282	22.7 22.0	$\frac{A274}{A244} = 2.44$
7	290	250	31.5	3.05
13	290	250	31.0	2.80

Table XVIII

Microbiological Assay

Sample	Concentration [a] mg/ml	Response to [b] L. casei	S. faecalis
		mg/ml	
5nPrTHF [c]	1.0	0.088	0.048
5nBuTHF [c]	1.0	0.095	0.106

[a] As determined by UV.

[b] Concentration as determined by assay using folic acid as standard.

[c] Determined as calcium salt.

Table XIX

Spectral Data of 5-n Butyltetrahydrofolic Acid Calcium Salt

pH	λ_{max} (mμ)	λ_{min} (mμ)	ϵ_{max} X 10^{-3}	Absorbance ratio A_{max}/A_{min}
1	272 287	240 283	20.5 19.5	$\frac{A270}{A240}$ = 2.18
7	288	245	29.5	2.8
13	288	244	29.5	2.5

defined peaks at $\tau = 6.4$, $\tau = 8.2$ and $\tau = 8.6$. There was no evidence of a methanol CH_3 at $\tau = 6.3$ and an acetate CH_3 at $\tau = 7.75$. See also Table XXI. T.L.C. data is given in Table XVI.

Preparation of 5,10-Methylene Tetrahydrofolic Acid

Experiment D1

The preparation was carried out as for 5-methyltetrahydrofolic acid (Experiment A9) but no sodium borohydride was added at the condensation stage. The product was found in tubes 60-120 and was characterised by an extinction maximum at 296 mμ and minimum at 250mμ. Tubes 60-120 were combined and freeze dried. The yield was 38% using an extinction coefficient of $31.7 \times 10^{-3}\ M^{-1}\ cm^{-1}$ in phosphate buffer pH 7.0 at 290 mμ.

The calcium salt was prepared and recrystallised as in Experiment C1. U.V. data is given in Table XX. Calculated for $C_{20}H_{21}N_7O_6$ Ca $4H_2O$; C, 42.25; H, 5.32; N, 17.26; found: C, 43.85; H, 5.01; N, 16.96. Proton magnetic resonance spectrometry in D_2O and T.F.A. were inconclusive.

Preparation of 5,-Isopropyltetrahydrofolic acid

Experiment E1

The preparation was carried out as for 5-methyltetrahydrofolic acid but replacing formaldehyde with acetone (7.3 ml., 100 mmoles). The product was found in tubes 40-135 and was characterised by an extinction maximum at 282 mμ and minimum at 251 mμ. Tubes 40-135 were combined and freeze-dried. The yield was 50% using an extinction coefficient of $31.7 \times 10^{-3}\ M^{-1}\ cm^{-1}$ in phosphate buffer pH 7.0 at 282 mμ.

The calcium salt was prepared and recrystallised as in Experiment C1.

Proton magnetic resonance spectrometry in T.F.A. failed to indicate an isopropyl splitting pattern and the spectrum closely resembled

Table XX

Spectral Data of 5,10-Methylene tetrahydrofolic Acid Calcium Salt

pH	λ_{max} (mμ)	λ_{min} (mμ)	ϵ_{max}	Absorbance ratio Amax/Amin
13	297	253	25.4	2.65
7	296	253	27.6	3.3
1[a]	292	245	22.1	2.78
1[b]	292, 278	245	ϵ292 = 20.7	A292/A245 = 2.6
1[c]	278, 292	245	ϵ278 = 18.2	A278/A245 = 2.08

[a] After 1 minute

[b] After 5 minutes

[c] After 30 minutes.

that of tetrahydrofolic acid, Table XXI. The U.V. data is shown in Table XXII with that of tetrahydrofolic acid. No isopropyl group was detected in the $N - CH\langle^{CH_3}_{CH_3}$ determination. Preliminary analytical figures were more consistent with the compound being tetrahydrofolic acid than with it being the isopropyl derivative. For $C_{22}H_{27}N_7O_6$ Ca $4H_2O$ (isopropyl derivative) calculated C, 44.21; N, 16.41; H, 5.90. For $C_{19}H_{21}N_7O_6$ Ca $4H_2O$ (tetrahydrofolic acid) calculated, C, 41.08; N, 17.68; H, 5.26; found: C, 43.32; N, 17.72; H, 4.79.

Preparation of 5-Benzyltetrahydrofolic acid

Experiment F1

The preparation was carried out as for 5-methyltetrahydrofolic acid by replacing formaldehyde with benzaldehyde (10 ml., 110 mmoles). The product was found in tubes 25-160 and was characterised by an extinction maximum at 278 mμ and minimum at 256 mμ. Tubes 25-160 were combined and freeze-dried. The yield was 83% using an extinction coefficient of 31.7×10^{-3} M^{-1} cm^{-1} in phosphate buffer, pH 7.0 at 278mμ. The calcium salt was prepared and recrystallised as in Experiment C1. Proton magnetic resonance spectrometry in T.F.A. failed to indicate benzyl protons and the spectrum closely resembled that of tetrahydrofolic acid (Table XXI). No benzyl group was detected in the N-benzyl determination. The U.V. data is shown in Table XXII with that of tetrahydrofolic acid. Preliminary analytical figures were more consistent with the compound being tetrahydrofolic acid than it being the benzyl derivative. For $C_{26}H_{27}N_7O_6$ Ca. $4H_2O$ (benzyl derivative) calculated C, 48.36; N, 15.19; H, 5.46. For $C_{19}H_{21}N_7O_6$ Ca. $4H_2O$ (tetrahydrofolic acid) calculated C, 41.08; N, 17.68; H, 5.26, found: C, 41.27; N, 18.45; H, 4.65.

Experiment F2

Experiment F1 was repeated using ethanol (150 ml.) to aid the

Table XXI

Proton Magnetic Resonance Data [a]

	EXPT	Aromatic	α CH	Glutamate	C_7 and C_9
F		1.7 1.78 1.93 2.02	4.83	7.12 7.18 7.40	
5CHOTHF		1.8 1.86 2.32 2.40	4.90	7.15 7.20 7.45	5.4 6.0
THF		1.8 1.85 2.35 2.40	4.85	7.15 7.42	5.45 5.9
5MeTHF	A15	1.90 1.96 2.55 2.62	4.85	7.15 7.40	5.45 5.9
5EtTHF	C1	1.93 2.00 2.60 2.70	4.90	7.15 7.40	5.45 5.9
	E1	1.82 1.90 2.35 2.40	4.90	7.15 7.20 7.40	5.45 5.9
	F1	1.80 1.90 2.35 2.40	4.90	7.15 7.20 7.4	5.45 5.9
5nPrTHF	G1	1.95 2.05 2.70 2.76	4.90	7.20 7.24 7.40	5.45 5.9
	H1	1.80 1.90 2.35 2.40	4.90	7.15 7.20 7.4	5.45 5.9
5nBuTHF	I1	2.00 2.08 2.72 2.80	4.90	7.20 7.24 7.4	5.45 5.9

[a] Abbreviations as in Table I.

Table XXII

U.V. Spectral Data of Product E1 and F1[a]

	pH	λ_{max} (mμ)	λ_{min} (mμ)
E1	1	283	244
	7	289	253
	13	291	253
F1	1	280 (shoulder 290)	244
	7	293	252
	13	293	251
THF	1	270, 294	244
	7	297	250
	13	297	250

[a] Abbreviations as in Table I.

solubility of benzaldehyde. The product was found in tubes 40-120 and was characterised by an extinction maximum at 282 mμ and minimum at 256 mμ. Tubes 40-120 were combined and freeze-dried. The yield was 82% using an extinction coefficient of 31.7×10^{-3} M^{-1} cm^{-1} in phosphate buffer pH 7.0 at 282 mμ.

The calcium salt was prepared and recrystallised as in Experiment C1.

Proton magnetic spectrometry in T.F.A. failed to indicate benzyl protons and the spectrum closely resembled that of tetrahydrofolic acid.

Experiment F3

Experiment F2 was repeated but allowing a condensation time of 2 hours before addition of sodium borohydride (12 g., 320 mmoles). The product was found in tubes 70-145 and was characterised by an extinction maximum at 280 mμ and minimum at 256 mμ. Tubes 70-145 were combined and freeze-dried. The yield was 72% using an extinction coefficient of 31.7×10^{-3} M^{-1} cm^{-1} in phosphate buffer pH 7.0 at 280 mμ.

The calcium salt was prepared and recrystallised as in Experiment C1.

Proton magnetic spectrometry in T.F.A. failed to indicate benzyl protons and the spectrum closely resembled that of tetrahydrofolic acid.

Experiment F4

Experiment F2 was repeated but allowing a condensation time of 2 hours before addition of sodium borohydride (1.1g., 30 mmoles). The product was found in tubes 15-105 and was characterised by an extinction maximum at 280 mμ and minimum at 256 mμ. Tubes 15-105 were combined and freeze-dried. The yield was 75% using an extinction coefficient of 31.7×10^{-3} M^{-1} cm^{-1} in phosphate buffer pH 7.0 at 280 mμ.

The calcium salt was prepared and recrystallised as in Experiment C1.

Proton magnetic resonance spectrometry in T.F.A. failed to indicate a benzyl splitting pattern and the spectrum closely resembled that of tetrahydrofolic acid.

Preparation of 5-β Phenylethyltetrahydrofolic Acid

Experiment H1

The preparation was carried out as for 5-methyltetrahydrofolic acid (Experiment A9) but replacing formaldehyde with phenylacetaldehyde (10 ml; 100 mmoles). The product was found in tubes 35-130 and was characterised by an extinction maximum at 278 mμ and minimum at 253 mμ. Tubes 35-130 were combined and freeze-dried. The yield was 80% using an extinction coefficient of $31.7 \times 10^{-3}\ M^{-1}\ cm^{-1}$ in phosphate buffer pH 7.0 at 278 mμ.

The calcium salt was prepared and recrystallised as in Experiment C1.

Proton magnetic resonance spectrometry in T.F.A. failed to indicate any splitting pattern and the spectrum closely resembled that of tetrahydrofolic acid (Table XXI).

Preparation of 5-Methyltetrahydromethotrexate

Experiment B1

Sodium borohydride (2 gm., 53 mmoles) in water (20 ml.) was added to a stirred solution of methotrexate (2 gm., 4 mmoles) in 0.066M Tris buffer, pH 8.0 (100 ml.), under nitrogen at room temperature. After 20 minutes, excess sodium borohydride was destroyed with 5N acetic acid and the solution adjusted to pH 7.8. Formaldehyde (2.7 ml., 37% w/v, 33 mmoles) was added, immediately followed by sodium borohydride (4 gm., 106 mmoles) in water 40 ml.

The mixture was incubated for 1 hour at $40^{\circ}C$ under a slow stream of nitrogen, cooled and mercaptoethanol (1.0 ml.) added. The pH was adjusted to 7.0 and the mixture diluted to 200 ml. with cold water.

The product was purified as in experiment A9 and was found in tubes 45-97 being characterised by an extinction maximum at 301 mμ and a minimum at 260 mμ. The extinction ratio was greater than 2.4. Tubes 45-97 were combined and freeze-dried. The yield of 5-methyltetrahydromethotrexate using an extinction coefficient of $\varepsilon = 30 \times 10^{3} M^{-1} cm^{-1}$ in phosphate buffer pH 7.0 at 300 mμ was about 25% based on methotrexate used.

The calcium salt was prepared and recrystallised as in Experiment A15. Calculated for $C_{21} H_{26} N_8O_5$ Ca $4H_2O$; C, 43.25; H, 5.92; N, 19.22; Cu, 6.86; N^5 CH_3, 4.98; N^{10} CH_3; 4.98. Found: C, 42.81; H, 5.75; N, 19.08; Ca. 5.87; NCH_3, 4.59. For methotrexate: calculated NCH_3 = 6.1, found NCH_3 = 2.92. U.V. data is given in Table XXIII. T.L.C. data is given in Table XXIV.

Proton magnetic resonance spectrometry in T.F.A. gave a characteristic N_5-CH_3 at $\tau = 6.65$. There was no evidence of a methanol CH_3 $\tau = 6.3$ or an acetate CH_3 $\tau = 7.75$ (In D_2O, N_5-CH_3 at $\tau = 7.5$). The product was biologically inhibitory for L. casei and S. faecalis

Experiment B2

Experiment B1 was repeated using methotrexate (5 gm). The product was found in tubes 6-100 being characterised by an extinction maximum at 301 mμ and minimum at 261 mμ. The extinction ratio were greater than 2.4. Tubes 6-100 were combined and freeze-dried. The yield of 5-methyltetrahydromethotrexate using an extinction coefficient of $\varepsilon = 30 \times 10^{-3} M^{-1} cm^{-1}$ in phosphate buffer pH 7.0 at 300 mμ was about 30% based on methotrexate used. The calcium salt was prepared and recrystallised as in Experiment A15.

Preparation of 5-Ethyltetrahydromethotrexate

Experiment K1

5-Ethyltetrahydromethotrexate was prepared essentially by the

Table XXIII

Spectral Data of Methotrexate Analogues [a]

Compound	pH	λ_{max}(mμ)	λ_{min}(mμ)	$\epsilon_{max} \times 10^{-3}$	Abs ratio $A_{max}:A_{min}$
5MeTHMTX	1	299	260	20.1	2.3
	7	301	258	30.0	3.44
	13	300	260	28.8	3.04
5EtTHMTX	1	300	258	20.0	2.1
	7	300	257	30.0	3.3
	13	300	258	28.6	3.0

[a] Abbreviations as in Table I and II.

[b] Spectra done on calcium salts.

Table XXIV

Rf Values of Methotrexate Derivatives in Various Solvent [a]

Solvent	5MeTHMTX	5EtTHMTX
1	0.65	0.65
2	0.13	0.13
3	0.75	0.75

[a] As in Table I and II.

method for 5-methyltetrahydromethotrexate (Experiment B2) but replacing formaldehyde with acetaldehyde (5.7 ml., 100 mmoles). After elution the product, 5-ethyltetrahydromethotrexate was found in tubes 30-110 and was characterised by an extinction maximum at 300 mμ and minimum at 260 mμ; the extinction ratio ($A_{300}:A_{260}$) was greater than 2.5. The yield of 5-ethyltetrahydromethotrexate using an extinction coefficient of $\epsilon = 30.0 \times 10^3\ M^{-1}\ cm^{-1}$ in phosphate buffer, pH 7.0 was about 30% based on methotrexate used.

The calcium salt was prepared and recrystallised as in Experiment A15. Calculated for $C_{22}H_{28}N_8O_5$ Ca. $4H_2O$; C, 44.29; H, 6.08; N, 18.78 %NCH_2CH_3 7.22; $N^{10}CH_3$, 4.87; found, C, 44.90; H, 5.80; N, 19.34; %NCH_2CH_3, 7.40. U.V. data is given in Table XXIII. T.L.C. data is given in Table XXIV.

Proton magnetic resonance spectrometry in T.F.A. gave a characteristic N_5-CH_2CH_3 splitting pattern. An unresolved triplet at $\tau = 8.5$ but the quartet which should appear at $\tau = 6.4$ is masked by $N_{10}CH_3$ at $\tau = 6.2$. There is no evidence of an acetate CH_3 at $\tau = 7.75$. The product was biologically inhibitory for L. casei and S. faecalis.

Preparation of 2-amino, 4-hydroxy-tetrahydropteridine

Experiment J1

Sodium borohydride (6 gm., 160 mmoles) in water (50 ml.) was added to a stirred suspension of 2-amino, 4-hydroxy-pteridine (5.3 gm, 32 mmoles) in 0.066 M Tris buffer, pH 9.0 (200 ml.), under nitrogen at room temperature. After 2 hours excess sodium borohydride was destroyed with 5N acetic acid and the solution adjusted to pH 7.8. Formaldehyde (8 ml., 37% w/v 100 mmoles) was added, followed by sodium borohydride (12 gm., 320 mmoles) in water (100 ml).

The mixture was incubated for 4 hours at 40°C under a slow stream of nitrogen and then cooled. The pH was adjusted to 7.0 and the precipitate filtered and washed with water (4.9 gm).

Proton magnetic resonance spectrometry in T.F.A. gave a singlet at $\tau = 1.2$ and a broad peak at $\tau = 1.6$ indicative of 2-amino, 4-hydroxy-pteridine. The filtrate was freeze-dried and then dissolved in water (20 ml). The pH was adjusted to 1.0 and the solution filtered. No precipitate was obtained. On T.L.C. the solution showed various pteridines.

RESULTS AND DISCUSSION

Preparation of 5-Methyltetrahydrofolic acid

The experiments were done as detailed in Experiments A1-A17. Initially DEAE cellulose (DE 22) was used. This however contained a soluble material which could not be removed by repeated washing with acetate buffer and water. The failure to remove this material resulted in the freeze-dried residues being contaminated with a yellow hygroscopic material.

Freeze drying proved a lengthy and tedious operation but no suitable alternative was available due to the nature of the compounds. Some experiments were conducted in the cold room at 4° to prevent decomposition as this material was very unstable. This however, had to be abandoned due to the instability of the control mechanism of the room. Later the reactions were done in the shortest possible time at room temperature, with all vessels routinely covered with aluminium foil.

The preparation of the free acid was hampered by its instability and improved purity was obtained by the preparation of the barium salt.

Success was achieved by use of DEAE cellulose (DE 52) which is a pre-washed micro granular cellulose and the barium salt of 5-methyltetrahydrofolic acid was prepared (Experiment A9). The calcium salt was also prepared. The free acid was prepared by repeated washing of the freeze-dried residue with alcohol. Comparison of yield versus pH is given in Table XXV and shows that the optimum pH is approximately 7.8 Table XXVI shows that while higher ratios of formaldehyde to folic acid are desirable, a reasonable yield is still achieved at a ratio of 1.1:1. The product was identical to that of Gupta and Huennekens (28) as shown by U.V. spectra. Comparison of the product with the starting material and other possible products is given in

Table XXV

pH of condensation[a]	% yield [b]
7	54, 49
7.8	75, 68, 62
8.5	39

[a] 7:1 formaldehyde: folic acid ratio and variable pH at condensation stage.

[b] Based on pure folic acid used.

Table XXVI

Mole ratio [a] formaldehyde : folic acid	% yield [b]
7 : 1	75, 68, 62
1.1 : 1	53
2 : 1	82

[a] pH 7.8 at condensation stage and variable formaldehyde: folic acid ratio.

[b] Based on pure folic acid used.

Tables XXVII and XXVIII.

These show that the product was 5-methyltetrahydrofolic acid. Reductive condensation must have taken place at N-5. See section on E.S.R. The P.M.R. showed the characteristic N-Me at $\tau = 7.5$. Microbiological assay showed the absence of other folates. T.L.C. showed the presence of a slight fluorescent impurity.

The P.M.R. spectrum in D_2O shows, in the case of the barium and calcium salts, no evidence of acetate CH_3 at $\tau = 8.0$ and the ethanol CH_3 at $\tau = 8.8$. The P.M.R. also shows absence of folic acid (C7 singlet at $\tau = 1.6$). In the case of the free acid a triplet at $\tau = 8.8$ and a quartet at $\tau = 6.3$ indicated the presence of ethanol. The ethanol appears to be tenaciously held within the molecule lattice and was not removed by prolonged drying under high vacuum over P_2O_5.

Similar phenomena have been demonstrated by Horwitz et al.,[34] with formaldehyde, Gupta and Huennekens,[28] with acetate and water and Whiteley and Huennekens[35] with hydrochloride. Zakrzewski[36] also showed that mercaptoethanol formed an adduct of unknown structure with tetrahydrofolic acid but no evidence was found for this, probably due to the low concentration of mercaptoethanol present. In the P.M.R. added mercaptoethanol gives characteristic additional triplets at $\tau = 7.3$ and $\tau = 6.3$.

Using Drieding models it was shown that 5-methyltetrahydrofolic acid could exist as a folded structure with the two aromatic rings parallel and over one another. This could explain the difficulty in removing the ethanol due to it being held within the fold.

Mechanism

This reductive condensation was thought to go via N_5, N_{10}-methylenetetrahydrofolic acid (V) Fig. VI. A more probable mechanism is given in Fig. VI.

Table XXVII

Spectral Data of Folate Analogues

Compound [a]	pH	λ_{max} (mμ)	λ_{min} (mμ)	$\epsilon_{max} \times 10^{-3}$
5MeTHF [b]	1	270, 294	242, 281	21.0, 20.0
	7	290	245	31.7
	13	290	245	30.8
F	7	282, 350	330	27, 7
DHF	7.5	283	250	21
THFA	7.5	298	245	22
10CHO-THF	7.5	260	240	17
5CHO-THF	13	282	245	32.6
5,10-Methenyl-THF	1	355	305	24.9
5,10-Methylene-THF	7.2	294	245	32
5-MeDHF	7	250, 290	230, 260	31

[a] Abbreviations as in Table I.

[b] As Ba Salt.

Table XXVIII

R_f Values in Various Solvents [a]

Solvent [b]	5MeTHF	5CHO-THF	F
I	0.84 (0.52) blue fluor. [c]	0.84	0.51
II	0.55 (0.37) blue fluor. [c]	0.36	0.15
III	0.68 (0.44) blue fluor. [c]	0.68	0.0

[a] These were detected as quenching spots unless otherwise indicated. Minimum detectable amounts about 5μg in 1 μl .

[b] As in "methods".

[c] Trace fluorescent impurity.

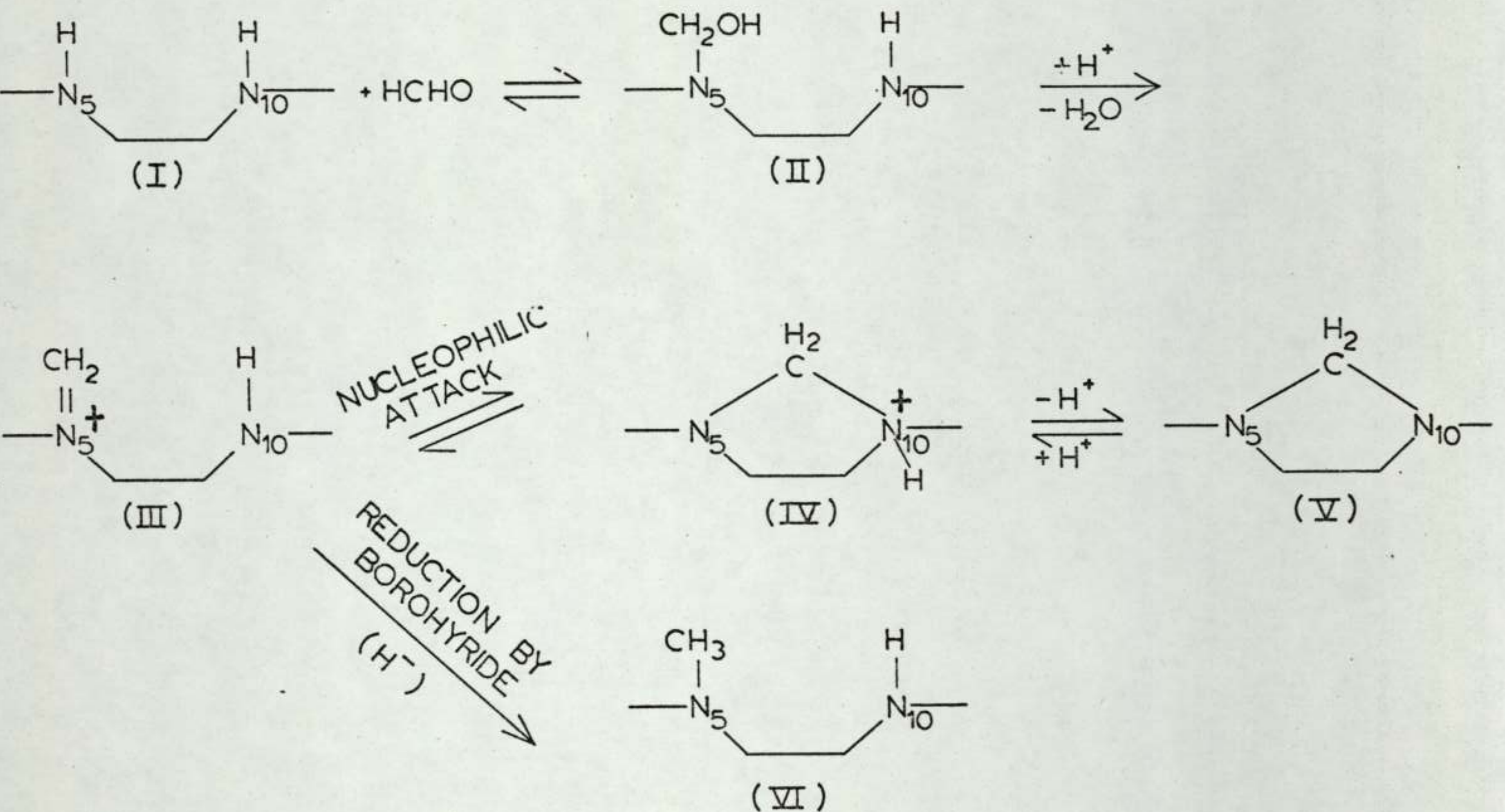


Fig. VI

Kallen and Jenks[38] have shown that the condensation of formaldehyde with tetrahydrofolic acid (I) proceeded via the imine (III). The work of Benkovic et al.,[39] using model compounds also supports this theory. The initial step was nucleophilic attack of tetrahydrofolic acid on formaldehyde to give the carbinolamine (II) which was then protonated and dehydrated to give (III).

Nucleophilic attack by N_{10} would give the 5,10-methylene compound (V) or by hydride ions from borohydride the 5-methyl compound (VI). Interconversion of (III) and (V) would explain the reduction of 5,10-methylenetetrahydrofolic acid to give 5-methyltetrahydrofolic acid[40]. That the presence of N_{10} was not required for reductive condensations was shown by Whiteley et al.,[43] in the preparation of 2-amino-4-hydroxy-5-6-dimethyltetrahydropteridine, and also by the reductive condensation of formaldehyde with tetrahydromethotrexate (Experiments B1 and B2).

Preparation of 5-Ethyl 5,-Propyl and 5 -Butyltetrahydrofolic Acids

The experiments were done as detailed in Experiments C1, G1 and I1. The calcium salts were prepared. The P.M.R. spectra showed evidence of the alkyl group in each case and the Ziesel determination confirmed this.

Microbiological assay showed that the compounds were inactive for L. casei and S. faecalis. The higher activity of the propyl and butyl derivatives was probably due to a small tetrahydrofolic acid contamination. T.L.C. data showed the presence of a slight fluorescent impurity but the tetrahydrofolate compounds were not separable from one another in the solvent systems used.

The P.M.R in T.F.A. showed the absence of an acetate CH_3 at $\tau = 7.75$ and methanol CH_3 at $\tau = 6.3$. The P.M.R. also showed the absence of folic acid (C7 singlet at $\tau = 0.9$). However in the case of the butyl compound there was evidence of small tetrahydrofolic acid

impurity (doublet at $\tau = 2.4$). A similar mechanism would apply to that for the 5-methyl derivative where the formaldehyde was replaced by acetaldehyde, propionaldehyde or butyraldehyde. The drop in yield along the series is probably due to an entropy effect.

Preparation of 5,10-Methylenetetrahydrofolic acid

The experiment was done as detailed in Experiment D1. The calcium salt was prepared. The compound was identified from U.V. data. In 0.1N HCl the compound degraded to tetrahydrofolic acid as shown by its shift in wavelength[42]. The P.M.R. in T.F.A. and D_2O were inconclusive probably due to decomposition. The compound was used as a standard.

The mechanism for formation is shown in Fig. VII. Nucleophilic attack by N_{10} on N_5 would give intermediate (IV) followed by loss of a proton to give 5-10, methylenetetrahydrofolic acid.

Preparation of 5, Isopropyltetrahydrofolic acid

The experiment was done as in Experiment E1 and the calcium salt was prepared. No evidence was obtained for the incorporation of the isopropyl group in the 5 position and U.V. and P.M.R. spectra resembled tetrahydrofolic acid. No isopropyl group was detected in the Ziesel determination and the analytical figures also resembled tetrahydrofolic acid.

The inability of acetone to condense with tetrahydrofolic was probably due to a rate phenomena as it is known that ketones condense less readily than aldehydes. The experiment could be repeated with a longer condensation time.

Preparation of 5-Benzyltetrahydrofolic acid

The experiments were done as detailed in Experiments F1-F4 and the calcium salts were prepared. No evidence was obtained for the incorporation of the benzyl group in the 5 position and U.V. and P.M.R.

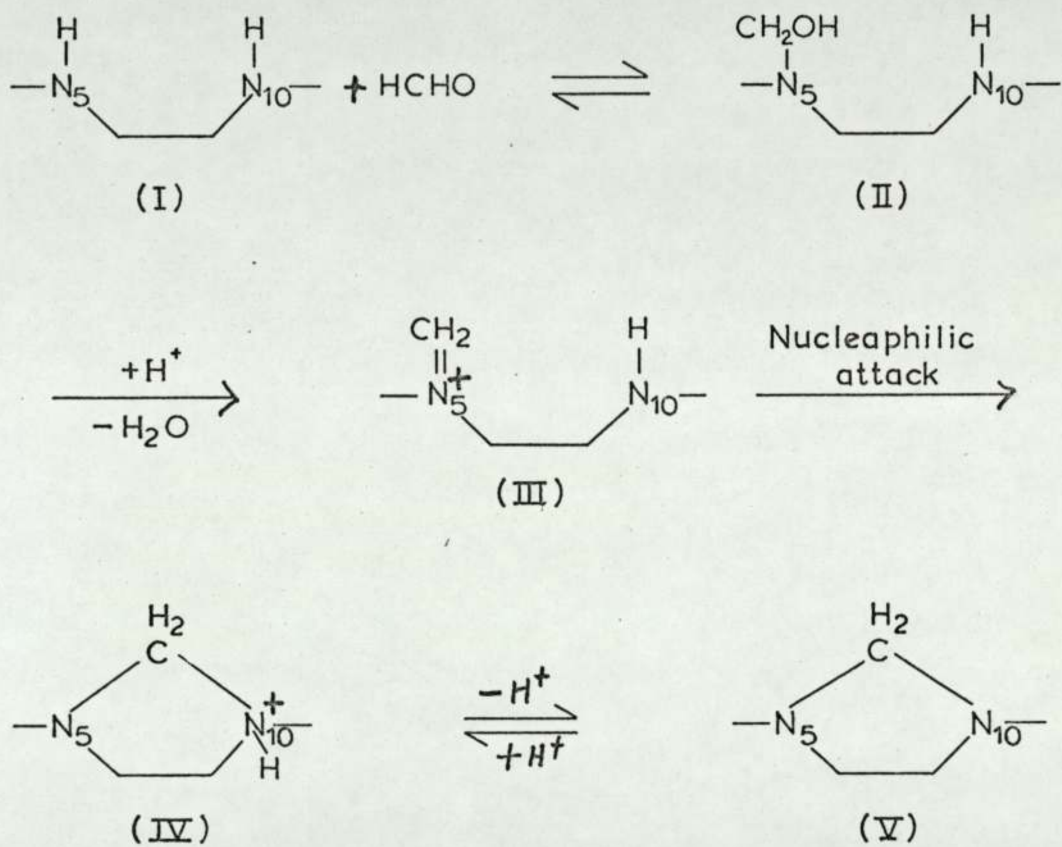


Fig. VII

spectra resembled tetrahydrofolic acid. The analytical figures also resembled tetrahydrofolic acid.

The inability of the benzylaldehyde to condense with tetrahydrofolic acid was probably due to entropy factors. At one time the solubility of benzylaldehyde was thought to be important but increasing the solubility with ethanol was unsuccessful.

Preparation of 5, β Phenylethyltetrahydrofolic acid

The experiment was done as detailed in Experiment H1 and the calcium salt was prepared. No evidence was obtained for the incorporation of the phenyl group in the 5 position and U.V. and P.M.R. spectra resembled tetrahydrofolic acid. The analytical figures also resembled tetrahydrofolic acid.

The inability of the phenylacetaldehyde to condense with tetrahydrofolic acid was again probably due to entropy factors, although increasing its solubility could be an important factor.

Preparation of 5-Methyl and 5-Ethyltetrahydromethotrexate

The experiments were done as detailed in Experiments B1, B2 and K1 and the calcium salts were prepared. Comparison of the products with the starting material and other possible products are given in Tables XXIX and XXX. By analogy with the tetrahydrofolic acid derivatives reductive condensation takes place at N_5 (see section on E.S.R.). This was substantiated by the P.M.R. spectrum which shows N_5-CH_3 (singlet) at $\tau = 7.5$ in D_2O and N_5CH_3 at $\tau = 6.65$ in T.F.A. as with the folate analogues. $N_{10}-CH_3$ appears at $\tau = 7.0$ in D_2O and at 6.2 in T.F.A. Also the CH_3 of $N_5-CH_2CH_3$ appears at $\tau = 8.5$ in T.F.A. as with the folate analogues. P.M.R. also shows the absence of methotrexate (C_7 singlet at $\tau = 0.9$ in T.F.A.). Microbiological assay showed that these compounds were inhibitory.

The formation of these methyl and ethyl derivatives substantiates

Table XXIX

Spectral Data of Methotrexate Analogues [a]

Compound [b]	pH	max (mμ)	min (mμ)	max x 10^{-3}	Absorbance ratio (max:min)
MTX	1	309,244	263,235	20.6,17.4	2.58, 1.07
	7	376,307,260 (shoulder 222)	345,274,240	9.4,24.5,24.0	1.22,1.32,1.56
	13	376,307,262	345,274,240	9.4,24.4,24.8	1.43,1.32,2.47
THMTX	0.3	306	-	15.2	-
	7	306	-	28.3	-
	13	306	-	28.3	-
5MeTHMTX	1	299	260	20.1	2.3
	7	301	258	30.0	3.44
	13	300	260	28.8	3.04
5EtTHMTX	1	300	258	20.0	2.1
	7	300	257	30.0	3.3
	13	300	258	28.6	3.0

[a] Spectra of methotrexate done as free acid and of 5 methyl and 5 ethyl derivatives as calcium salts.

[b] Abbreviations as in Table I.

Table XXX

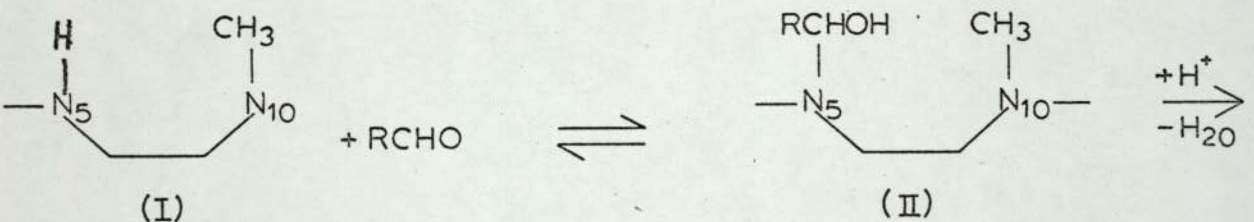
Rf Values in Various Solvents [a]

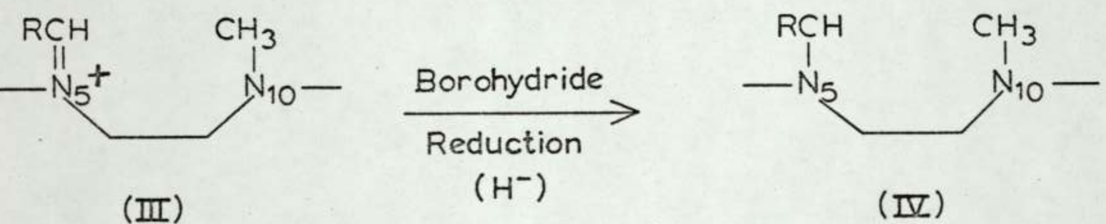
Solvent [b]	MTX	5MeTHMTX	5EtTHMTX
1	.78	.65	.65
2	.40	.13	.13
3	.68	.75	.75

[a]These were detected as quenching spots. Minimum detectable amount about 5μg in 1μl. Abbreviations as in Table I and II.

[b]As in "methods".

the proposal that the reductive condensation proceeds via reduction of the imine (III) in Fig. VIII. The Ziesel determination depends on the basicity of the nitrogen bearing the alkyl. The N_{10} in methotrexate is weakly basic and this explanes the low value obtained. However in the reduced derivatives the basicity of the N_{10} is still further reduced[38] and the value obtained is probably only that due to the alkyl on N_5.





$R = H$ or CH_3

Fig. VIII

Proton Magnetic Resonance Spectrometry

The proton magnetic resonance spectra of starting materials, possible impurities and of the products formed were determined in D_2O using T.M.S. as external reference (Table XXXI) and in T.F.A. using T.M.S. as internal reference (Table XXXII).

It was shown that proton magnetic resonance spectrometry would not only indicate the presence of the various alkyl derivatives but also the purity of them, as any gross impurity would show in the aromatic region. Hence in the case of the n butyl derivative it was possible to see a small doublet at $\tau = 2.37$ (T.F.A.) indicative of tetrahydrofolic acid.

When using deuterium oxide difficulty was encountered due to insolubility and this was overcome to a limited extent by the addition of small quantities of ammonia. However in the case of the calcium salts of the higher analogues useful spectra could not be obtained. Hence the spectra were repeated using trifluoroacetic acid.

Here solubility was greatly enhanced (compounds dissolved forming red solution) but difficulty was encountered due to line broadening. This, it was thought, was due to the formation of free radicals and is discussed in more detail in the section on electron spin resonance.

It was seen that there was some shift to low field with increasing dilution. There could be two possible effects taking place, a hydrogen bonding effect or a inter molecular interaction. If the hydrogen bonding effect was important one would expect an upfield shift on dilution where in fact the shift is downfield. If the inter molecular interaction was important the shift would be downfield. This has also been observed in the purine series by Hruska et al.[44]. Interaction of the π bonds of the aromatic rings could account for this downfield shift. This would be indicative of some kind of charge transfer complex of the nature of which is unknown and could be the subject of further work.

Table XXXI

Proton Magnetic Resonance Spectra in Deuterium Oxide

	C_6 C_7	Ar	GluαCH	$GluCH_2-CH_2$	$N_{10}CH_3$	N_5CH_3
Glu			6.1	7.7		
pNH_2BGlu		2.1 2.2 2.75 2.9	5.6	7.7		
$2NH_2 4OHPt$	1.4 1.6	(J=2.5)Two doublets				
FA	1.74	2.37 2.51 3.48 3.61	5.6	7.66		
Meth	1.6	2.30 2.40 3.35 3.42	5.6	7.7	7.0	
5MeTHFA		2.20 2.30 3.20 3.28	5.5	7.7		7.5
5MeTHMeth		2.10 2.15 3.05 3.15	5.5	7.7	7.0	7.5
5EtTHFA						

Acetate CH_3 at τ = 8.0

Methanol CH_3 at τ = 6.5

Ethanol CH_2 at τ = 8.8 Triplet CH_3 at τ = 6.3 Quartet

Mercaptoethanol CH_2 at τ = 6.3 Triplet CH_3 at τ = 7.3 Triplet

Table XXXII

Proton Magnetic Resonance Spectra in Trifluoroacetic Acid

	C_6,C_7	C_2NH_2	Ar	Glu α CH	$GluCH_2$-CH_2	C_9CH_2	
pNH_2BGlu [a]			1.81 1.90 2.15 2.24	4.90	7.15 7.20 7.45		
$2NH_2$, 4OHPt	0.85	1.25					
$2NH_2$,4,6OHPt	1.22	1.55					
F	0.90	1.20	1.70 1.78 1.93 2.02	4.83	7.12 7.20 7.45	4.65	
MTX	0.90		1.90 2.30	4.50	7.15 7.45	4.86	$N_{10}CH_3$ 6.2
THF			1.80 1.85 2.40	4.85	7.15 7.42		
5MeTHF			1.90 1.96 2.55 2.62	4.85	7.17 7.40		6.65 N_5CH_3
5EtTHF			1.93 2.00 2.60 2.70	4.90	7.15 7.40		6.1 (quartet) N-CH_3
5nPrTHF			1.95 2.05 2.70 2.76	4.90	7.20 7.24 7.40		6.1 N-CH_3
5nBuTHF			2.00 2.08 2.72 2.80	4.90	7.20 7.24 7.40		6.1 N-CH_3
5CHOTHF			1.80 1.86 2.32 2.40	4.90	7.15 7.20 7.45		N_5CHO 0.35
5MeTHMTX			1.70 1.90	4.85	7.15 7.20 7.45		N_5CH_3 6.65, $N_{10}CH_3$ 6.2
5EtTHMTX			1.75 1.93	4.85	7.15 7.20 7.45		N-CH_3 6.1

Acetate CH_3 at $\tau = 7.75$ Methanol CH_3 at $\tau = 6.30$ Ethanol CH_2 at $\tau = 8.75$ (triplet), CH_3 at $\tau = 6.3$ (quartet)

[a] As in Table I.

Experiments were performed to determine the chemical shifts of the aromatic protons in folic acid and p-amino benzoyl-L-glutamic acid and the C_7 proton in folic acid and the C_7 and C_6 proton in 2-amino-4 OH pteridine with change in concentration.

Table XXXIII

Chemical Shift of C_7 Proton of Folic Acid with Concentration [a]

Concentration mg/ml	Molarity	τ
600	1.36	1.78
150	0.34	1.48
75	0.17	1.37
37	0.08	1.31

[a] Solvent D_2O. External reference T.M.S.

Table XXXIV

Chemical Shift of α and β Aromatic Proton of Folic Acid with Concentration[a]

Concentration	molarity	τ α-proton	τ β-proton
600	1.36	2.44	3.54
150	0.34	2.32	3.31
75	0.17	2.25	3.14
37	0.08	2.23	3.11

[a] Solvent D_2O. External reference T.M.S.

Table XXXV

Chemical Shift of α and β Aromatic Protons of pNH_2 Benzoyl L-Glutamic Acid with Concentration [a]

Concentration mg/ml	molarity	τ α-protons	τ β-protons
600	2.18	2.37	3.23
150	0.54	2.31	3.13
75	0.27	2.26	3.05
37	0.13	2.24	3.03

[a] Solvent D_2O. External reference T.M.S.

Table XXXVI

Chemical Shift of C_6 and C_7 protons of $2NH_24OH$ Pteridine with Concentration [a]

Concentration mg/ml	molarity	C_6 proton	C_7 proton
200	1.23	1.40	1.6
100	0.62	1.41	1.61
50	0.31	1.35	1.54
25	0.15	1.27	1.48
12.5	0.075	1.26	-

[a] Solvent D_2O. External reference T.M.S.

Electron Spin Resonance Spectrometry

In applying E.S.R. to folate chemistry one must consider the role of the folate derivatives in nature. The folic acid coenzymes act as electron donor acceptor reagents.

Perault and Pullman[45] have shown that these properties were accounted for in terms of the energies of their highest filled and lowest empty molecular orbitals.

Table XXXVII

Energies of Molecular Orbitals

Compound	Energy of highest occupied M.O.	Energy of lowest unoccupied M.O.
FA	+ 0.45	-0.65
Pt fraction THFA	+ 0.05	-1.07

It can be seen from Table XXXVII that folic acid should be a moderate donor or acceptor of electrons. Its moderate electron acceptor properties have been demonstrated by Fujimori,[46] who has showed that folic acid was able to form a charge transfer complex with tryptophane. Also tetrahydrofolic acid should be a good electron donor but its electron acceptor properties should have vanished. Fujimori[46] confirms that it was unable to form a charge transfer complex with tryptophane.

The electron donating properties of tetrahydrofolic acid have been investigated by Bobst[47] who has shown by E.S.R. that one electron oxidations in trifluoroacetic acid/methanol mixtures resulted in an unstable cationic radical of red colour which could be trapped at liquid nitrogen temperature.

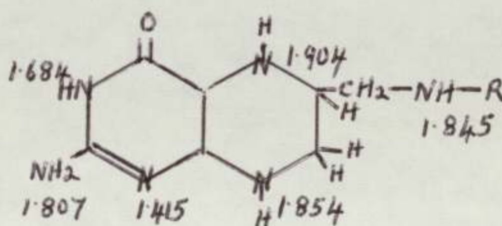
Bobst[48] has also shown that one electron oxidation of tetrahydropteridine in trifluoroacetic acid/methanol mixtures produced E.S.R. signals with hyperfine structure. Deuterium-hydrogen exchange and perturbation of the tetrahydropteridine molecule by methyl groups in different positions allowed the interpretation of the observed splitting and has shown that the free-spin of the cationic tetrahydropteridine radical was strongly localised in position N_5.

Comparing the tetrahydropteridine radical cation with the tetrahydrofolate radical cation it was feasible that free-spin was also localised on position N_5 in tetrahydrofolate. This was confirmed by theoretical calculations carried out by Pullman (Fig IX) who calculated the electron charge distribution on the nitrogen atoms of tetrahydrofolic acid. It was shown that the smallest formed positive charge carried by N-H group was on the N_5 (FigIX), ($2\varepsilon - 1.904\varepsilon = 0.096\varepsilon$). Thus the N_5 conserved the greatest fractions of its lone pair and so any group leaving a N atom would leave the N_5 preferentially, similarly it was at the N_5 that any one carbon unit would attack.

Thus it could be expected that in the N_5 alkyltetrahydrofolates a positive charge would be localised at the N_5 position and that these would be powerful electrophilic substitution reagents.

The E.S.R. technique was used to study the oxidation of the N_5 alkyl-tetrahydrofolates, to see if any stable characterisable radical species were generated, the location of these species and if, in each instance, they were the same.

Fig IX



Results of E.S.R. Determinations

Folinic Acid

From Table XXXVIII it can be seen that no radicals were detected. The -CHO group is strongly electron attracting, resulting in the compound being reluctant to form radical species.

Tetrahydrofolic Acid (Table XXXIX)

The radicals found were stable over a period of 5 hours. The presence of a radical confirms the work of Bobst[42] but attempts to resolve the signal into its hyperfine structure were unsuccessful.

5-Methyltetrahydrofolic Acid (Table XL)

Compared with tetrahydrofolic acid a more intense initial absorption was obtained. However the signal became broad, probably due to oxidation of the radicals formed.

5-Ethyltetrahydrofolic Acid (Table XLI)

The absorption was similar to 5-methyltetrahydrofolic acid, the radicals being present over a period of time.

Sample E1 (Table XLII)

The absorption was very broad being similar to that for tetrahydrofolic acid.

Sample F1 (Table XLII)

The absorption was very broad being similar to that for tetrahydrofolic acid.

Attempts to resolve the E.S.R. signals into hyperfine splitting failed. Thus a method of determining the identity of the atom on which the lone electron resided was lost.

However, this electron was located by means of its "g" factor.

Table XXXVIII

E.S.R. of Folinic Acid

Sample	Observation
solid folinic acid + standard DPPH	No absorption
aq. folinic acid phosphate buffer pH 7	"
aq. folinic acid 0.1N NaOH pH 13	"
folinic acid in CF_3COOH	"

Table XXXIX

E.S.R. of Tetrahydrofolic Acid

Sample	Observation
solid THFA + standard DPPH	some absorption (masked by standard peak)
solid THFA	broad absorption
aq. THFA phosphate buffer pH 7.0	no absorption

Table XL

E.S.R. of A9 (5-Methyltetrahydrofolic Acid)

Sample	Observation
solid 5MeTHFA [a]	clearly defined absorption (10 gauss)
aq. 5MeTHFA phosphate buffer pH 7.0	no absorption
aq. 5MeTHFA 0.1N NaOH pH 13.0	no absorption
5MeTHFA in CF_3COOH	no absorption

[a] 5-Methyltetrahydrofolic acid.

Table XLI

E.S.R. of C1 (5 Ethyltetrahydrofolic Acid)

Sample	Observation
solid 5EtTHFA [a]	sharp absorption ($7\frac{1}{2}$ gauss)
aq. 5Et THFA pH 1.0	no absorption
aq. 5Et THFA pH 7.0	"
aq. 5Et THFA pH 10	"
aq. 5Et THFA pH 13	"

[a] 5-Ethyltetrahydrofolic acid.

Table XLII

E.S.R. of sample E1 and F1

Sample	Observation
E1 solid	Broad absorption (100 gauss)
F1 solid	Broad absorption (70 gauss)

The "g" factor was characteristic of the particular radical species and was determined by using a standard of known "g" factors. Values of "g" were determined for D.P.P.H. as standard and the samples, by varying the field until resonance was achieved.

For $g = \frac{hV}{\beta H}$ where $\frac{h}{\beta} = 0.71458 \times 10^{-6}$ gauss. sec.

The "g" value ranges from 2.00417 to 2.00436. This was typical of nitrogen radicals. The close proximity of the values suggests that the same nitrogen was involved in each case, this being the N_5 as predicted by molecular orbital theory. (Table XLIII).

Table XLIII

Values of "g" for the 5-alkyltetrahydrofolic acids

Sample	H Mc/sec	V Mc/sec	g
DPPH	14.161	9337	2.00357
	14.120	9336	2.00355
THFA	14.242	9337	2.00422
	14.287	9336	2.00436
5MeTHFA	14.241	9338	2.00432
	14.292	9336	2.00428
5EtTHFA	14.281	9336	2.00425
	14.241	9337	2.00420
E1	14.260	9335	2.00417
	14.240	9337	2.00420
F1	14.253	9335	2.00423
	14.242	9337	2.00420

Biological Testing of Folate Derivatives

As already discussed 5-methyltetrahydrofolic acid would in theory reduce the overall lethal effects of methotrexate. Work was carried out to test this hypothesis and also to compare its effectiveness with that of 5-formyltetrahydrofolic acid. For all experiments male C57BL/BcrXIF/BcrF_1 hybrid mice were used. They were housed in plastic (perspex) boxes containing 4 mice, and were fed cube diet 41B and tap water _ad libitum_.

Groups comprised of 8 mice, each weighing 27-29 g. at the time of the first injection. They were weighed several times before starting the experiments, and daily during experiments. Deaths were also recorded daily. All mice which survived beyond day 11 recovered, and were killed 2-3 weeks after the last injections.

In all experiments methotrexate was administered on 5 consecutive afternoons (days 1-5), and the test compounds were injected 5 hours earlier into the opposite flanks of the animals.

For experiments I and II (Table XLIV) methotrexate sodium parenteral was used after dilution to the appropriate concentrations of methotrexate and sodium chloride. Subsequently, pure methotrexate dihydrate was added to 0.9 per cent sodium chloride and dissolved by adding the minimum amount of solid sodium bicarbonate (final pH approximately 8). 5-Methyltetrahydrofolic acid was administered within an hour of dissolution in 0.9 per cent sodium chloride. 5-Formyltetrahydrofolic acid was administered in 0.9 per cent sodium chloride. All test solutions were administered subcutaneously with the dose per kg. body-weight dissolved in 10 ml., i.e., the volume injected was 0.3 ml. per 30 g. mouse. Control mice received the same volume of saline solution. The dosages and results are summarised in Tables XLIV and XLV.

It was found that 5-methyltetrahydrofolic acid, calcium salt and 5-formyltetrahydrofolic acid calcium salt, had no toxic effects at any

dose used. However 5-methyltetrahydrofolic acid barium salt, although not toxic at doses sufficient to protect against methotrexate toxicity, with single injections of 50 mg. per kg caused some paralysis of the hind legs and diarrhoea. These effects were seen after 15-30 minutes and lasted 2-3 hours. The mice used survived single injections of 100 and 200 mg. of methotrexate per kg. with only slight temporary loss of weight, but 5 consecutive daily injections of 20 mg. per kg. resulted in the death of nearly all the animals within a few days of the last injections. The LD_{50} with this dose schedule lay between 10 and 15 mg. per kg. daily.

The preliminary experiment (Table XLIV) showed that 20 or 60 mg. of 5-methyltetrahydrofolic acid barium salt per kg. strongly protected mice against the toxicity of 5 daily injections of methotrexate (20 mg. per kg.) when given 5 hours before each injection; deaths were reduced from 7/8 in the control mice to 0/8 in each of the test groups. While control mice dropped over 8 g. in weight before dying between 5 and 9 days after the first treatment with methotrexate, mice on the lower dose of 5-methyltetrahydrofolic acid dropped only 1.5 g. and then recovered. A rather larger drop of 3.5 g. at the higher dose was attributed to the toxicity of the barium salt used.

Subsequent experiments (II, III, V) showed that as little 1 mg. of 5-methyltetrahydrofolic acid per kg. gave appreciable protection against methotrexate at 20 or 25 mg. per kg., while 3 mg. per kg. was completely protective.

When the daily dose of methotrexate was doubled to 50 mg. per kg. (III, IV), 5-methyltetrahydrofolic acid at 3 or 5 mg. per kg. still gave appreciable but not complete, protection. With a further doubling to 100 mg. of methotrexate per kg. (VI), however, some deaths still occurred even with 20 or 40 mg. of 5-methyltetrahydrofolic acid per kg.

Direct comparisons between the effects of 5-methyltetrahydrofolic

Table XLIV

Effect of 5-Methyltetrahydrofolic Acid and Citrovorum Factor on the Survival of Methotrexate-treated Mice: Inhibitor and Methotrexate Injected on Days 1,2,3,4 and 5.

EXPT.	TEST COMPOUND	Dose (mg./kg.)	Methotrexate dose (mg./kg.)	Survivors at 14 days	Mice dead on days:	Fall in av. wt. (g.)
I	Control	-	20	1	6,8,8,8,8,8,8	8.5
	$5CH_3THFNa$	20	20	8		1.5
		60	20	8		3.5
II	Control	-	20	2	8,8,8,8,9,9	7
	$5CH_3THF$-Na	1	20	6	8,8	3
		3	20	8		0.5
		10	20	8		0.5
		30	20	8		1
III	$5CH_3THF$ Ca	3	25	8		0.5
		3	50	7	9	3
		3	100	0	6,7,7,7,7,7,8,8	8
IV	Control	-	50	0	6,7,7,7,7,7,8,8	9
	MTHF-Ca	5	50	3	7,8,8,8,8	5
	5CHO THF-Ca	5	50	8		3

Continued

Table XLIV Continued

Expt.	Test Compound	Dose (mg/kg)	Methotrexate dose (mg./kg.)	Survivors at 14 days	Mice dead on days:-	Fall in av. wt. (g.)
V	Control	-	25	0	7,7,7,7,7,7,7,8	8
	$5CH_3THF$-Ca	0.37	25	2	7,8,8,9,9,10	8
		1.1	25	7	8	2
		3.3	25	8		1
		10	25	8		0
	5CHOTHF-Ca	0.37	25	0	6,7,7,7,8,8,8,9	7
		1.1	25	8		3
		3.3	25	8		1
		10	25	8		0
VI	Control	-	100	0	6,6,6,6,6,6,6,6	8
	$5CH_3THF$-Ca	10	100	0	6,8,8,8,8,8,9,9	7
		20	100	6	9,9	5
		40	100	6	9,10	4
	5CHOTHF-Ca	10	100	1	7,8,8,9,9,9,9	6.5
		20	100	7	8	3.5
		40	100	7	8	4

acid and 5-formyltetrahydrofolic acid were made under the same conditions. The first comparison (Table XLIV,IV) indicated that the 5-methyl compound afforded the mice appreciably less protection then did the same dose of 5-formyl compound. Experiment VI, using large doses of inhibitors and methotrexate, led to a similar conclusion when based on the number of mice surviving though at the two higher levels of inhibitor deaths occurred 1-2 days earlier with the 5-formyltetrahydrofolic acid treated mice. With very small levels of inhibitor (0.37 mg. per kg; experiment V) 5-methyltetrahydrofolic acid was slightly more effective than 5-formyltetrahydrofolic acid. Thus it was found that the two tetrahydrofolic acids did not differ greatly in their ability to counteract the toxicity of methotrexate.

Comparison of the effect of one single to three smaller consecutive daily injections is given in Table XLV. Experiment III showed that although 3 mg. of 5-methyltetrahydrofolic acid per kg. daily protects mice strongly against 25 or 50 mg. of methotrexate per kg., the same total amount given on day 5 only had very little protective effect. Even with the dose increased to 50 mg. per kg. (II) the protective effect was still small; the maximum survival (3/8) occurred with administration on day 3. With three consecutive daily doses of 15 mg. per kg (III) the protective effect of 5-methyltetrahydrofolic acid was greatest (5/8 survivors) when given on days 3, 4 and 5 of methotrexate administration. Injections starting on days 1, 2, 4 or 5 failed to save more than 1 out of 8 animals. Reduction in the frequency of 5-methyltetrahydrofolic acid administration thus greatly reduces its protective effect against methotrexate toxicity.

Table XLV

Effect of 5-Methyltetrahydrofolic Acid on the Survival of Methotrexate-treated Mice when Administered on 1 or 3 days of Methotrexate Treatment only

Expt.	Dose of MTHF-Ca (mg./kg.)	Injected on day(s):-	Methotrexate dose (mg./kg.)	Survivors at 14 days	Mice dead on days:-	Fall in av. wt. (g.)
I	15	5	25	3	7,7,8,8,8	7
	15	5	50	0	7,7,7,7,7,7,8,8	7
	15	5	100	0	5,6,6,7,7,8,8,8	8
II	Control	-	50	0	6,7,7,7,7,8,8,9	10
	50	1	50	1	7,8,8,8,9,9,10	6
	50	2	50	2	7,9,9,9,9,10	5.5
	50	3	50	3	9,9,10,10,10	6
	50	4	50	1	7,7,7,7,8,8,11	8.5
	50	5	50	0	6,6,7,7,8,8,8,10	5
III	Control	-	50	0	6,7,7,7,7,7,8,8	9
	15	1,2,3	50	1	7,7,7,8,8,9,11	5
	15	2,3,4	50	1	6,8,8,8,8,8,11	6.5
	15	3,4,5	50	5	7,7,9	7
	15	4,5,6	50	1	6,7,7,7,7,7,7	5.5
	15	5,6,7	50	0	6,6,6,6,7,7,7,8	9

CONCLUSION

It has been shown that relatively small daily amounts of 5-methyltetrahydrofolic acid gives complete protection as judged by survival and body-weight against lethal doses of methotrexate. This protective action is attributed to direct conversion and utilisation of the 5-methyltetrahydrofolic acid without involvement of dihydrofolic acid (see Fig. V). It was found that small daily doses were much more effective than fewer larger doses and that 5-methyltetrahydrofolic acid and 5-formyltetrahydrofolic acid did not differ greatly in their ability to inhibit methotrexate toxicity.

Experiments with Higher Folate Analogues

Further experiments were carried out with other folate analogues (Table XLVI, Experiments II-V).

5-Ethyltetrahydrofolic acid afforded limited protection at the doses used. This was true also for folic acid itself and for the small dose of dihydrofolic acid (0.02 ml./kg.). With 0.1 ml. per kg. completed protection was afforded.

For the 5 n-butyltetrahydrofolic acid complete protection was afforded at 25 mg./kg. but this result was thought to be possibly due to the small tetrahydrofolic acid contaminant and the result is being further investigated.

Experiments using R_1 lymphoma

Experiments have been carried out by Dr. T.A. Connors of the Chester Beatty Research Institute on mice bearing the R_1 lymphoma.

The R_1 lymphoma is obtained by subcutaneous injection of 2×10^6 ascites cells. It disseminates rapidly and untreated mice die consistently at about 9 days. Treatment was begun three days later and was given on five consecutive days intraperitoneally. Table XLVII

Table XLVI

Effect of Folate Analogues on the Survival of Methotrexate-treated Mice: Inhibitor and Methotrexate Injected on Days 1,2,3,4,5

EXPT.	TEST COMPOUND	Dose (mg/kg)	Methotrexate dose (mg/kg)	Survivors at 14 days	Mice dead on days	Fall in av. wt.(g)
I	control		25	1	6,7,7,7,8,9,9	8.25
II	5EtTHF [a]	25	25	6	8,9	2.75
III	5nBuTHF	25	25	8		0.25
IV	FA	25	25	6	6,9	3.5
V	DHF	(0.02ml/kg)	25	2	7,7,8,8,9,9	4
		(0.10ml/kg)	25	8		2.25

[a] Abbreviations as in Table I and II.

Table XLVII

Effect of 5-Methyltetrahydrofolic acid and methotrexate on survival: Mice with R_1 lymphoma

Expt.	Methotrexate dose mg/kg.	Dose mg/kg.	Mean Survival Days
Control			9.5
I	0.188		10.6
II	0.375		11.0
III	0.75		13.6
IV	1.5		16.0
V	3.0		16.4
VI	6.0		16.2
VII	12.0		12.2
VIII	24.0		9.0
IX	3.0	1	16.6
X	3.0	5	16.2
XI	3.0	10	14.2
XII	3.0	15	14.0
XIII	16.0	40	14.8
XIV	32.0	40	14.6
XV	64.0	40	14.6

shows that the optimum dose of methotrexate is 3.0 mg./kg. and the above 6.0 mg./kg. drug toxicity becomes important. Using the optimum dose of methotrexate low dose levels of 5-methyltetrahydrofolic acid have no effect on the tumour while high dose levels reduce the potency of the inhibitor.

Using doses of methotrexate at which the animal would succumb to drug toxicity, combinations of methotrexate and 5-methyltetrahydrofolic acid give improved survival times. It has been also shown that large doses of 5-methyltetrahydrofolic acid do not affect the growth of the tumour. Further experiments are in progress.

ACKNOWLEDGEMENTS

The author thanks Dr. J. A. Blair for supervision and encouragement; Dr. T. Connors, Chester Beatty Research Institute and Dr. C. E. Searle, University of Birmingham for the animal testing; Mr. R. J. Leeming, General Hospital, Birmingham for the microbiological assays; to Mr. E. Hartland, University of Aston in Birmingham, for the N.M.R. spectra and Mr. P.J.P. Doyle, University of Aston for the E.S.R. determinations.

The author is also grateful to the Birmingham Branch, British Empire Cancer Campaign for financial assistance.

REFERENCES

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

2. Wills, L., and Stewart, A., Brit.J.Exptl.Pathol., 16, 444 (1935).

3. Day, P.L., Langston, W.C., and Darby, W.J., Proc.Soc.Exptl.Biol. Med., 38, 860 (1938).

4. Stokstad, E.L.R., and Manning, P.D.V., J.Biol.Chem., 125, 687 (1938).

5. Hogan, A.G. and Parrott, E.M., J.Biol.Chem., 132, 507 (1940).

6. Pfiffner, J.J., Binkley, S.B., Bloom, E.S., Brown, R.A., Bird, O.D., Emmett, A.D., Hogan, A.G., and O'Dell, B.L., Science 97, 404 (1943).

7. Snell, E.E., and Peterson, W.H., J Bacteriol., 39, 273 (1940).

8. Mitchell, H.K., Snell, E.E., and Williams, R.J., J.Am.Chem.Soc., 63, 2284 (1941).

9. Mitchell, H.K., Snell, E.E., and Williams, R.J., J.Am.Chem.Soc., 66, 267 (1944).

10. Angier, R.B., Boothe, J.H., Hutchings, B.L., Mawat, J.H., Semb, J., Stokstad, E.L.R., Subba Row, Y., Waller, C.W., Cosulich, D.B., Fahrenbach, M.J., Hultquist, M.E., Kuh, E., Northey, E.H., Seeger, D.R., Sickels, J.P. and Smith, J.M., Jr., Science 103, 667 (1946).

11. Franklin, A.L., Stokstad, E.L.R., and Jukes, T.H., J.Biol.Chem., 169, 427 (1947).

12. Wagner, A.F., and Folkers, K., "Vitamins and Coenzymes," Interscience Publishers, John Wiley and Sons, N.Y., 123 (1964).

13. Hortman, S.C., and Buchanan, J.M., Ann.Rev.Biochem., 28, 368 (1959).

14. Oleson, J.J., Hutchings, B.L., and Subba Row, Y., J.Biol.Chem., 175, 359 (1948).

15. Franklin, A.L., Stokstad, E.L.R., and Jukes, T.H., Proc.Soc. Exptl.Biol.Med., 67, 398 (1948).

16. Philips, S.F., and Thiersch, J.B., J. Pathol.Exptl.Therap., 95, 303 (1949).

17. Franklin, A.L., Belt, M., Stokstad, E.L.R., and Jukes, T.H., J.Biol.Chem., 177, 621 (1949).

18. Goldin, A., Venditti, J.M., Kline, J., and Mantel, N., Nature, 212, 1548 (1966).

19. Schworzenberg, L., Mathe, G., Hayat, M., De Vassal, F., Amiel, J. L., Cattan, A., Schneider, M., Schlumberger, J.J., Rosenfeld, C., Jasmin, C., and Ngo Minh Man, La Presse Medicale, 77, 385 (1969).

20. Rueckert, R.R., and Mueller, G.C., Cancer Res., 20, 1584 (1960).

21. O'Brien, J.S., Cancer Res., 22, 267 (1962).

22. Blakley, R.L., "Biochemistry of Folic Acid and Related Pteridines", Amsterdam, (North Holland Publishing Co)

23. Donaldson, K.O., and Keresztesy, J.C., J.Biol.Chem., 234, 3235 (1959)

24. Keresztesy, J.C., and Donaldson, K.O., Biochem.Biophys.Res.Commun. 5, 286 (1960)

25. Donaldson, K.O., and Keresztesy, J.C., Biochem.Biophys.Res.Commun. 5, 289 (1960).

26. Donaldson, K.O., and Keresztesy, J.C., Fed.Proc., 20, 453 (1961).

27. Sakami, W., and Ukstins, I., J.Biol.Chem., 236, PC 50 (1961).

28. Gupta, V.S., and Huennekens, F.M., Arch.Biochem.Biophys., 120, 712 (1967)

29. O'Dell, B.L., Vanderbelt, J.M., Bloom, E.S., and Pfiffner, J.J., J.Am.Chem.Soc., 69, 250 (1947)

30. Hatefi, Y., Talbert, P.T., Osborn, M.J., and Huennekens, F.M., Biochem.Prep., 7, 89 (1960)

31. Scrimgeour, K.G., and Vitals, K.S., Biochemistry, 5, 1438 (1966).

32. Silverman, M., and Noronha, J.M., Biochem.Biophys.Res.Commun.,

4, 180 (1961).

33. Chanarin, I., and Perry, J., Biochem.J., 105, 633 (1967).

34. Horwitz, S.B., Kwak, G., Wilson, L., and Kisliuk, R.L., J.Med. Chem., 12, 49 (1969).

35. Whiteley, J.M., and Huennekens, F.M., Biochem., 6, 2620 (1967).

36. Zakrzewski, S.F., Federation Proc., 22, 231 (1966).

38. Kallen, R.G., and Jencks, W.P., J. Biol.Chem., 241, 5851 (1966).

39. Benkovic, S.J., Benkovic, P.A., and Chrzanawski, R., J.Am. Chem.Soc., 92, 523 (1970).

40. Gupta, V.S., Drais, J.H., and Huennekens, F.M., Arch.Biochem. Biophys., 133, 436 (1969).

41. Osborn, M.J., Talbert, P.T., and Huennekens, F.M., J.Am.Chem.Soc. 82, 4921 (1960).

42. Horwitz, S.B., and Kisliuk, R.L., J.Med.Chem., 11, 907 (1968).

43. Whiteley, J.M., Drais, J.H., and Huennekens, F.M., Arch.Biochem. Biophys., 133, 436 (1969).

44. Hruska, F.E., Bell, C.L., Victor, T.A., and Danyluk, S.S., Biochem., 7, 3721 (1968).

45. Perault, A., and Pullman, B., Biochim.Biophys.Acta., 44, 251 (1960).

46. Fujimori, E., Proc.Natl.Acad.Sci. U.S., 44, 1197 (1958).

47. Bobst, A., Helv.Chim.Acta., 50, 2222 (1967).

48. Bobst, A., Helv.Chim.Acta., 51, 607 (1968).

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ANALYTICAL BIOCHEMISTRY 41, 332–337 (1971)

Preparation of 5-Alkyltetrahydrofolic Acids

(*dl*-5-Alkyl-5,6,7,8-tetrahydropteroyl-L-monoglutamic Acids)

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Received July 29, 1970

5-Methyltetrahydrofolate (*dl*-5-methyl-5,6,7,8-tetrahydropteroyl-L-monoglutamic acid) has been prepared directly from folic acid without intermediate isolation of tetrahydrofolic acid (1). We now report the preparation of the ethyl, *n*-propyl, and *n*-butyltetrahydrofolates as their calcium salts.

EXPERIMENTAL

General

Chemicals were obtained from the following sources: folic acid, Koch Light Ltd., Colnbrook, Bucks; sodium borohydride, acetaldehyde, butyraldehyde, propionaldehyde, Tris buffer, Hopkin and Williams Ltd.; and DEAE-cellulose (D.E. 52), W. and R. Balston Ltd., Maidstone, Kent.

Ultraviolet spectra were determined in aqueous solution at pH 1.0, 7.0, and 13.0. Thin-layer chromatography was performed with 0.25 mm depth cellulose powder MN300, F254 (Macherey, Nagel and Co., Duren, Germany) and developed at room temperature in the dark.

The following solvent systems were used;

(a) 0.1 *M* phosphate buffer, pH 7.0, containing mercaptoethanol (0.5% v/v).

(b) *n*-Propanol/water/ammonia (200:100:10 v/v) containing mercaptoethanol (0.5% v/v).

(c) The organic phase of 1-butanol/acetic acid/water (4:1:5 v/v) containing mercaptoethanol (0.5% v/v). Compounds were detected as quenching or fluorescent spots when viewed under ultraviolet light emitting at either 254 or 365 mμ.

Proton magnetic resonance spectra were recorded with a Perkin-Elmer R10 spectrometer using tetramethylsilane (TMS) as internal reference.

DEAE-cellulose was provided preswollen and required no further washing.

Preparation of 5-Ethyltetrahydrofolic Acid

5-Ethyltetrahydrofolic acid was prepared by the method of Blair and Saunders (1) replacing formaldehyde with acetaldehyde. Sodium borohydride (6 gm, 160 mmoles) in water (50 ml) was added to a stirred solution of folic acid (6 gm, 15 mmoles) in 0.066 M Tris buffer, pH 7.8 (200 ml), under nitrogen at room temperature. After 15 min, excess sodium borohydride was destroyed with 5 N acetic acid and the solution adjusted to pH 7.8. Acetaldehyde (5.7 ml, 100 mmoles) was added, immediately followed by sodium borohydride (12 gm, 320 mmoles) in water (100 ml). The mixture was incubated for 1 hr at 45°C under a slow stream of nitrogen and cooled, and mercaptoethanol (1.5 ml) added. The pH was adjusted to 7.0 and the mixture diluted to 500 ml with cold water.

The pale yellow solution was passed through a 3.8×45 cm column of DEAE-cellulose previously equilibrated with ammonium acetate (3 liters, 0.13 M, pH 7.0) containing mercaptoethanol (0.2 M). Elution was initiated with ammonium acetate (2 liters, 0.13 M, pH 7.0) followed by a gradient of ammonium acetate (0.13–0.4 M, pH 7.0, 1 liter each) and completed with ammonium acetate (2 liters, 0.4 M, pH 7.0). All solutions contained mercaptoethanol (0.2 M). The 0.13 M buffer eluate (2 liters) was collected in bulk and discarded. After 200 ml of the ammonium acetate gradient had passed through the column, the eluate was collected in 15 ml fractions and the extinction at 290 mμ was determined after dilution with 0.1 M phosphate buffer, pH 7.0. A complete spectrum was recorded on every tenth fraction.

The product, 5-ethyltetrahydrofolic acid, was found in tubes 50–130 and was characterized by an extinction maximum at 290 mμ and a minimum at 250 mμ; the extinction ratio ($A_{290}:A_{250}$) was greater than 2.5. The contents of tubes 50–130 were bulked and freeze-dried. The yield of 5-ethyltetrahydrofolic acid using an extinction coefficient of $\epsilon = 31.0 \times 10^{-3}\ M^{-1}\ cm^{-1}$ at 290 mμ as for 5-methyltetrahydrofolic acid (1) was about 70% based on folic acid used.

Preparation of Calcium Salt. To the freeze-dried chromatographically purified 5-ethyltetrahydrofolic acid (2.5 gm) was added deaerated water (20 ml) containing sodium chloride (500 mg). The pH was adjusted to 7.0 (0.1 N NaOH) and the solution filtered. Calcium chloride solution (12 ml of 10 gm $CaCl_2 \cdot 6H_2O$/100 ml water) and mercaptoethanol sufficient to give a final concentration of 10^{-2} M was added to the filtrate. The calcium salt was precipitated by the addition of methanol (500 ml)

and the solution cooled overnight. The calcium salt was centrifuged at 5°, washed with 75% aqueous methanol (20 ml), followed by methanol (20 ml) and dried rapidly under high vacuum over phosphorus pentoxide at room temperature. Yield 1.2 gm. purity 90%.

The calcium salt was recrystallized from deaerated water containing mercaptoethanol (10^{-3} M) and diluting slowly with methanol to give a crystalline precipitate which was centrifuged, washed, and dried rapidly to yield the calcium salt of 5-ethyltetrahydrofolate.

Calc. for $C_{21}H_{25}N_7O_6Ca \cdot 4H_2O$: C 43.22, H 5.70, N 16.80, Ca 6.87; NCH_2CH_3 4.97. Found: C 43.88, H 5.22, N 16.30, Ca 6.10, NCH_2CH_3 4.19.

UV data are given in Table 1.

Proton magnetic resonance spectrometry in trifluoroacetic acid gave a N—CH_2CH_3 splitting pattern with an unresolved triplet at $\tau = 8.5$ and a poorly defined quartet at $\tau = 6.1$. There was no evidence of a methanol CH_3 at $\tau = 6.3$ or an acetate CH_3 at $\tau = 7.75$.

The product was biologically inactive for *Lactobacillus casei* and *Streptococcus faecalis*.

Preparation of 5-n-Propyltetrahydrofolic Acid

The preparation was carried out as for the ethyl derivative but replacing acetaldehyde with propionaldehyde (7.3 ml, 100 mmoles). After elu-

TABLE 1
Spectral Data of Folate Analogs[a]

Compound	pH	λ_{max} (mμ)	λ_{min} (mμ)	$\epsilon_{max} \times 10^{-3}$	Absorbance ratio	Ref.
5 Me THFA[b]	1	270, 294	242, 281	24.0, 23.2	$A_{270}/A_{242} = 2.60$	Unpub. data
	7	290	245	32.0	3.85	2
	13	290	245	31.6	3.40	Unpub. data
5 Et THFA[c]	1	273, 294	245, 284	21.5, 19.6	$A_{273}/A_{245} = 2.35$	This paper
	7	292	250	31.4	2.8	"
	13	291	249	29.6	3.0	"
5 Pr THFA[d]	1	274, 290	244, 282	22.7, 22.0	$A_{274}/A_{244} = 2.44$	"
	7	290	250	31.5	3.05	"
	13	290	250	31.0	2.8	"
5 Bu THFA[e]	1	272, 287	240, 283	20.5, 19.5	$A_{272}/A_{240} = 2.18$	"
	7	288	245	29.5	2.8	"
	13	288	244	29.5	2.5	"

[a] All spectra done on calcium salts.
[b] 5-Methyltetrahydrofolic acid.
[c] 5-Ethyltetrahydrofolic acid.
[d] 5-*n*-Propyltetrahydrofolic acid.
[e] 5-*n*-Butyltetrahydrofolic acid.

tion, the product, 5-propyltetrahydrofolic acid was found in tubes 35–135 and was characterized by an extinction maximum at 290 mμ and a minimum at 250 mμ; the extinction ratio ($A_{286}:A_{250}$) was greater than 2.5. The yield of 5-propyltetrahydrofolic acid using an extinction coefficient of $\epsilon = 31.0 \times 10^3 \ M^{-1} \ cm^{-1}$ at 290 mμ as for 5-methyltetrahydrofolic acid (1) was about 80%.

Preparation of Calcium Salt. The calcium salt was prepared and recrystallized as for the ethyl derivative.

Calc. for $C_{22}H_{27}N_7O_6Ca \cdot 3H_2O$: C 45.59, H 5.74, N 16.92, Ca 6.91, $NCH_2CH_2CH_3$ 9.84. Found: C 45.30, H 5.70, N 17.86, Ca 8.1, $NCH_2CH_2CH_3$ 7.08.

UV data are given in Table 1.

Proton magnetic resonance spectrometry in trifluoroacetic acid gave a N—CH_2—CH_2—CH_3 splitting pattern with an unresolved triplet at $\tau = 9.0$, and poorly defined peaks at $\tau = 6.1$ and $\tau = 8.1$.

Preparation of 5-n-Butyltetrahydrofolic Acid

The preparation was carried out as for the ethyl derivative but replacing acetaldehyde with butyraldehyde (9.0 ml, 100 mmoles). After elution, the product, 5-butyltetrahydrofolic acid, was found in tubes 45–130 and was characterized by an extinction maximum at 286 mμ and minimum at 250 mμ; the extinction ratio ($A_{286}:A_{250}$) was greater than 1.5. The yield of 5-butyltetrahydrofolic acid using an extinction coefficient of $\epsilon = 31.0 \times 10^3 \ M^{-1} \ cm^{-1}$ at 290 mμ as for 5-methyltetrahydrofolic acid (1) was about 60%.

Preparation of Calcium Salt. The calcium salt was prepared as for the ethyl derivative.

Calc. for $C_{23}H_{29}N_7O_6Ca \cdot 4H_2O$: C 45.16, H 6.10, N 16.03, *n*-butyl 11.63. Found: C 44.00, H 5.95, N 17.30, *n*-butyl 4.25.

UV data are given in Table 1.

Proton magnetic resonance spectrometry in trifluoroacetic acid gave a N—CH_2—CH_2—CH_2—CH_3 splitting pattern with an unresolved triplet at $\tau = 9.1$ and poorly defined peaks at $\tau = 6.1$, $\tau = 8.2$, and $\tau = 8.6$. There was no evidence of a methanol CH_3 at $\tau = 6.3$ or an acetate CH_3 at $\tau = 7.75$.

RESULTS AND DISCUSSION

Tetrahydrofolate analogs have been prepared from folic acid without isolation of the intermediate tetrahydrofolic acid, which is unstable.

Microbiological assay shows the absence of folic acid. TLC data are given in Table 2 and show the presence of a light fluorescent impurity.

TABLE 2
R_f Values in Various Solvents[a]

Solvent[b]	5 Me THFA	5 Et THFA	5 Pr THFA	5 Bu THFA
a	0.84	0.84	0.84	0.84
	(0.52) bl. fl.[c]	(0.52) bl. fl.[c]	(0.52) bl. fl.[c]	(0.52) bl. fl.[c]
b	0.55	0.55	0.55	0.55
	(0.37) bl. fl.[c]	(0.37) bl. fl.[c]	(0.37) bl. fl.[c]	(0.37) bl. fl.[c]
c	0.68	0.68	0.68	0.68
	(0.44) bl. fl.[c]	(0.44) bl. fl.[c]	(0.44) bl. fl.[c]	(0.44) bl. fl.[c]

[a] These were detected as quenching spots unless otherwise indicated. Minimum detectable amounts about 5 μg in 1 μl. Abbreviations as in Table 1.
[b] As in "Experimental."
[c] Trace fluorescent impurity. Bl. fl. = blue fluorescence.

The compounds were not separable in the solvent systems used. The PMR shows the absence of an acetate CH_3 at $\tau = 7.75$ and methanol CH_3 at $\tau = 6.3$. The PMR also shows the absence of folic acid (C 7 singlet at $\tau = 0.9$). However, in the case of the butyl compound there is evidence of a small tetrahydrofolic acid impurity (doublet at $\tau = 2.4$).

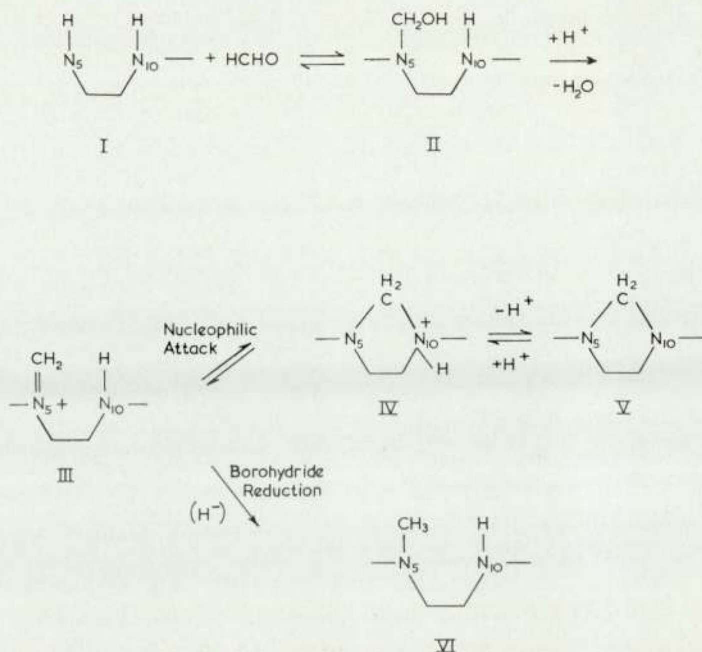

FIGURE 1

This reductive condensation has been thought to go via N_5,N_{10}-methylenetetrahydrofolic acid (V, Fig. 1). A more probable mechanism is given in Fig. 1.

Kallen and Jencks (2) have suggested that condensation of formaldehyde with tetrahydrofolic acid (I) proceeds via the imine (III). The initial step is a nucleophilic attack of tetrahydrofolic acid on formaldehyde to give the carbinolamine (II), which is then protonated and dehydrated to give (III). Nucleophilic attack by N_{10} would give the 5,10-methylene compound (V) or by hydride ions from borohydride the 5-methyl compound (III). Interconversion of (III) and (V) would explain the reduction of 5,10-methylenetetrahydrofolic to 5-methyltetrahydrofolic acid (3). A similar mechanism would apply where formaldehyde is replaced by acetaldehyde, propionaldehyde, or butyraldehyde.

That the presence of N_{10} is not required for reductive condensations is shown by Whiteley *et al.* (4) in the preparation of 2-amino-4-hydroxy-5,6-dimethyltetrahydropteridine. Successful condensations can also be obtained when a substituent is present at N_{10} (5).

SUMMARY

The ethyl, *n*-propyl, and *n*-butyltetrahydrofolic acids have been prepared as their calcium salts by a method similar to that for 5-methyltetrahydrofolic acid.

ACKNOWLEDGMENT

We are grateful to the Birmingham branch, British Empire Cancer Campaign, for financial assistance and to Mr. R. J. Leeming, General Hospital, Birmingham, for the microbiological assays.

REFERENCES

1. Blair, J. A., and Saunders, K. J., *Anal. Biochem.* **34,** 376 (1970).
2. Kallen, R. G., and Jencks, W. P., *J. Biol. Chem.* **241,** 5851 (1966).
3. Gupta, V. S., and Huennekens, F. M., *Arch. Biochem. Biophys.* **120,** 712 (1967).
4. Whiteley, J. M., Drais, J. H., and Huennekens, F. M., *Arch. Biochem. Biophys.* **133,** 436 (1969).
5. Blair, J. A., and Saunders, K. J., unpublished results. (1970).

Reprinted from ANALYTICAL BIOCHEMISTRY, Volume 41, Number 2, June 1971
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ANALYTICAL BIOCHEMISTRY 41, 482–486 (1971)

Preparation of Analogs of *dl*-Tetrahydromethotrexate (4-Amino-4-deoxy-N^{10}-methyl-*dl*-5,6,7,8-tetrahydropteroyl-L-monoglutamic Acid)

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Received August 28, 1970

5-Alkyl derivatives of *dl*-tetrahydromethotrexate have been prepared by reduction of methotrexate by sodium borohydride followed by reductive condensation with aldehyde and sodium borohydride and formation of the calcium salt (1–3).

MATERIALS AND METHODS

Chemicals were obtained as follows: methotrexate, Lederle Laboratories; sodium borohydride, formaldehyde, acetaldehyde, Tris buffer, Hopkin and Williams Ltd; and DEAE-cellulose (D.E. 52), W. and R. Balston Ltd.

Ultraviolet spectra were determined in aqueous solution at pH 1.0, 7.0, and 13.0. Thin-layer chromatography was performed with 0.25 mm depth cellulose powder MN 300 F254 (Macherey, Nagel and Co., Duren, Germany) and developed at room temperature in the dark.

The following solvent systems were used:

(*a*) 0.1 *M* phosphate buffer, pH 7.0, containing mercaptoethanol (0.5%, v/v).

(*b*) *n*-Propanol/water/0.88 sp.gr. aqueous ammonia (200:100:10, v/v) containing mercaptoethanol (0.5%, v/v).

(*c*) The organic phase of 1-butanol/acetic acid/water (4:1:5, v/v) containing mercaptoethanol (0.5%, v/v). Compounds were detected as quenching or fluorescent spots when viewed under ultraviolet light emitting at either 254 or 365 mμ.

Proton magnetic resonance (PMR) spectra were recorded with a Perkin-Elmer R10 spectrometer using tetramethylsilane (TMS) as internal reference in the case of trifluoroacetic acid (TFA) and as external reference in the case of D_2O. DEAE-cellulose was provided preswollen and required no further washing. N—CH_3 and N—CH_2CH_3 determinations were carried out by the Ziesel method.

Preparation of 5-Methyltetrahydromethotrexate

Sodium borohydride (6 gm, 160 mmoles) in water (50 ml) was added to a stirred solution of methotrexate (6 gm, 13 mmoles) in 0.066 *M* Tris buffer, pH 8.0 (200 ml), under nitrogen at room temperature. After 20 min, excess sodium borohydride was destroyed with 5 *N* acetic acid and the solution adjusted to pH 7.8. Formaldehyde (8 ml, 37% w/v, 100 mmoles) was added, immediately followed by sodium borohydride (12 gm, 320 mmoles) in water (100 ml). The mixture was incubated for 1 hr at 40°C under a slow stream of nitrogen and cooled, and mercaptoethanol (1.5 ml) added. The pH was adjusted to 7.0 and the mixture diluted to 500 ml with cold water. The product was purified as previously (2) and was found in tubes 35–100, being characterized by an extinction maximum at 301 mμ and a minimum at 260 mμ; the extinction ratio $A_{310}:A_{248}$ was greater than 2.4. Tubes 35–100 were combined and freeze-dried. The yield of 5-methyltetrahydromethotrexate using an extinction coefficient of $\epsilon = 30 \times 10^3\ M^{-1}\ cm^{-1}$ at 301 mμ was about 30% based on methotrexate used.

Preparation of Calcium Salt

To the freeze-dried chromatographically purified 5-methyltetrahydromethotrexate (2.0 gm) was added deaerated water (40 ml) containing sodium chloride (400 mg). The pH was adjusted to 7.0 (0.1 *N* NaOH) and the solution filtered. Calcium chloride solution (10 ml of 10 gm $CaCl_2 \cdot 6H_2O$/100 ml water) and mercaptoethanol sufficient to give a final concentration of $10^{-2}\ M$ were added to the filtrate. The calcium salt was precipitated by addition of methanol (400 ml), and dried rapidly over P_2O_5 under high vacuum at room temperature (yield 1.2 gm). The calcium salt was recrystallized from deareated water containing mercaptoethanol ($10^{-3}\ M$) and diluting slowly with methanol to give a crystalline precipitate that was filtered, washed, and dried rapidly to yield the calcium salt of 5-methyltetrahydromethotrexate. UV data are given in Table 1.

Proton magnetic resonance spectrometry in TFA gave a characteristic N^5CH_3 at $\tau = 6.65$. There is no evidence of methanol CH_3 $\tau = 6.3$ or an acetate CH_3 $\tau = 7.75$ (in D_2O, N^5CH_3 at $\tau = 7.5$).

Calculated for $C_{21}H_{26}N_8O_5 \cdot Ca \cdot 4H_2O$: C 43.25, H 5.92, N 19.22, Ca 6.86, N^5CH_3 4.98, $N^{10}CH_3$ 4.98. *Found:* C 42.81, H 5.75, N 19.08, Ca 5.87, NCH_3, 4.59.

For methotrexate, *calculated* $NCH_3 = 6.1$; *found* $NCH_3 = 2.92$. $N^{10}CH_3$ appears at $\tau = 6.2$ in PMR in TFA.

Preparation of Ethyl Derivative

The preparation was carried out as for the methyl derivative but replacing formaldehyde with acetaldehyde (5.7 ml, 100 mmoles). After

elution the product, 5-ethyltetrahydromethotrexate, was found in tubes 30–110 and was characterized by an extinction maximum at 300 mμ and minimum at 260 mμ; the extinction ratio ($A_{300}:A_{260}$) was greater than 2.5. The yield of 5-ethyltetrahydromethotrexate using an extinction coefficient of $\epsilon = 30.0 \times 10^3 M^{-1}$ cm^{-1} was about 30%.

Preparation of Calcium Salt

The calcium salt was prepared as for the methyl derivative. UV data are given in Table 1.

Proton magnetic resonance spectrometry in TFA gave a characteristic N_5—CH_2CH_3 splitting pattern with an unresolved triplet at $\tau = 8.5$. The quartet which should appear at $\tau = 6.1$ is masked by N_{10}—CH_3 at $\tau = 6.2$. There is no evidence of an acetate CH_3 at $\tau = 7.75$.

Calculated for $C_{22}H_{28}N_8O_5Ca \cdot 4H_2O$: C 44.29, H 6.08, N 18.78, $N^5CH_2CH_3$ 7.22, $N^{10}CH_3$ 4.87. *Found:* C 44.90, H 5.80, N 19.34, $N^5CH_2CH_3$ 7.40.

RESULTS AND DISCUSSION

5-Methyl and 5-ethyltetrahydromethotrexates have been prepared from methotrexate without the isolation of the intermediate tetrahydromethotrexate.

TABLE 1
Spectral Data of Methotrexate Analogs[a]

Compound	pH	λ_{max} (mμ)	λ_{min} (mμ)	$\epsilon_{max} \times 10^{-3}$	Absorbance ratio (max:min)
MTX[b]	1	309,244	263,235	20.6,17.4	2.58,1.07
	7	376,307,260 (shoulder 222)	345,274,240	9.4,24.5,24.0	1.22,1.32,1.56
	13	376,307,262	345,274,240	9.4,24.4,24.8	1.43,1.32,2.47
THMTX[c] (4)	0.3	306	—	15.2	—
	7	306	—	28.3	—
	13	306	—	28.3	—
5MeTHMTX[d]	1	299	260	20.1	2.3
	7	301	258	30.0	3.44
	13	300	260	28.8	3.04
5EtTHMTX[e]	1	300	258	20.0	2.1
	7	300	257	30.0	3.3
	13	300	258	28.6	3.0

[a] Spectra of methotrexate done as free acid and of 5-methyl and 5-ethyl derivatives as calcium salts.

[b] Methotrexate.

[c] Tetrahydromethotrexate (4).

[d] 5-Methyltetrahydromethotrexate.

[e] 5-Ethyltetrahydromethotrexate.

TABLE 2
R_f Values in Various Solvents[a]

Solvent[b]	MTX	5MeTHMTX	5EtTHMTX
a	0.78	0.65	0.65
b	0.40	0.13	0.13
c	0.68	0.75	0.75

[a] These were detected as quenching spots. Minimum detectable amount about 5 μg in 1 μl. Abbreviations as in Table 1.
[b] As in "Methods."

Comparison of the product with starting material and other possible products is given in Tables 1 and 2.

By analogy with the tetrahydrofolate derivatives reductive condensation takes place at N_5 (Fig. 1).

This is substantiated by the PMR spectrum which shows N_5—CH_3 (singlet) at $\tau = 7.5$ in D_2O and N_5—CH_3 at $\tau = 6.65$ in TFA as with the folate analogs (2,5). N_{10}—CH_3 appears at $\tau = 7.0$ in D_2O and at $\tau = 6.2$ in TFA.

Also the CH_3 of N_5—CH_2CH_3 appears at $\tau = 8.5$ in TFA as with the folate analog (3).

PMR also shows the absence of methotrexate (C7 singlet at $\tau = 0.9$ in TFA).

The formation of these methyl and ethyl derivatives substantiates the

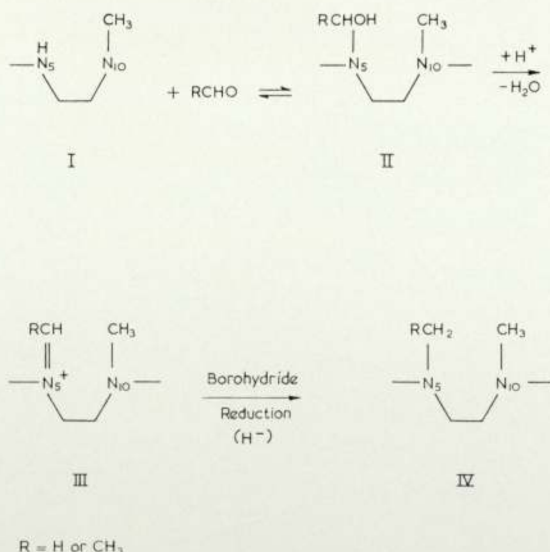

FIGURE 1

proposal that the reductive condensation proceeds via reduction of the imine (III in Fig. 1) (3).

The Ziesel determination depends on the basicity of the nitrogen bearing the alkyl. The N_{10} in methotrexate is weakly basic and this explains the low value obtained. However in the reduced folate derivatives the basicity of the N_{10} is still further reduced (6) and the value obtained is probably only that due to the alkyl on N_5.

SUMMARY

5-Methyl and 5-ethyltetrahydromethotrexates have been prepared as their calcium salts by a method similar to that for 5-methyltetrahydrofolic acid.

ACKNOWLEDGMENT

We are grateful to the Birmingham branch, British Empire Cancer Campaign for financial assistance.

REFERENCES

1. Blair, J. A., and Saunders, K. J., British Patent Application 14027/69 (1969).
2. Blair, J. A., and Saunders, K. J., *Anal. Biochem.* **34**, 376 (1970).
3. Blair, J. A., and Saunders, K. J., *Anal. Biochem.* **41**, 332 (1971).
4. Horwitz, S. B., and Kisliuk, R. L., *J. Med. Chem.* **11**, 907 (1968).
5. Blair, J. A., and Saunders, K. J., unpublished data.
6. Kallen, R. G., and Jencks, W. P., *J. Biol. Chem.* **241**, 5845 (1966).