Synthesis and Reactions of 5-Alkyltetrahydrofolates

by

Anthony Joseph Robb

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in
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SUMMARY.

5-Methyltetrahydrofolic acid, 5-n-butyltetrahydrofolic acid and tetrahydromethotrexate have been successfully prepared.

The solubility of 5-methyltetrahydrofolic acid was determined in the pH range 1 to 7; also, the pK value for the ionisation of the 3,4-amide function was measured by a spectrophotometric method.

An attempt was made to separate the diastereoisomers of 5-methyltetrahydrofolic acid on a DEAE cellulose column and also by fractional crystallisation.

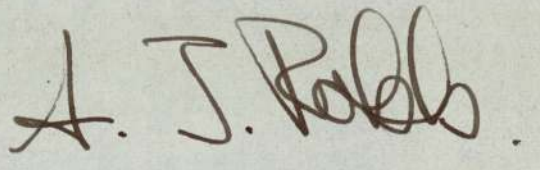
A detailed kinetic study of the autoxidation of 5-methyltetrahydrofolic was carried out using a simple manometric method and the rate of oxidation was found to have first order dependence on both 5-methyltetrahydrofolic acid concentration and oxygen partial pressure.

The effects of pH and various catalysts and inhibitors were studied. The rate was found to increase on going from pH 9 to pH 13 but decreased markedly from pH 5 to pH 1. Copper ions were found to increase the reaction rate by a factor of 10, whereas phenol (a free-radical scavenger) halved the reaction rate.

Product analysis was undertaken using n.m.r., t.l.c. u.v., i.r., e.s.r., and microbiological assay techniques and the major products of oxidation were found to be 5-methyl-5,6-dihydrofolic acid and 8-dehydro-4a-hydroxy-5-methyltetrahydrofolic acid. A search for observable intermediates proved fruitless.

A mechanism in keeping with the above observations is proposed.

This work was carried out between 1970 and 1973 at the University of Aston in Birmingham. It has been done independently and has not been submitted for any other degree.



A. J. Robb.

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It may be a weed instead of a fish that,
after all my labour, I may at last pull up.

MICHAEL FARADAY
(1791 - 1867)

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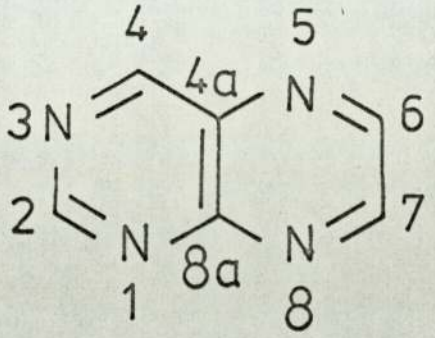
SPECTRA (Cont.)

CHAPTER I

Introduction.

I Structure, Nomenclature and Abbreviations.

The systematic name for the pteridine ring system is pyrimido [4,5-b] -pyrazine. The approved[1] numbering system is: -



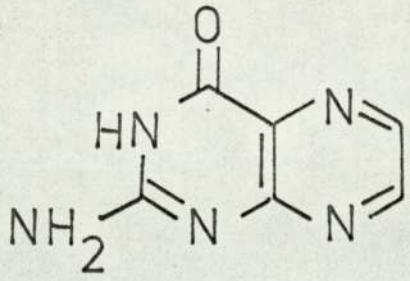
(I)

Early workers used pterin indifferently to describe a group of natural products, mainly butterfly and insect pigments,[2,3] based on this structure, but it is now used solely as a trivial name for 2-amino-4-hydroxy pteridine (II). This is a widespread pteridine derivative and is found in reptiles, amphibia,[4] fish[5] and mammals[6].

Although termed 2-amino -4-hydroxy pteridine the following structures depict the 4-oxo form. Evidence suggests that the choice between the hydroxy and oxo form lies in favour of the latter, except in the case of O-methylated derivatives[7-17]. In this thesis all pterin derivatives will be described in the oxo form. The most important group of pterins in nature are those with substituents at C(6). A simple example is tetrahydrobiopterin (III), a cofactor in the enzymic

hydroxylation of phenylalanine to tyrosine (see later). It was work on the mechanism of reduced pterin hydroxylation cofactors which heightened interest in the oxidation of reduced pterins in general. A more complex 6-substituted derivative is folic acid (IV). In nature this may exist with up to seven glutamate moieties joined by γ -peptide bonds (V) (see later).

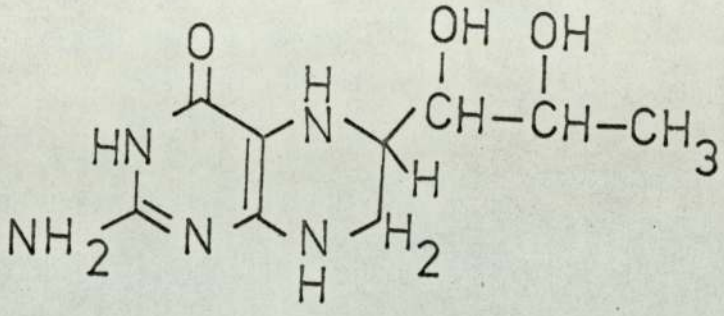
Folates perform their major metabolic function as a single carbon atom carrier in the tetrahydro state (VI). This thesis is concerned with 5-alkyl derivatives of (VI), especially 5-methyltetrahydrofolic acid (VII) the major mammalian folate monoglutamate. Other important derivatives referred to in this work are shown VIII —— XVI. The general term folate will be used to cover all derivitives of folic acid.



(II)

2-Amino-4-hydroxypteridine

(PTERIN)

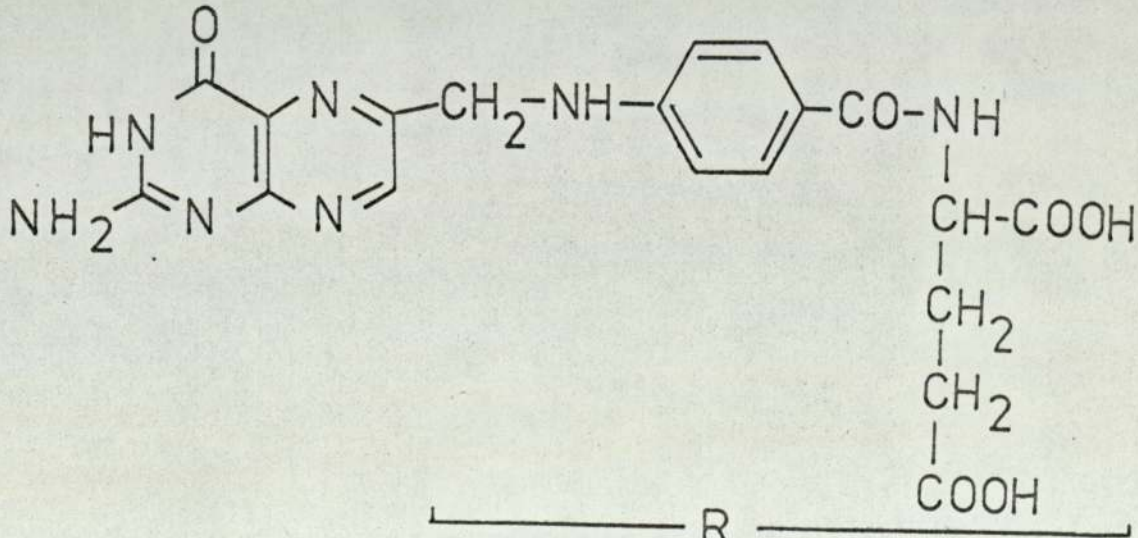


(III)

6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin

(TETRAHYDROBIOPTERIN)

(THB)



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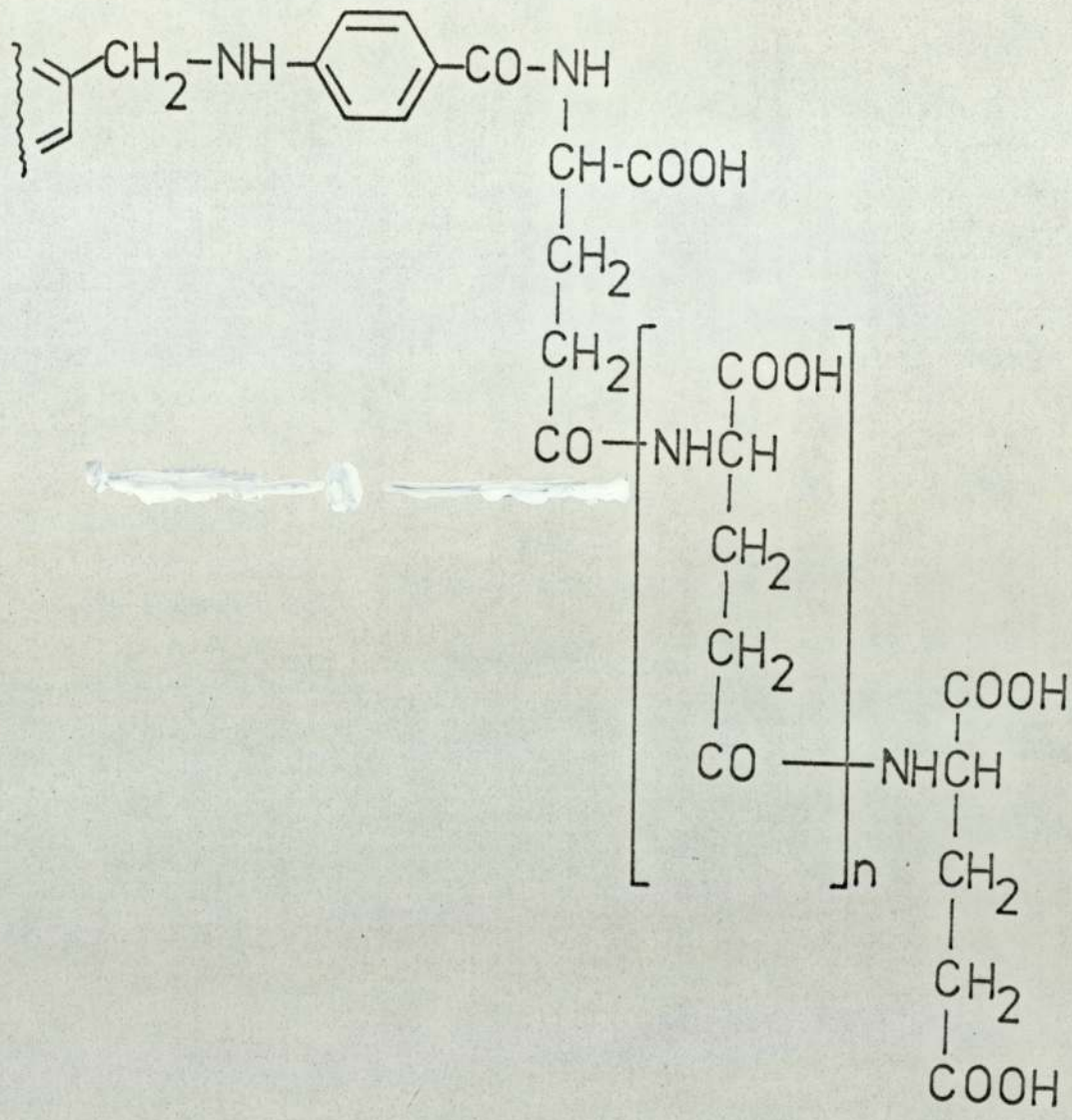
Pteroyl-L-monoglutamic acid

(FOLIC ACID)

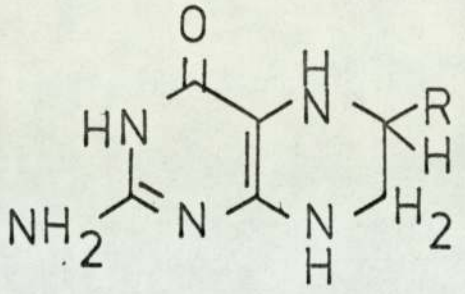


(V)

a) Folyldiglutamic acid (n = 1)

b) Folylhexaglutamic acid (n = 5)

(FOLIC ACID POLYGLUTAMATE.)

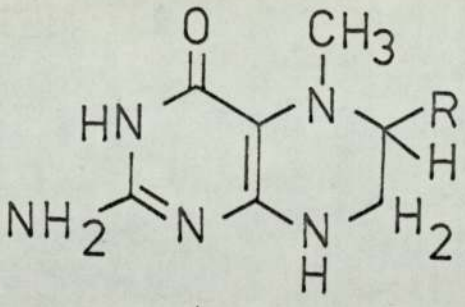


(VI)

5,6,7,8-Tetrahydropteroyl-L-monoglutamic acid

(TETRAHYDROFOLIC ACID)

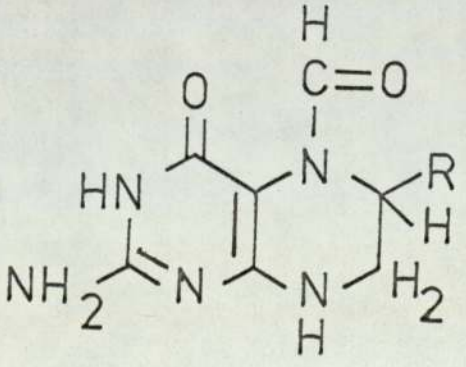
(THF)



(VII)

5-Methyl-5,6,7,8-tetrahydropteroyl-L-monoglutamic acid

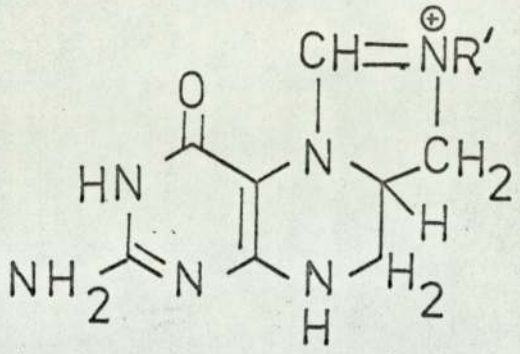
(5-METHYL THF)



(VIII)

5-Formyl-5,6,7,8-tetrahydropteroyl-L-monoglutamic acid

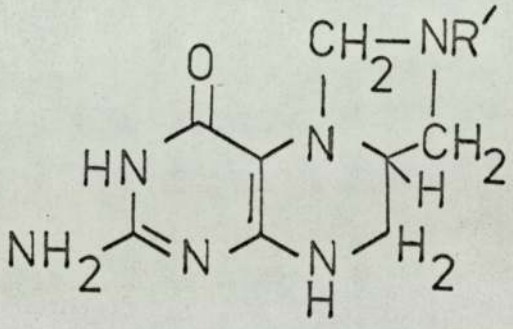
(5-FORMYL THF) (CITROVORUM FACTOR)



(IX)

5,10-Methenyl-5,6,7,8-tetrahydropteroyl-L-monoglutamic acid

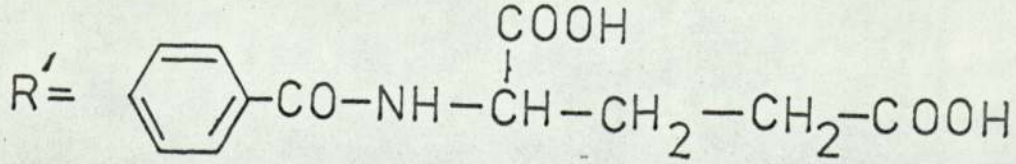
(5,10-METHENYL THF)



(X)

5,10-Methylene-5,6,7,8-tetrahydropteroyl-L-monoglutamic acid

(5,10-METHYLENE THF)



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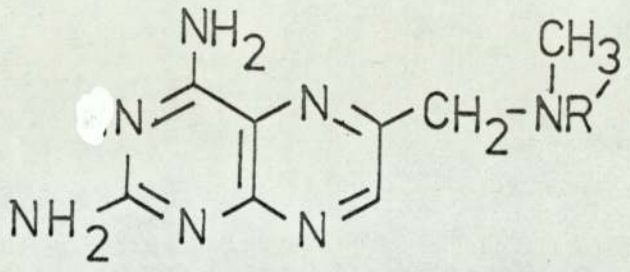
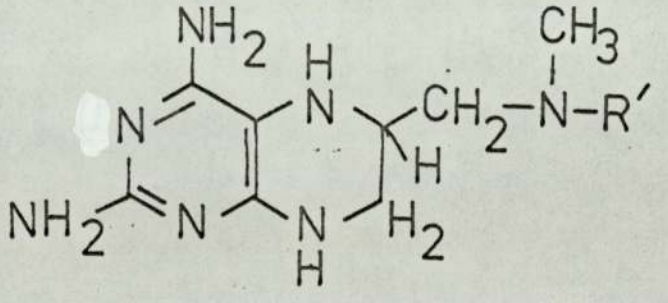


(XI)

4-Amino-10-methyl-4-deoxypteroyl-L-monoglutamic acid

(AMETHOPTERIN)

(METHOTREXATE)

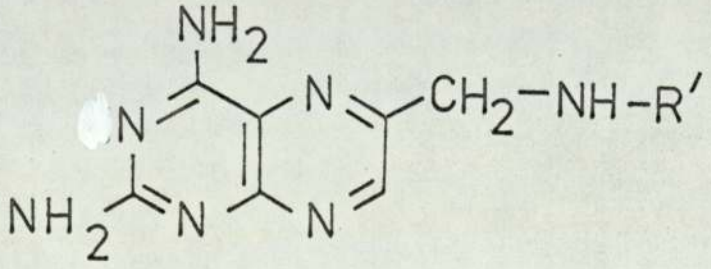


(XII)

4-Amino-10-methyl-4-deoxy-5,6,7,8-tetrahydropteroyl-L-monoglutamic acid

(5,6,7,8-TETRAHYDROMETHOTREXATE)

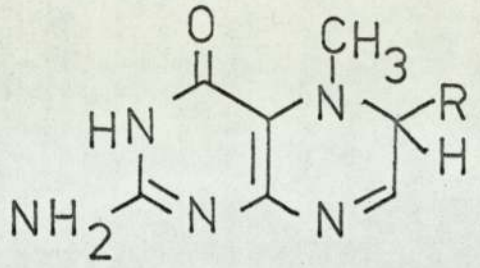
(THM)



(XIII)

4-Amino-4-deoxypteroyl-L-monoglutamic acid.

(AMINOPTERIN)

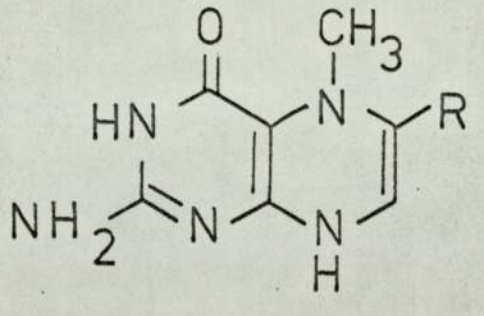


(XIV)

5-Methyl-5,6-dihydropteroyl-L-monoglutamic acid.

5-METHYL-5,6-DIHYDROFOLIC ACID

(5-Methyl-5,6-DHF).

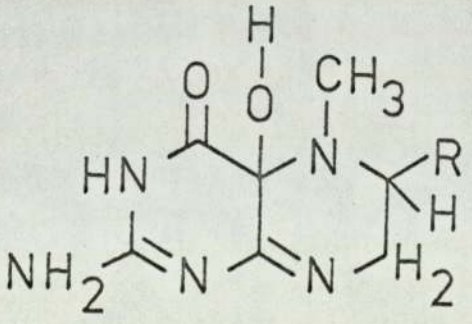


(XV)

5-Methyl-5,8-dihydropteroyl-L-monoglutamic acid.

5-METHYL-5,8-DIHYDROFOLIC ACID

(5-Methyl-5,8-DHF)



(XVI)

8-Dehydro-4a-hydroxy-5-methyl-5,6,7,8-tetrahydropteroyl-L-monoglutamic acid.

(4a-HYDROXY-5-METHYL THF)

ABBREVIATIONS.

* THF: 5,6,7,8-Tetrahydrofolic acid.

* DHF: 7,8-Dihydrofolic acid

5-Methyl THF: 5-Methyl-5,6,7,8-tetrahydrofolic acid.

4a-OH-5-Methyl THF: 8-dehydro-4a-hydroxy-5-methyl 5,6,7,8-tetrahydrofolic acid.

5-Methyl-5,6-DHF: 5-Methyl-5,6-dihydrofolic acid.

DEAE: Diethyl aminoethyl cellulose

TEAE: Triethyl aminoethyl cellulose

Tris: Tri-(hydroxymethyl)-amino methylhydrochloride.

THB: Tetrahydrobiopterin.

THM: Tetrahydromethotrexate.

PABG: p-Aminobenzoylglutamic acid.

* Used by Rabinowitz[18] in 1960 and now generally accepted in Biochemical literature.

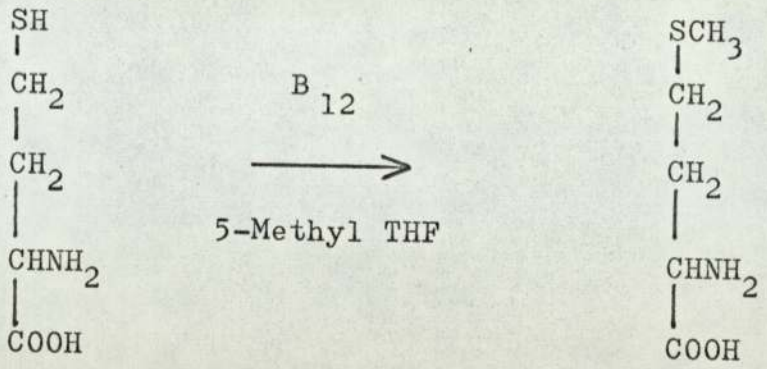
2. Historical background.

Vitamin Bc, observed and designated by Hogan and Parrot,[19] was isolated by Pfiffner et al.[20] in 1943. Shortly afterwards evidence for the presence of a pyrimidopyrazine ring in its molecular structure was published[21] and in 1946 Angier et al[22] found its structure by synthesis to be N-[4-{ [(2-amino-4-hydroxy-6-pteridinyl] methyl] amino} benzoyl] glutamic acid (IV). These workers named the compound pteroylglutamic acid.

The term 'folic acid' was proposed by Mitchell et al.[23,24] for a compound isolated from spinach and defined "as the material responsible for growth stimulation of Streptococcus Lactis R on a given medium". Folic acid is now specifically applied to structure (IV). The successful catalytic hydrogenation of vitamin Bc in dilute alkali over palladium to yield a dihydro derivative or in glacial acetic acid over platinum to give a tetrahydro derivative was reported by O'Dell et al.[25] in 1947. They also observed the ease of aerial oxidation of these reduced pteridines and suggested that these compounds may be involved in oxidation - reduction enzyme systems. They were to be proved correct (see later).

In 1959 Donaldson and Keresztesy[26] demonstrated the existence of a new form of naturally-occurring folic acid, prefolic A. They isolated it in essentially pure form as a barium salt from horse liver[27] and synthesised it by addition of formaldehyde to THF and subsequent reduction with borohydride.[28] These workers were able

to show that prefolic A was 5-methyl THF.[29-33] At about this time the prevalence of 5-methylTHF in blood[34,35] and its part with B_{12} in methionine methyl synthesis,

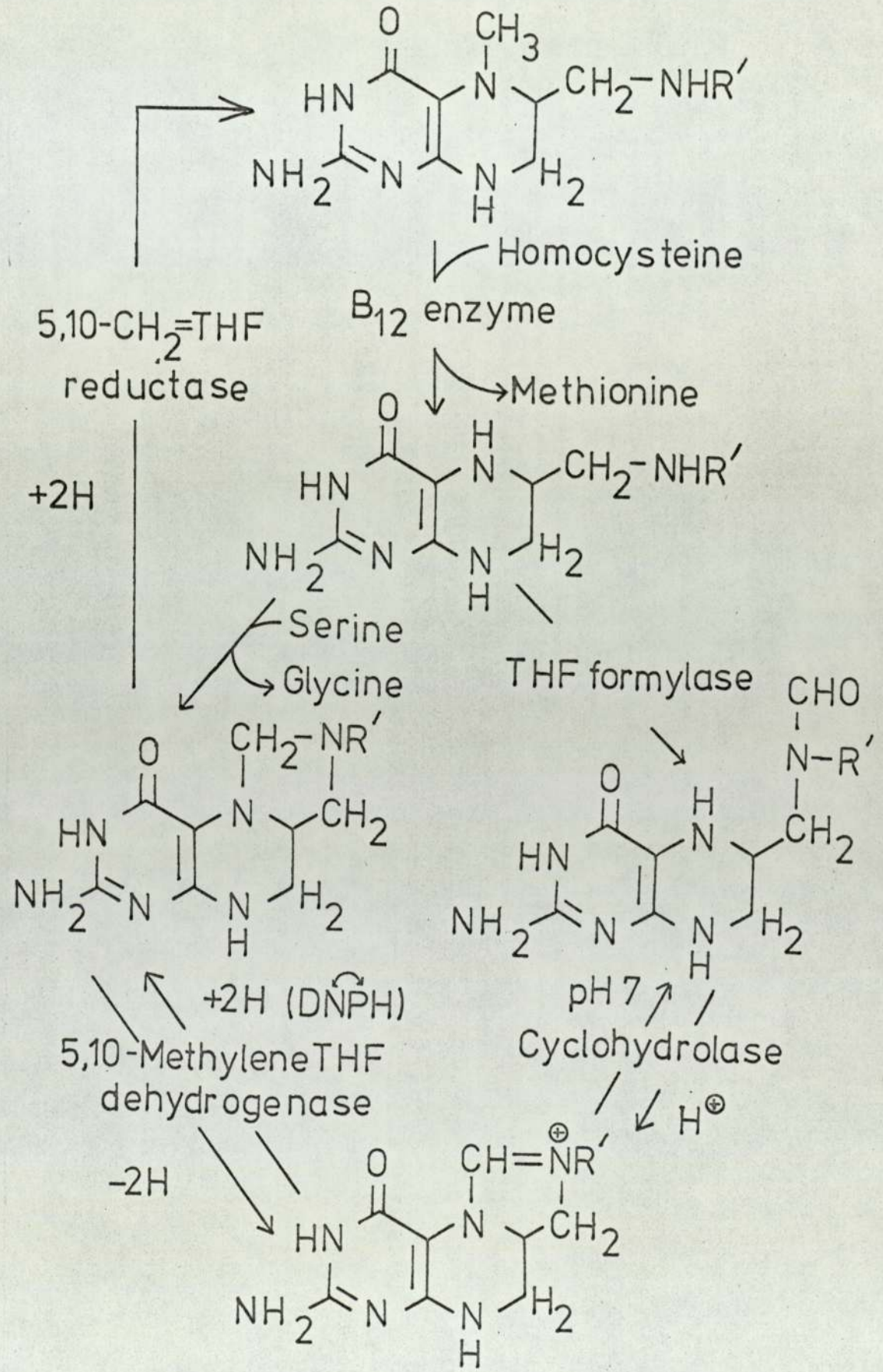


Homocysteine Methionine

were established.[36-39] The latter reaction is an example of the involvement of methyl and other derivatives of THF in the biological transfer of one carbon atom units at the oxidation levels of formate, formaldehyde and methanol, and the interconversion of these various states (see Scheme I) This is the major role of folates.[40,41]

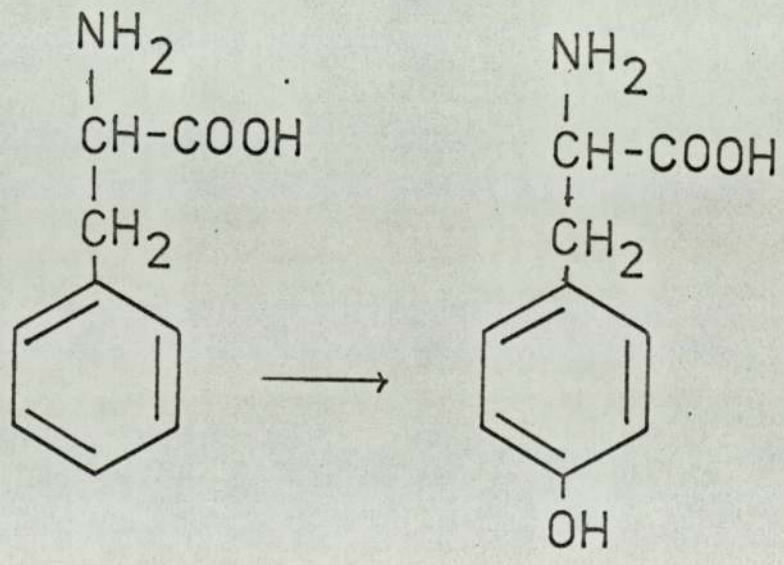
SCHEME I.

Biological interconversion of some tetrahydrofolates.



Interest in folates had already been aroused by the partially successful use of methotrexate (XI) and aminopterin (XIII) to treat leukaemia. These folate antagonists inhibit the enzyme dihydrofolate reductase[42], resulting in a deficiency of THF and THF derivatives which in turn blocks thymidylate synthesis, with a consequent failure in DNA synthesis and arrest of cell division. A more potent folate antagonist is tetrahydromethotrexate (XII), which is believed to directly block thymidylate synthesis[43,44]. Unfortunately, these drugs lack specificity and although cancer cell division is stopped, so is the growth of other rapidly dividing cells such as those in bone marrow and intestinal mucosa. This toxicity severely limits the use of these analogues. Early workers attempted, with limited success, to reverse this toxicity by administration of folic acid in large quantities[45-47]. Use of 5-formyl THF has proved rather more successful;[48,49] recently 5-methyl THF was shown to produce as favourable results as 5-formyl THF and it was proposed that the pharmaceutical effects of citrovorum factor were due to its conversion to 5-methyl THF[50]. The realisation of the importance of these compounds initiated much work on the synthesis of tetrahydrofolate derivatives. The first part of this project involves a brief survey of a novel synthesis of 5-methyl THF[51] and related analogues[52,53] in an attempt to extend the application of the synthesis and also to generate supplies of 5-methyl THF for an investigation

of its autoxidation. This is of great interest for two reasons: (i) 5-methyl THF is not only one of the most important folate derivatives but also one of the most abundant, yet because of its lability was not isolated until 18 years after the isolation of folic acid. This raises the obvious questions of rate and products of oxidation, especially with relevance to its biological stability. (ii) Mentioned earlier was the idea that reduced pteridines play an important role in biological red-ox systems. The most important is the hydroxylation of phenylalanine (XVII) to tyrosine (XVIII).



XVII XVIII

The reaction can be expressed:

Phenylalanine + NADPH + H^+ + O_2

$\rightarrow$ $NADP^+$ + H_2O + tyrosine

It was hoped that a kinetic and qualitative investigation into the oxidation of 5-methyl THF would throw some light

on the mechanism of oxidation of reduced pteridines in general and tie in with other recent studies on the oxidation of tetrahydrobiopterin and THF.[54,55].

3. Microbiological Assay.

Despite many elaborations and recent innovations, modern chemical techniques cannot compare in sensitivity with microbiological assay. Many folates, even in concentrations as low as 10^{-9}M, can be characterised by combining this technique with column chromatography or with t.l.c. by bioautography.[56-59] A table of the response of the three most commonly used organisms is given (TABLE I). Folates exist in nature either as monoglutamates,[60] folate-protein complexes,[61] or as conjugates with two or more glutamic acid moieties linked by γ-peptide bonds.[59] There are reports of polyglutamates with up to seven glutamates residues.[62, 63] These 'bound' folates must be freed by incubation with conjugase (a γ-glutamyl carboxy peptidase found in the intestinal mucosa, kidney, spleen and brain of various animals) before they are assayed.[64] Using this technique, it was discovered that most of 5-methyl THF in red blood cells is 'bound', while that in serum is 'free'.[35]

Great confusion is apparent in the literature before 1960 because care was not taken to prevent (i) autolysis or (ii) autoxidation of samples before or during the assay procedure. Care is now taken in the preparation of samples, and antioxidants are used routinely with this technique.

TABLE I

Response of L. casei, P. cerevisiae and S. faecalis to naturally occurring folate derivatives.[64]

Compound	Growth Activity [a] for		
	L.casei	P.cerevisiae	S.faecalis
Folic Acid	+	-	+
DHF	+	-	+
THF	+	+	+
5-Formyl THF	+	+	+
10-Formyl Folic Acid	+	-	+
10-Formyl DHF	+	-	+
10-Formyl THF	+	+	+
5-Methyl THF	+	-	-
F diglutamate[b]	+	-	-
F hexaglutamate[c]	-	-	-
THF diglutamate	+	+	-

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

b folyldiglutamate

c folylhexaglutamate

CHAPTER II

PART A. Preparation of 5-alkyl tetrahydrofolates

PART B. Solubility determination of 5-methyl THF.

PART C. Determination of pKa value for ionisation of 3,4-amide group in 5-methyl THF.

PART D. Attempted resolution of diastereoisomers of 5-methyl THF.

PART A. Preparation of 5-Methyl tetrahydrofolate.

(i) Introduction.

The method of preparing 5-methyl THF by condensing formaldehyde and tetrahydrofolic acid at neutral pH with subsequent reduction by sodium borohydride and purification on a DEAE cellulose column was first reported by Donaldson and Keresztesy[28]. This method has been slightly modified by Sakami and Ukstins[33] and used successfully by Gupta and Huennekens[65]. Ways of preparing THF are described shortly. The drawback with the above methods is that they involve isolation of THF which is extremely labile. A single stage preparation has been described by Chanarin and Perry[66], starting from commercially prepared folinic acid (VIII). They formed 5,10-methenyl THF (IX) from folinic acid at pH 1 and reduced this with borohydride at pH 7. The product gave an ultra violet spectrum similar to 5-methyl THF λmax = 289 nm, λmin = 245 nm at pH 7. The ratio of these was 1.5:1. This large amount of absorption at λ 250 nm is indicative of impurities, the minimum ratio in the following work was 3.0:1.

A convenient method of preparing 5-methyl THF in quantity from folic acid without the isolation of the intermediate tetrahydrofolic acid has been devised by Blair and Saunders[51]. They extended this synthesis to cover higher analogues[52] and to prepare N(5) alkyl derivatives of tetrahydro methotrexate (XII)[53].

It is this method of preparing N(5) alkyl tetrahydrofolates that is used and studied in this work.

(ii) Reduction of Folic Acid.

Folic acid and many of its derivatives can easily be reduced chemically by a variety of reagents to the corresponding dihydro or tetrahydro compounds.

The method of O'Dell et al[25] mentioned earlier is still a popular method for THF. If glacial acetic acid is used as the solvent in this preparation the resulting THF is isolated as the diacetate[67,68]. Reduction of folic acid at elevated temperatures with dithionite yields THF[69,70]; at room temperature DHF is produced. This can be precipitated by lowering the pH in the presence of an antioxidant[71-74] (ascorbate or mercaptoethanol). THF is also produced by reducing folic acid with a large excess of borohydride[75-77]. This method is incorporated into the preparation of 5-methyl THF used here.

Materials and Methods.

U.v. spectra were recorded on a Perkin-Elmer PE 137 or Unicam SP 700 spectrophotometer, nuclear magnetic resonance spectra on a Perkin-Elmer R 14 or Varian HA100D spectrometer. Thin layer chromatograms were run on cellulose powder MN300 F254 (Macherey, Nagel and Co., Duren, Germany). The following solvent systems were used.

(i) 0.1M phosphate buffer, pH 7.0.

(ii) n-Propanol/water/0.88 s/g aqueous ammonia (200:100:1,v/v)

(iii) The organic phase of 1-butanol/acetic acid/water (4:1:5 v/v).

Samples were observed as absorbing or fluorescing spots when viewed in u.v. light (λ = 254 n m).

(iii) Preparation of 5-methyl THF.

Method. (Blair & Saunders[51]).

Sodium borohydride (6 gm, 160 mmoles) in water (50 ml) was added to a stirred solution of folic acid (6g.) 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 5N 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 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 x 4.5 cm. column of DEAE cellulose previously equilibriated with ammonium acetate (3 litres, 0.13 M pH 7.0) containing mercaptoethanol (0.2M). Elution was initiated with ammonium acetate (2 litres, 0.13M, pH 7.0) followed by a gradient of ammonium acetate (0.13 - 0.4M, pH 7 1 litre each) and completed with ammonium acetate (2 litres, 0.4M, pH 7.0). All solutions contained mercaptoethanol (0.2M). The 0.13M buffer eluate(2 litres) 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 nm was determined after dilution with

0.1M phosphate buffer, pH 7.0. A complete spectrum was run on every tenth fraction. Fractions having absorbance maxima at 290 nm and minima at 248 nm were pooled and lyophilised. In later preparations the condensation stage was allowed to proceed for 2 minutes before the addition of borohydride. Seven batches of 5-methyl THF were made in this way. In the last three preparations mercaptoethanol was omitted for the final stage below.

Isolation as barium salt.

Crude 5-methyl THF (1.4g) was dissolved in distilled water (20 ml) containing sodium chloride (300 mg). The pH was adjusted to 7 with .5N NaOH. Barium chloride (10 ml of 22 gm $BaCl_2 . 2H_2O$ in 100 ml water) and mercapto-ethanol (1% solution) were added. The barium salt was precipitated with absolute ethanol (150 ml) and cooled overnight. After centrifuging, the solid was washed with 50% aqueous ethanol (20 ml) followed by absolute ethanol (15 ml), then dried under vacuum at room temperature. The barium salt was recrystallised by warming in deaerated water containing mercaptoethanol (10^{-3}M) and diluting slowly with ethanol to reprecipitate. This was again centrifuged, washed, and dried rapidly. The calcium salt was prepared as above by substituting calcium chloride solution (10 ml of 10 gm $CaCl_2 . 6H_2O$/100 ml water) for barium chloride solution.

Samples were identified by t.l.c., u.v. and n.m.r. spectroscopy. % purity was calculated from $\Sigma\lambda_{max}$,

using Σ_{max} (mol.) = 30.8 x 10^3 in 0.1M phosphate buffer pH 7.0 Ratios of $\Sigma\lambda_{max}$: $\Sigma\lambda_{min}$ varied between 3.1:1 and 3.3:1.

A selection of spectra for compounds in this section is given in the appendix.

TABLE II

Rf. Values of 5-methyl THF with standards.

T.l.c. solvent	Sample	Observation	Rf.
(i)	5-methyl THF	Abs.	0.87
"	"	Fl.	0.59
"	"	Fl.	0.20
"	5-formyl THF	Abs.	0.87
"	PABG	Abs.	0.90
"	Folic acid	Abs.	0.50
(ii)	5-methyl THF	Abs.	0.55
"	"	Fl.	0.20
"	5-methyl THF (std)	Abs.	0.55
"	"	Fl.	0.20
"	5-formyl THF	Abs.	0.48
(iii)	5-methyl THF	Abs.	0.70
"	"	Fl.	0.50
"	PABG	Abs.	0.80
"	5-formyl THF	Abs.	0.75
"	Folic acid	Abs.	0.00

Abs = absorbing spot.

Fl. = fluorescing spot.

TABLE III

U.V. Data for 5-methyl THF and reported data for other folates.

Compound	pH	λ max. nm	λ min. nm
5-methyl THF	1	270, 292	243, 282
"	7	292	248
"	13	288	247
Folic acid	7	282, 350	330
DHF	7.5	283	250
THF	7.5	298	245
10-CHO-THF	7.5	260	240
5-CHO-THF	13	282	245
5,10-methenyl THF	1	355	305
5,10-methylene THF	7.2	294	245
5-methyl-5,6-DHF	7.0	250, 290	230, 260
4a-hydroxy-5-methyl THF	7.0	281	240

NMR Data see spectra 1,2,3)

(i) ^{1}H NMR

(a) Folic Acid

τ 7.4 (2H,b) β-CH_2, τ 7.15 (2H, d) γ-CH_2,

τ 4.8 (1H,b) α-CH, τ 4.65(2H,S) C_9,

τ 1.95 (2H,d) τ 1.7 (2H,d) (AB quartet) aromatic protons,

τ 1.15 (2H,b) NH_2, τ .85 (1H,s) C_7.

(b) 5-Methyl THF.

τ7.8 (1H,s) acetate impurity, τ 7.4 (2H,b) βCH_2

τ7.2 (2H,d) γ CH_2, τ 6.6 (3H,s) N(5) CH_3

τ6.0 (2H,b) C_7, τ 5.8 (2H,s) C_9, τ 5.7(1H,b) C_6,

τ4.8 (1H,b) αCH, τ 2.4(2H,d) τ, 1.8(2H,d) (AB quartet) aromatic protons.

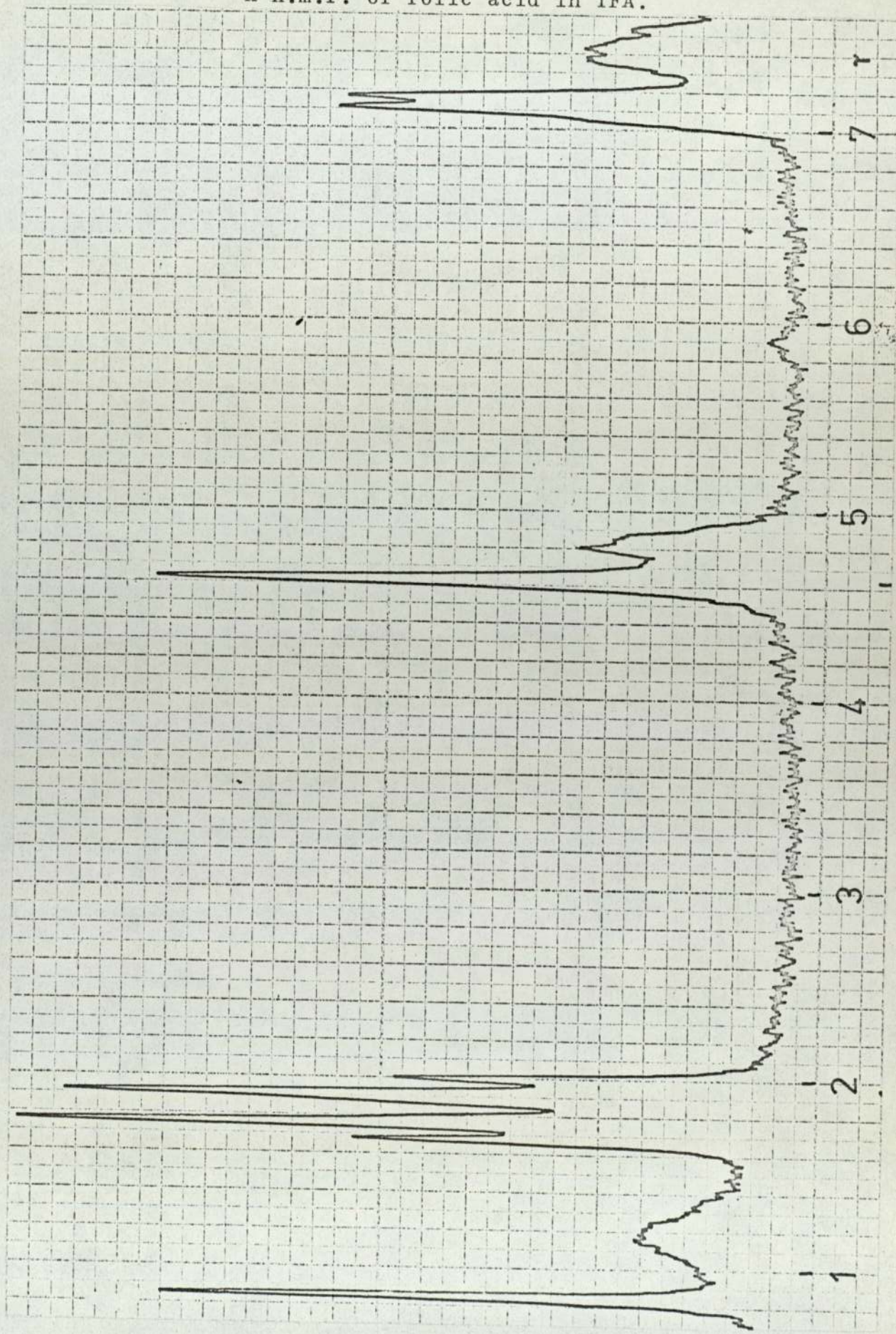
(ii) ^{13}C NMR

A ^{13}C magnetic resonance spectrum was recorded for 5-methyl THF on a Varian XL 100 spectrometer. The sample was made up as a concentrated solution in a deaerated mixture of NaOD/D_2O (2%). Dioxan was used as an internal reference (peak 12, spectrum 3.) Comparison with the spectrum for folic acid (spectrum 4) shows five new peaks in the region 600 Hz to 2,000 Hz. Of these, the two largest (1,833 Hz and 826 Hz) are absent in an earlier

spectrum run on a sample prepared without antioxidants and hence are due to mercaptoethanol impurity. The other three peaks correspond to N(5)-CH_3, C_7 and C_6. Unfortunately, easy assignment of these is impossible and can be done only by comparison with deuterated samples.

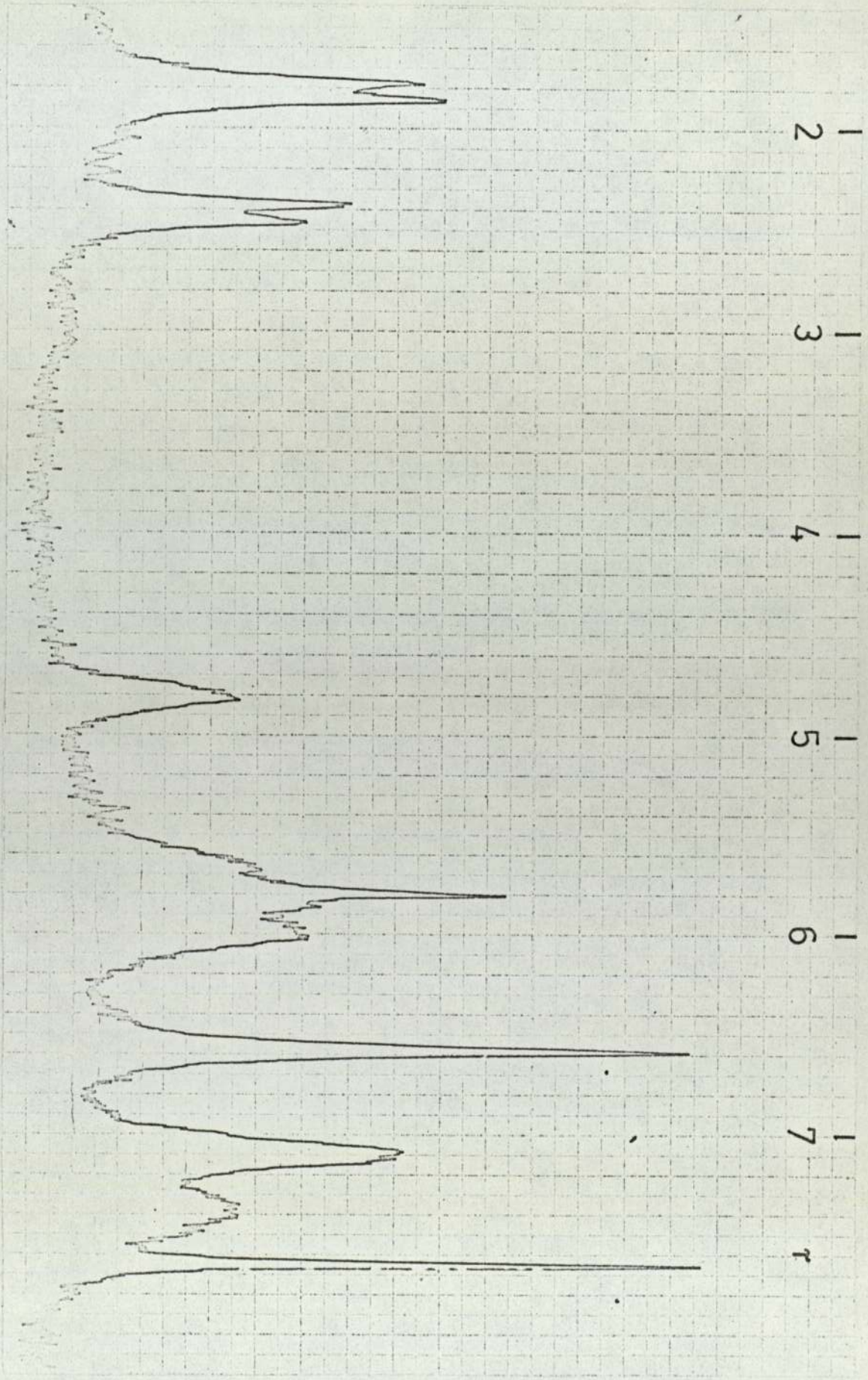
SPECTRUM 1.

^{1}H n.m.r. of folic acid in TFA.



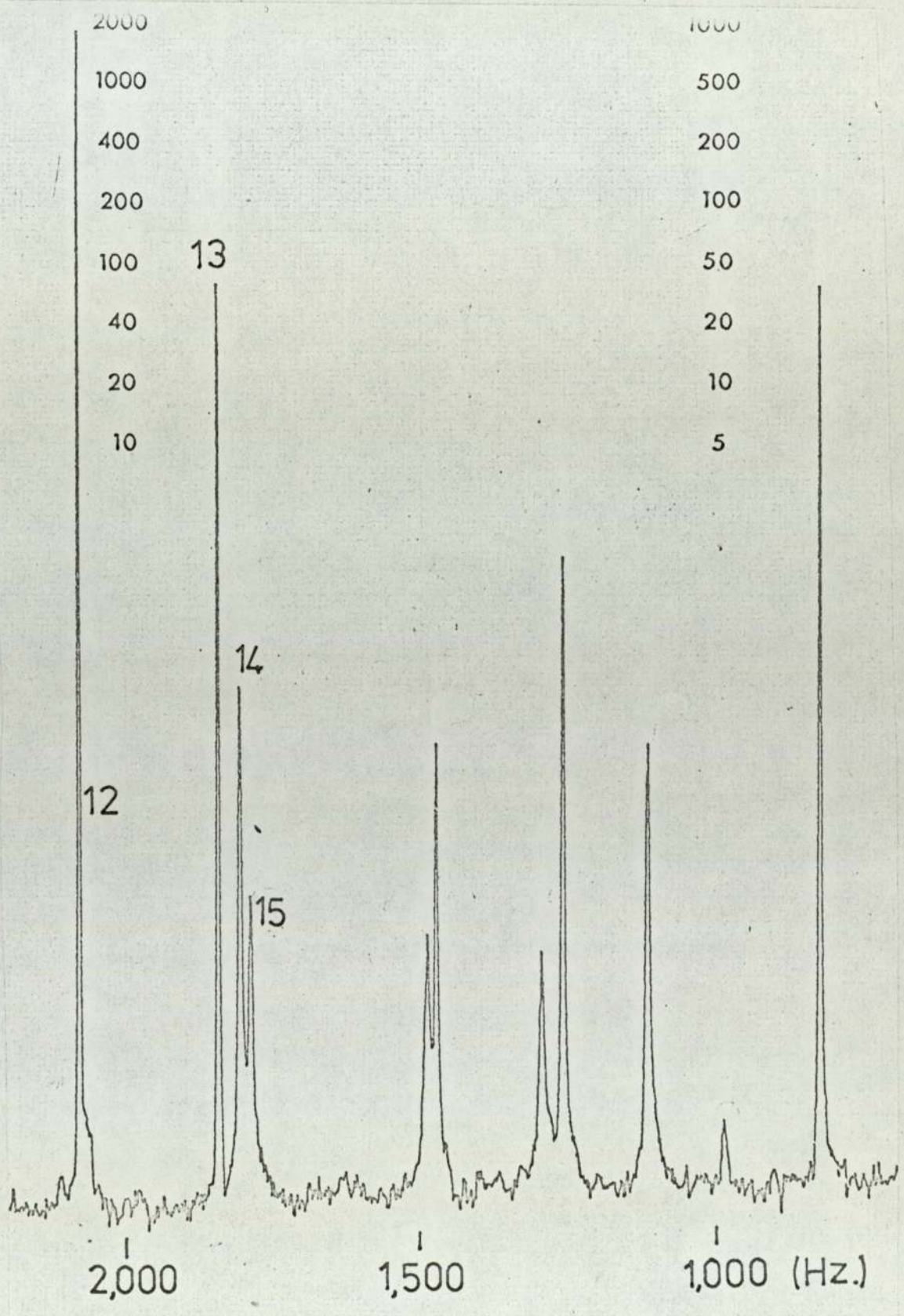
SPECTRUM 2.

^{1}H n.m.r. of 5-methyl THF in TFA.



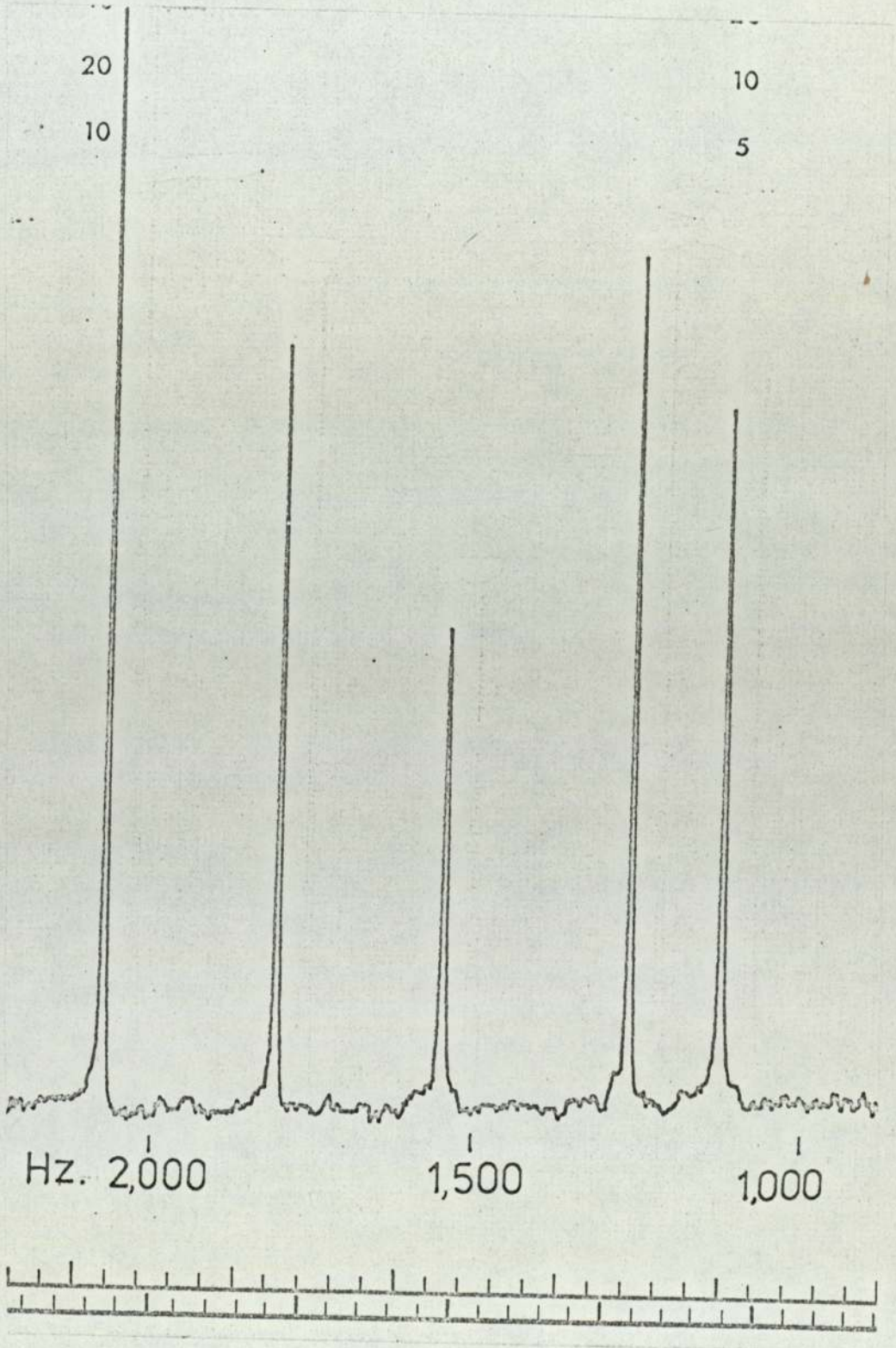
SPECTRUM 3.

^{13}C n.m.r. of 5-methyl THF in NaOD.



SPECTRUM 4.

^{13}C n.m.r. of Folic acid in NaOD.



PART A.2. Preparation of other 5-alkyl tetrahydro folates.

(i) 5-n-butyl THF.

This was prepared by a slight modification of the method given for 5-methyl THF. Re-distilled n-butyraldehyde (B.P. 74°C, lit. B.P. 74°C) (8 ml.) was substituted for formaldehyde and the time allowed for the condensation stage to take place was extended to 45 minutes. Isolation as the barium salt was as above.

(ii) 5-isobutyl THF.

As above, using isobutyraldehyde in place of n-butyraldehyde. The product was isolated as the barium salt, a sample of which was tested for reversal of methotrexate toxicity in mice by Dr. Searle, Department of Cancer Studies, Birmingham University. (Experimental given in Appendix).

(iii) 5-benzyl THF.

This was attempted by replacing formaldehyde with benzaldehyde. Homogeneity was achieved by the addition of ethanol (100 ml). The condensation stage was allowed to proceed for 30 minutes. Purification on a DEAE column gave a solidly yellow column. No material came off the column within 24 hours. This strong adsorption on the column is indicative of unreduced folate. The preparation was abandoned.

TABLE IV.

T.L.C. Data of Products.

T.L.C. Solvent.	Sample	Observation.	Rf.
(i)	5-n-butyl THF	Abs.	0.90
"	"	Fl.	0.21
"	5-methyl THF	Abs.	0.95
"	"	Fl.	0.21
"	Folic acid	Abs.	0.50
"	5-isobutyl THF	Abs.	0.85
"	"	Fl.	0.25
"	5-methyl THF	Abs.	0.85
"	"	Fl.	0.21
"	Folic acid	Abs.	0.50
(ii)	5-n-butyl THF	Abs.	0.80
"	"	Fl.	0.05
"	5-methyl THF	Abs.	0.50
"	"	Fl.	0.05
"	Folic acid	Abs.	0.20
"	5-isobutyl THF	Abs.	0.45
"	"	Fl.	0.15
"	5-methyl THF	Abs.	0.45
"	"	Fl.	0.10
"	Folic acid	Abs.	0.15
(iii)	5-n-butyl THF	Abs.	0.50
"	"	Fl.	0.45
"	5-methyl THF	Abs.	0.64
"	"	Fl.	0.45
"	Folic acid	Abs.	0.00
"	5-isobutyl THF	Abs.	0.50
"	"	Fl.	0.10
"	5-methyl THF	Abs.	0.50
"	"	Fl.	0.10
"	Folic acid	Abs.	0.00

TABLE V.

U.V. Data of Products.

Compound	pH	λ max. n m		λ min. n m.	
5-n-butyl THF	1	271	292	244	285
"	7		292	248	
"	13		289	248	
5-isobutyl THF	1	274	291	244	284
"	7		290	248	
"	13		288	250	

TABLE VI

Results of Biological Tests (Dr. Searle).

Compound	Dose mg/Kg	Dead after 8 days.
Controls	-	15/16
Folic Acid	5	1/8
"	15	2/8
"	45	3/8
5-isobutyl THF	5	0/8
"	15	0/8
"	45	1/8

^{1}HMR Data of Products.

(iii) 5-n-butyl THF.

τ9.05(3H,t)5-n-butyl CH_3, τ 8.55(2H,m)-n-butyl CH_2
τ8.1(2H,m)-n-butyl CH_2, τ 7.7(1H,s) acetate impurity
τ7.45(2H,m)βCH_2, τ 7.15(2H,d)γ CH_2,
τ6.35(2H,m) N(5) CH_2-n-butyl, τ 6.1(2H,b)C_7
τ5.9(2H,d) C_9, τ5.45(1H,b)C_6, τ 4.9(1H,t)αCH
τ2.7(2H,d), τ1.95(2H,d) AB quartet aromatic protons,
τ2.35($\frac{1}{2}$H,d) impurity.

(iv) 5-isobutyl THF.

τ9.5($\frac{1}{2}$H,s) impurity, τ 8.9(4 H,d) CH_3gps(isobutyl).
τ7.7($\frac{1}{4}$H,s) acetate impurity, τ 7.4(2H,b)βCH_2
τ7.15(2H,d)γ CH_2, τ 6.0(1H,b)$^{N(5)}CH_2$ isobutyl,
τ5.8 (2H,d)C_7, τ 5.7(2H,d)C_9, τ 5.4(1H,b)C_6
τ4.8 (1H,b)αCH, τ2.3(2H,d), τ1.8(2H,d)(AB quartet)
aromatic protons. τ 2.5(1H,d) impurity.

PART A3 Preparation of Tetrahydromethotrexate.

Method.

Methotrexate (6g) was reduced by borohydride as in the initial reduction stage for the preparation of 5-methyl THF. The pH was then adjusted to 7 and the reaction mixture chromatographed as usual. The product was isolated as a barium salt.

TABLE VII

T.l.c. Data.

TLC Solvent	Sample	Observation	Rf.
(i)	THM	Abs.	0.75
"	"	Fl.	0.40
"	Methotrexate	Abs.	0.75
(ii)	THM	Abs.	0.35
	Methotrexate	Abs.	0.55
(iii)	THM	Abs.	0.65
"	"	Fl.	0.34
"	Methotrexate	Abs.	0.80

TABLE VIII

U.v. Data.

Compound	pH	λ max.n m	λ min.n m
Methotrexate	1	244, 307	235, 263
"	7	260,302,371	240,272,340
"	13	257,302,370	238,268,338
Tetrahydromethotrexate	1	300	262
"	7	297	260
"	13	297	259

^{1}H NMR Data

(b) Methotrexate.

τ 7.5(H,S) acetate impurity, τ7.4(2H,b)βCH$_2$

τ7.1(2H,d)γ CH$_2$, τ6.15(3H,s) N(10)CH$_3$

τ4.8(1H,b)αCH, τ 4.4(2H,s)C$_9$,

τ1.85(2H,d), τ1.65(2H,d) (AB quartet) aromatic protons

τ0.8(1H,s)C$_7$.

(ii) Tetrahydromethotrexate.

τ 7.8(4H,s) acetate impurity, τ7.5(2H,b)βCH$_2$

τ 7.2(2H,d)γ CH$_2$, τ6.4(3H,s) N(10)CH$_3$

τ6.3(2H,d)C$_7$, τ 5.85(2H,d)C$_9$, τ 5.3(1H,d)C$_6$

τ 4.85(1H,b)αCH, τ 1.8(2H,d), τ1.55(2H,d)

(AB quartet) aromatic protons.

PART A.4. Preparation of 2-(^{14}C)-5-methyl THF.

Introduction.

Despite their biological importance, folates are not synthesised in the human body and must be obtained from food[78]. Folate deficiency can be fatal[79] and is caused either by lack of folates in the diet or malabsorption across the intestine. High specific activity 2-(^{14}C) folic acid is readily available and much work has been done on its transport across the gut wall. Discussion on the mechanism of transport is rife, but the transport of 5-methyl THF has received little attention.

In order to study this, the preparation of 2-(^{14}C)-5-methyl THF was attempted.

(a) Small scale high specific activity (20mCi/Mole).

Pt O_2(1mg.) was reduced to Pt under H_2 at room temperature and pressure, in glacial acetic acid (0.1 ml). Folic acid (1 mg.), 2-(^{14}C) folic acid (100mμCi. 1 mg) and ascorbate (20 mg.) all suspended in glacial acetic acid (0.1 ml) were added and reduced until the stoichiometric amount of the H_2 had been taken up. The residue after lyophilisation was dissolved in 0.066 M. Tris buffer pH 7.8, formaldehyde (0.1 ml, 37% w/v) was added and the mixture allowed to stand for 15 minutes under nitrogen. After further reduction for 2 hrs. under H_2 the platinum was centrifuged down and the product was placed on a 10 ml. DEAE cellulose column previously equilibriated with 0.2M

phosphate buffer pH 7 and washed with 250 ml. 0.1M ammonium acetate pH 7. Elution was performed with a concentration gradient of ammonium acetate(0.1M in the mixing chamber 0.4M in the reservoir.) 16 drop fractions were collected automatically. The radio activity in each tube was monitored by counting a 2µl sample of each tube in 5 ml. NE 220 scintillator. Three peaks of radioactivity were obtained, corresponding to tubes 25 - 33; A, 34 - 42; B, 44 - 60; C. After lyophilisation the residues were subjected to t.l.c. in three solvent systems.

Results.

TABLE IX

T.l.c. data.

T.l.c. Solvent	Sample	R_f
(i)	5-methyl THF Std.	0.87
(i)	C	0.87
(i)	C	0.51
(i)	B	0.91
(i)	B	0.77
(ii)	5-methyl THF Std.	0.51
(ii)	C	0.45
(ii)	B	0.42
(ii)	A	0.35
(iii)	5-methyl THF Std.	0.55
(iii)	C	0.53
(iii)	C	0.40
(iii)	B	0.57
(iii)	B	0.35
(iii)	A	6.25
(iii)	A	0.35

b) Large Scale Low Specific Activity (10μCi/mole).

Method.

This was a modification of that devised by Blair and Saunders (p. 23). 2-(^{14}C) folic acid (100 mμCi) were added to 5g. cold folic acid before the first borohydride reduction. Fractions (8 ml.) were collected automatically and the UV spectrum of every tenth fraction was recorded. 2-(^{14}C)-5-methyl THF was found in tubes 11 - 250. After lyophilisation the product was isolated as the barium salt.

TABLE X. t.l.c.

T.l.c. solvent.	Sample	Obs.	R_f
(i)	5-methyl THF	Abs.	0.90
(i)	"	Fl.	0.35
(i)	Product	Abs.	0.90
(i)	"	Fl.	0.35
(ii)	5-methyl THF	Abs.	0.67
(ii)	"	Fl.	0.51
(ii)	Product	Abs.	0.67
(ii)	"	Fl.	0.50
(iii)	5-methyl THF	Abs.	0.65
(iii)	"	Fl.	0.45
(iii)	"	Fl.	0.31
(iii)	Product	Abs.	0.65
(iii)	"	Fl.	0.45

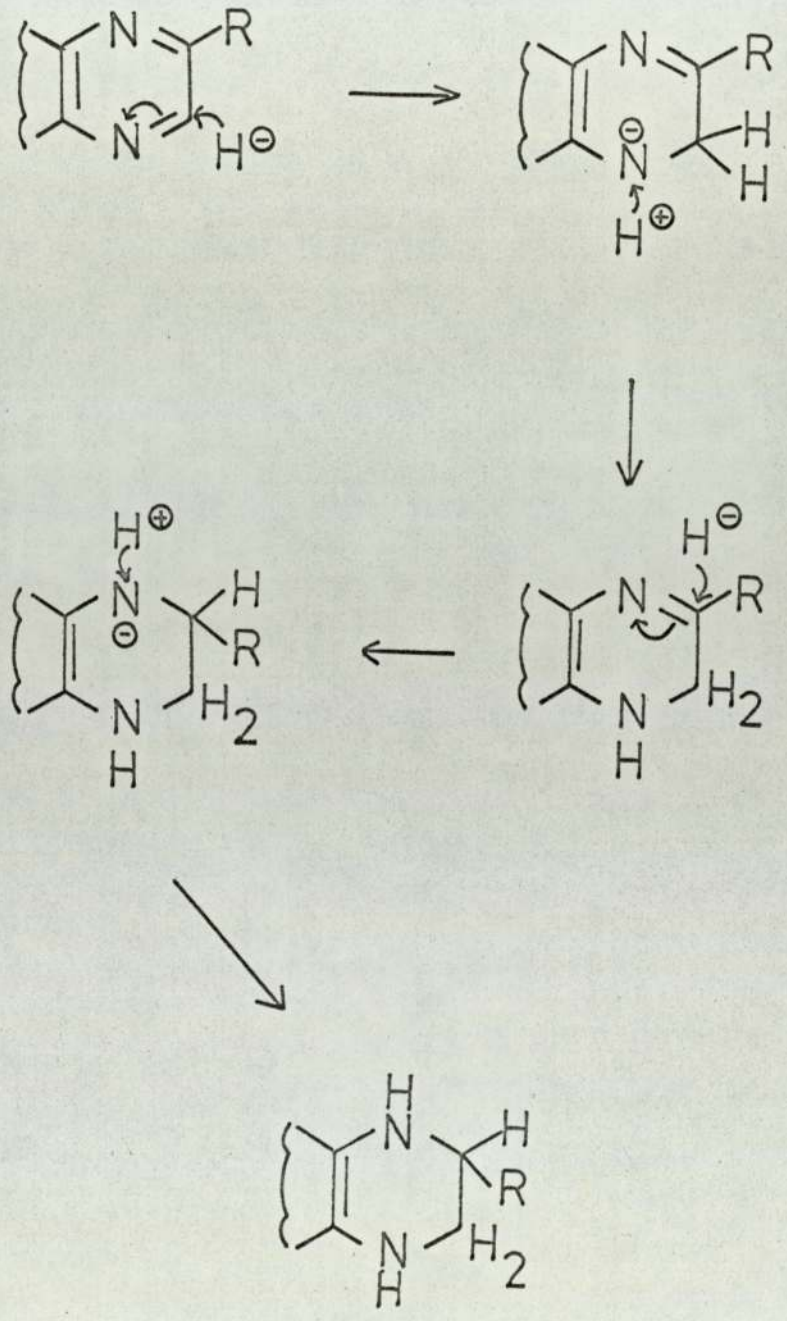
TABLE XI.

u.v. Data of Product.

pH	λ max nm	λ min nm
1	270, 295	248, 282
7	292	248
13	290	248

SCHEME II

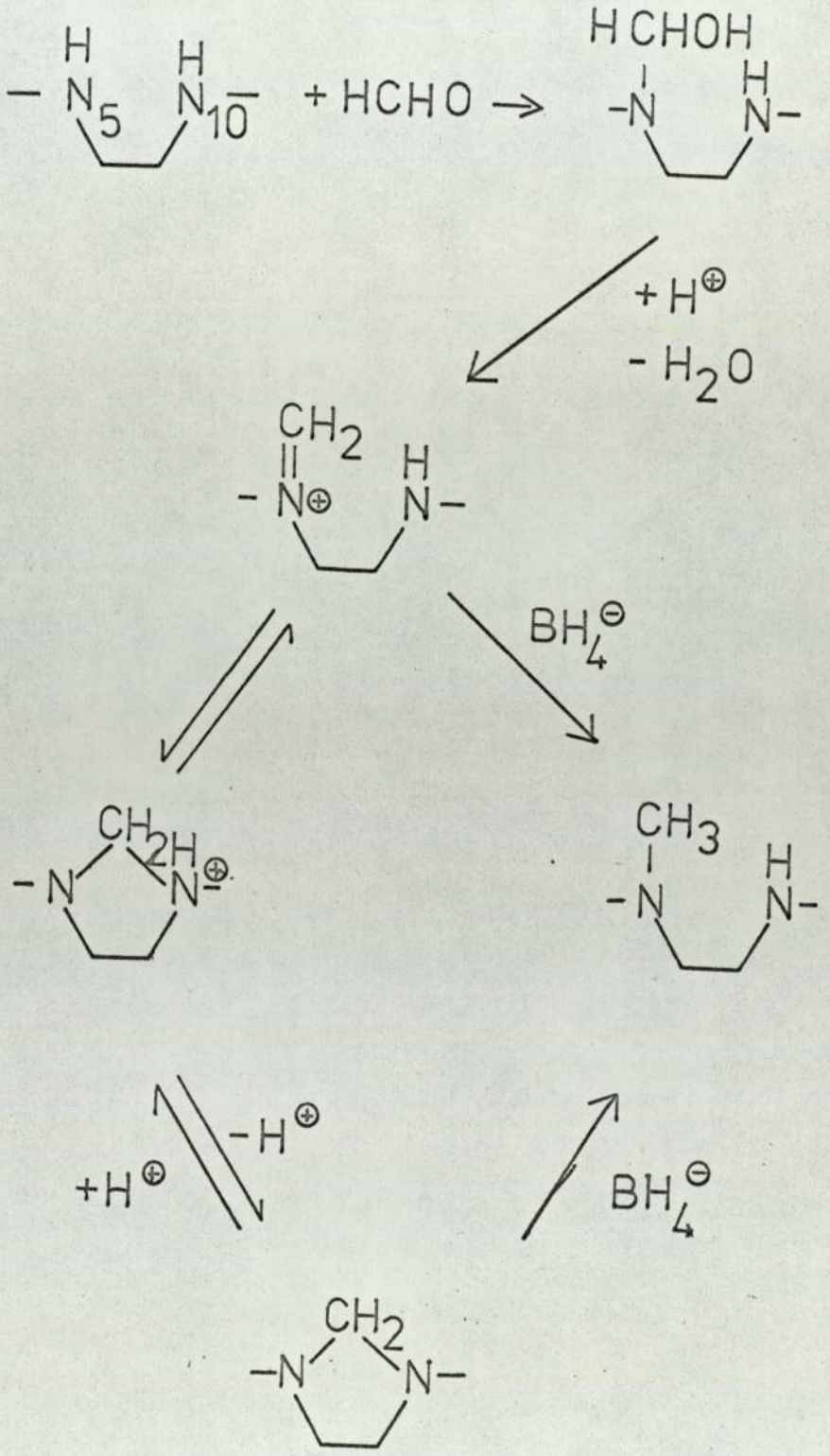
Mechanism of reduction of folic acid by borohydride.



This is in agreement with the observation that 7,8-dihydrofolic acid is the first product of reduction[76].

Blair and Saunders[52] have proposed the following mechanism for the condensation of THF with formaldehyde: -

SCHEME III

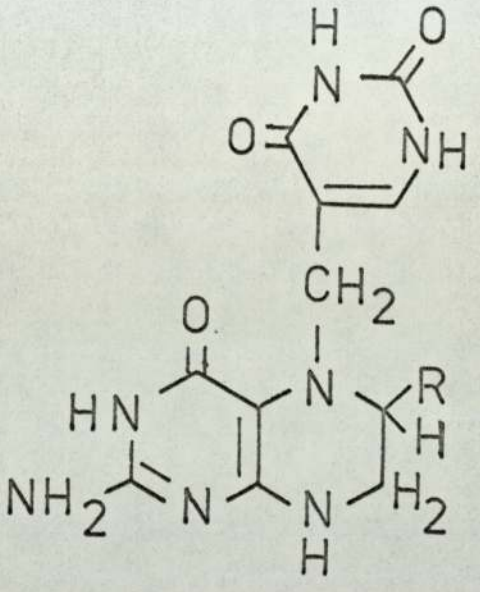


Preferential attack at N(5) is predicted by charge density calculations[81]. The bell-shaped rate profile observed for the condensation[80] is easily explained by competition between OH^- and THF for nucleophilic attack on formaldehyde at higher pHs and competition between protonation of N(5) and electrophilic attack by formaldehyde at lower pHs.

Discussion.

Seven batches of 5-methyl THF as Ba and Ca salts were prepared in good yield (50-60%) and reasonable purity (80 - 95%), using the method of Blair and Saunders[51]; so too were 5-n-butyl THF and tetrahydromethotrexate (yields and purities within the above range). 5-Isobutyl THF was difficult to prepare - the product was always isolated as a mixture of 5-isobutyl THF and THF judged by u.v. and n.m.r. spectra. The ability to reverse methotrexate toxicity (Table VI) could be due to THF impurity. Also contaminated with THF was the small-scale high specific activity 2^{14}C-5-methyl THF preparation. The original method of preparation devised by Beavon[56] did not yield any 2^{14}C-5-methyl THF. A modified method yielded 30% 5-methyl THF and 60% THF, judged by radioactivity under the peaks in the elution profile. An enzymic preparation of N(5)^{14}C and ^{3}H labelled 5-methyl THF has been reported[82] since the above work was carried out. This enzymic method has the advantage that only the biologically active diastereoisomer is produced (see Section D). Samples prepared by the method of Blair and Saunders usually contained small amounts of impurity, observable on t.l.c. as fluorescing spots when viewed under u.v. light (λ254 or λ356 nm). These slight impurities can be separated from 5-methyl THF by column chromatography on A25 DEAE sephadex, see Diagram (IV) p. 120. Trace amounts of THF are often present in above preparations. As THF is more labile than 5-methyl THF (see conclusions Chapter IV), the fluorescent impurities are probably oxidation products of the former. The lack of success in preparing 5-isopropyl-,[83]

5-isobutyl and 5-benzyl THF seems to be due to a high activation energy for the condensation stage rather than steric hindrance. Gupta and Huennekens[84] have reported easy formation of 5-thyminyl tetrahydrofolate(XIX) from THF and 5-chloromethyl uracil.



(XIX)

At this stage it was decided to limit the preparative work to producing 5-methyl THF for oxidation studies.

PART B. Determination of Solubility of 5-Methyl THF.

Introduction.

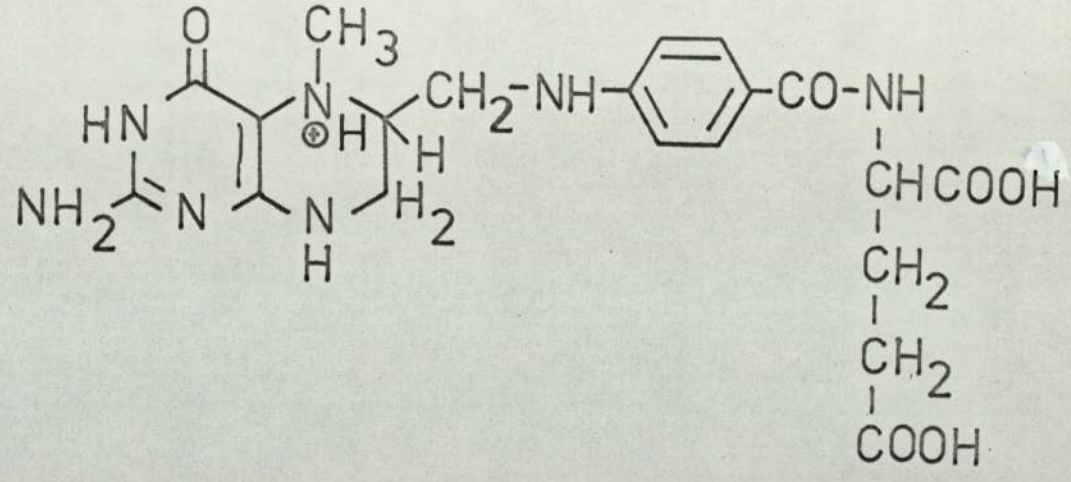
Folic acid uptake across rat intestine has been found to level off when the concentration of folic acid in the incubation medium is 10^{-5}M or greater.[85] This observation can be explained in terms of an acidic micro-climate or unstirred layer (pH3) close to the intestinal wall, as the maximum solubility of folic acid at this pH is 10^{-5}M. Indeed this observation is proposed as evidence for the existence of such a layer. However, studies on 5-methyl THF uptake did not exhibit any saturation phenomena at this concentration. The solubility of 5-methyl THF was determined in the range pH 7 → pH 2.

Method.

5-Methyl THF (Ba salt, 400 mg.) was stirred into 0.1M phosphate buffer (10 ml.) pH 7 at 25°C. The suspension was filtered and an aliquot of filtrate was diluted with 0.1M phosphate buffer pH 7 (0.2 ml. in 250 ml.) The absorbance at 290 nm. was recorded and the pH remeasured. Excess solid 5-methyl THF was added to the filtrate and after a second filtration the absorbance at 290 nm. was again recorded. 10 N HCl was used to adjust the pH in small increments to give various pH values in the range pH 7 to pH 2. The above procedure was repeated for each value of pH. Results are given in Table XII.

Calculation of 5-methyl THF zwitterion concentration.

The cation of 5-methyl THF (XX) can be regarded as a triprotic acid with the following pK values pK' 5.2, N(5); pK' 4.8, γ - carboxyl, pK' 3.5, α-carboxyl[86]. (These values are for model compounds and may not be strictly accurate for 5-methyl THF.)



(XX)

For acid AH_3 the equilibria are:

$$[H^+]\ [AH_2^-] = Ka_1\ [AH_3] \quad - (1)$$

$$[H^+]\ [AH^{2-}] = Ka_2\ [AH_2^-] \quad - (2)$$

$$[H^+]\ [A^{3-}] = Ka_3\ [AH^{2-}] \quad - (3)$$

and we can write

$$[AH_3] + [AH_2^-] + [AH^{2-}] + [A^{3-}] = C \quad - (4)$$

where C is the analytical concentration of the acid. The fraction of the acid present as each species (α) is the ratio of the concentration of that species to the analytical concentration. The index on α gives the number of protons attached to the molecule.

By comparison with glycine the monoanionic form will be much less abundant than the zwitteranionic form and is therefore ignored for the purposes of the calculation

It is more convenient to calculate the reciprocal of α as this is directly expressible in terms of the mass balance (4): e.g.

$$\frac{1}{\alpha_3} = \frac{C}{[AH_3]} = 1 + \frac{[AH_2^-]}{[AH_3]} + \frac{[AH^{2-}]}{[AH_3]} + \frac{[A^{3-}]}{[AH_3]} \quad - (5)$$

Equation (1) gives the second term directly:

$$\frac{[AH_2^-]}{[AH_3]} = \frac{Ka_1}{[H^+]} \quad - (6)$$

Multiplying (1) by (2) to eliminate $[AH_2^-]$ gives the third term

$$\frac{[AH^{2-}]}{[AH_3]} = \frac{Ka_1 \; Ka_2}{[H^+]^2} \quad - (7)$$

Multiplying (1), (2) and (3) together gives the fourth term:

$$\frac{[A^{3-}]}{[AH_3]} = \frac{Ka_1 \; Ka_2 \; Ka_3}{[H^+]^3} \quad - (8)$$

Substituting (6), (7) and (8) in 5 gives

$$\alpha_3 = \frac{[AH_3]}{C} = \left[1 + \frac{Ka_1}{[H^+]} + \frac{Ka_1 \, Ka_2}{[H^+]^2} + \frac{Ka_1 \, Ka_2 \, Ka_3}{[H^+]^3} \right]^{-1} \quad - (9)$$

Combining (9) and (6), we have

$$\alpha_2 = \frac{[AH_2^-]}{C} = \alpha_3 \frac{Ka_1}{[H^+]} \qquad - (10)$$

Combining (9) and (7) gives

$$\alpha_1 = \frac{[AH^{2-}]}{C} = \alpha_3 \frac{Ka_1 Ka_2}{[H^+]^2} \qquad - (11)$$

Combining (9) and (8) gives

$$\alpha_o = \frac{[A^{3-}]}{C} = \alpha_3 \frac{Ka_1 Ka_2 Ka_3}{[H^+]^3} \qquad - (12)$$

Knowing the relevant pK' values (3.5, 4.8 and 5.2 in this case), it is possible to calculate % concentrations of all 3 species at a given pH. The computer programme used to calculate these is given in the appendix.

TABLE XII

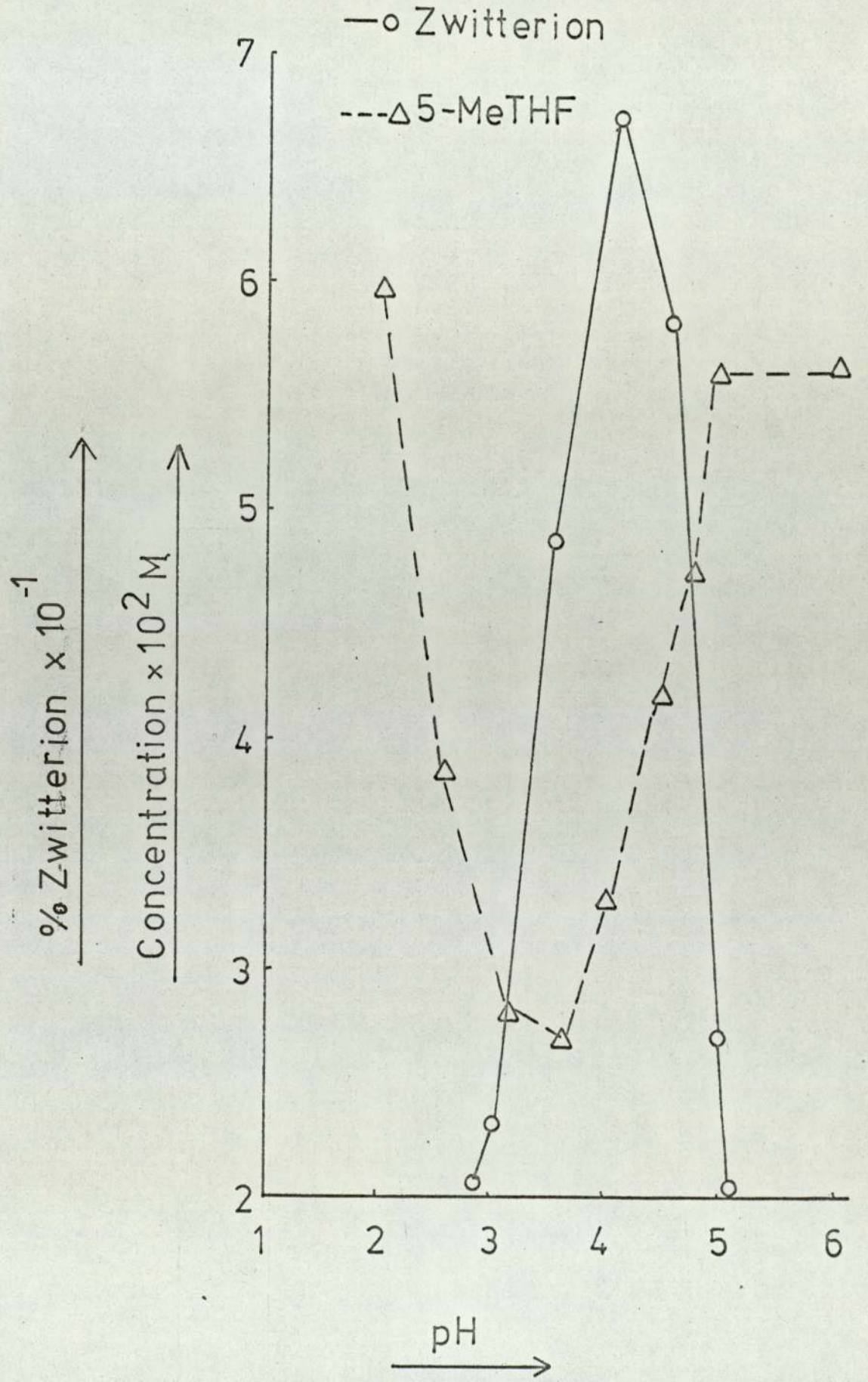
Solubility of 5-methyl THF, % 5-methyl THF Zwitterion and % Folic Acid Unionised species at various pH values.

pH	% Unionised Folic Acid	% 5-methyl THF Zwitterion	Solubility of 5-methyl THF $\times 10^{2}$M.
2.0	96.9	3.0	6.2
2.6	90.8	11.1	3.9
3.2	66.0	33.1	2.8
3.6	42.7	53.8	2.7
4.0	21.4	67.3	3.3
4.5	6.2	58.7	4.2
4.7	3.3	43.6	4.7
5.2	0.7	16.5	5.6
6.0	1.8×10^{-3}	0.5	5.6

A diagramatic representation of the results is given in Diagram (I).

DIAGRAM I.

Solubility curve for 5-methyl THF.

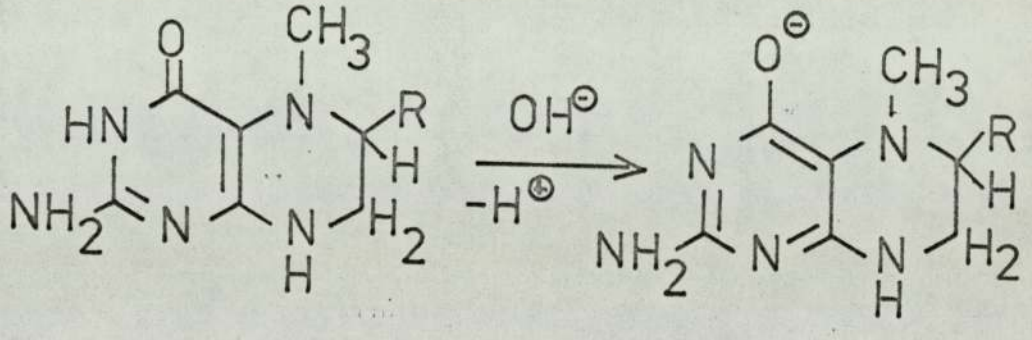


Discussion.

As expected, the solubility of folic acid undergoes a marked decrease at low pH values, due to protonation of its carboxyl groups to form a neutral species. Because of enhanced basicity at N(5) in the case of tetrahydro derivatives, protonation occurs and very little neutral species is ever present. The form nearest approaching a neutral species is the internally neutralised zwitterion formed by protonation of N(5) and loss of a proton from one of the carboxyl groups. It can be seen from diagram (I) that the solubility of 5-methyl THF is fairly well related to the lack of this species in solution. The minimum solubility of 5-methyl THF over the range studied is 2×10^{-2}M, explaining why no saturation occurs in uptake across the gut wall at 10^{-5}M.

PART C. Determination of the pKa for Ionisation of the 3,4-Amide Function.

Previous studies[54,55] on the oxidation of tetrahydropterins have demonstrated a first order dependence on imino-enolate anion (XXl) concentration. In order to investigate this possibility for 5-methyl THF the pKa for the reaction (see below) was determined.



(VII) (XXl)

The absorbance maxima for N(5) substituted tetrahydrofolates exhibits a bathochromatic shift on lowering the pH from 13 to 7. This was not reported by Saunders[51] but lack of the phenomena with THM, a compound which does not contain a 3,4-amide function, supports this idea. A similar though larger shift occurs in the case of THF; Kallen and Jencks[86] attributed this to the said ionisation. Although the shift is small in the case of 5-methyl THF, it is large enough for the ionisation constant to be measured. The shift for 5-methyl THF is of the order of 3 nm while that for THF is 7 nm.

Method.

pH was measured at room temperature using an E.I.L. 3010 pH meter. 5-Methyl THF (1 mg. Ba salt) was dissolved in deaerated distilled water, then the pH quickly adjusted to 8.5 with 0.1M NaOH. The ultra violet spectrum was immediately recorded in the range 285→300 nm. and the pH of the solution was re-checked. Nine incremental aliquots of 0.1M NaOH were added, and the pH and u.v. spectrum were recorded as above for each one. The experiment took 15 minutes to complete, in which time no appreciable oxidation took place (estimated $\frac{1}{2}$ life under these conditions 8 hrs.). The variation of λmax with pH was plotted and the ionization constant was obtained from the inflection point. The above procedure was repeated for 5-methyl-5,6-DHF (for preparation see Chapter III).

Results.

TABLE XIII

For 5-methyl THF.

pH	λ max (n m)
8.5	292.5
10.2	292.5
10.5	292.0
10.7	291.5
11.1	291.0
11.4	290.5
11.7	290.0
12.0	290.0
12.3	290.0

DIAGRAM II.

pKa Determination for 3,4-amide function of 5-methyl THF.

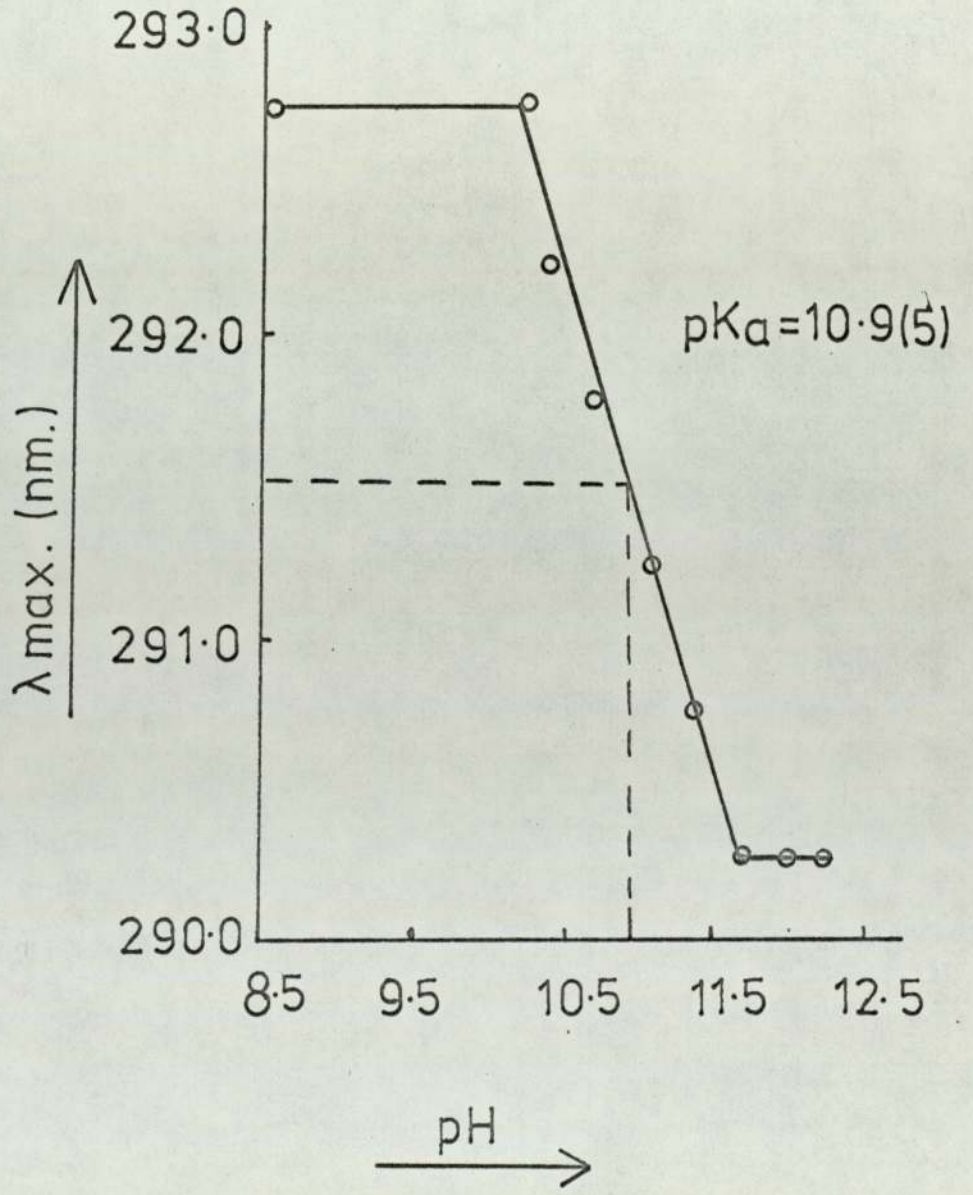


DIAGRAM III.

pKa Determination for 3,4-amide function of 5-methyl-5,6-DHF

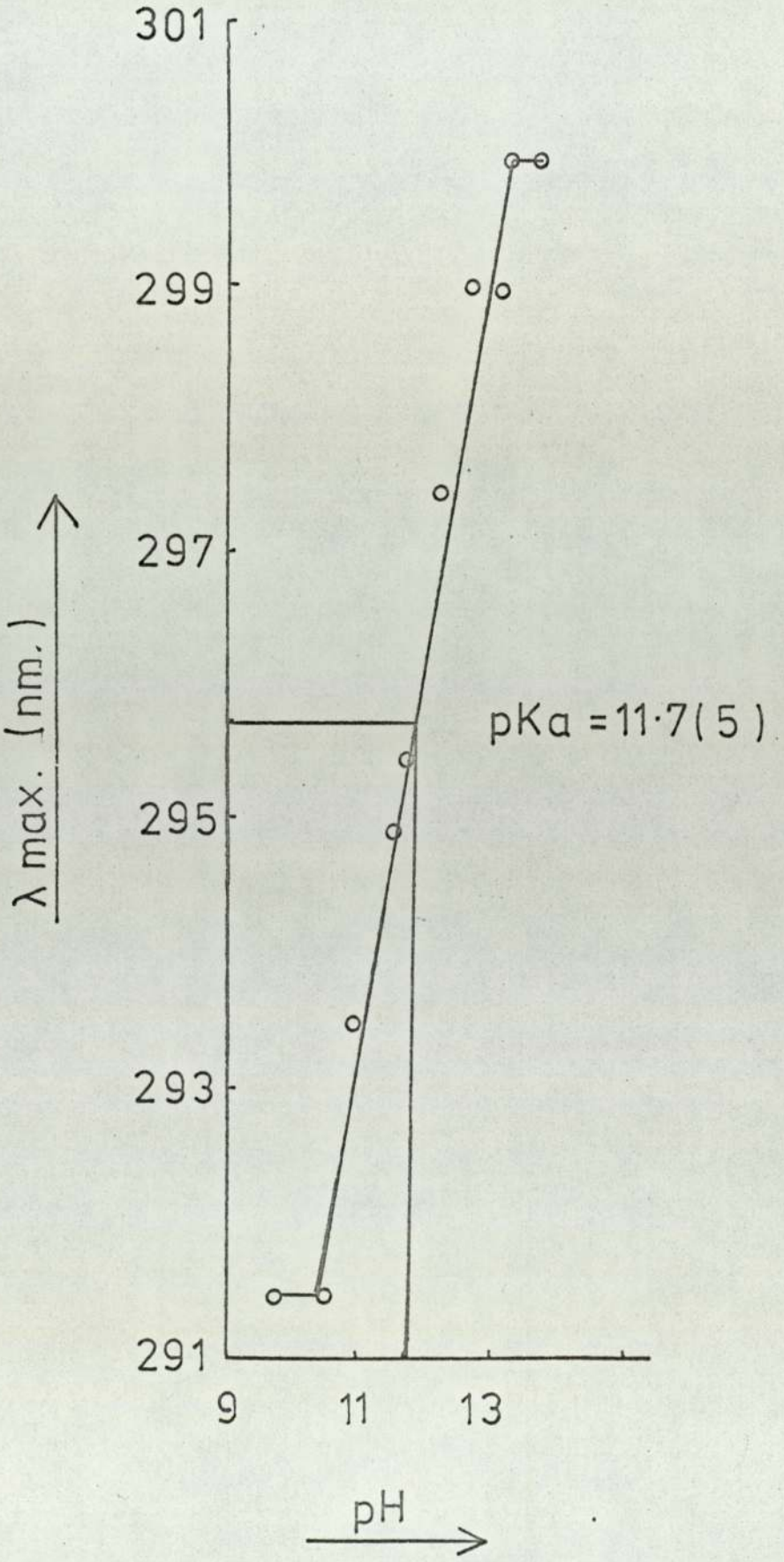


TABLE XIV.

For 5-methyl-5,6-DHF.

pH	λ max n m
10.2	291.5
10.8	293.5
11.4	295.0
11.7	295.5
12.0	297.5
12.5	299.0
12.8	299.0
13.1	300.0
13.2	300.0

Discussion.

The pKa for the 3,4 amide group in 5-methyl THF was found to be 10.9 and that for 5-methyl-5,6-DHF 11.7. The higher value for dihydro species is in line with observations on other tetrahydro and dihydro pterins. Pfleiderer and Zondler reported the pKa value for 7,8-dihydro-6,7-dimethyl pterin to be 11.09, while that for the corresponding tetrahydro compound is reported to be 10.4 [87], and Pearson has obtained pKa values of 10.8 and 11.3 for

THF and DHF respectively[54,114].

The importance of these ionisation constants is discussed in Chapter IV.

PART D. Resolution of Diastereoisomers of 5-methyl THF.

Introduction.

Chemical reduction of pteroyl-L-glutamic acid produces a new asymmetric centre at C (6). Consequently, the resulting THF and derivatives prepared from it are mixtures of diastereoisomers, only one of which will be biologically active. This has to be taken into account when evaluating microbiological assay data (all such data have been corrected where appropriate in this text). Separation of the diastereoisomers of dl-5,10-methylene THF by fractional crystallisation has been described by Cosulich et al.[88], and resolution on DEAE and TEAE cellulose columns has been reported by Kaufman, Donaldson and Kerestesy[89], and also by Ramasastri and Blakley[90]. Horwitz et al.[91] have claimed to separate diastereoisomers of formaldehyde derivatives of THF on a DEAE column. They demonstrated that of two peaks corresponding to 1.5 moles of formaldehyde per mole of THF, only one was biologically active. This result, though interesting, should be viewed with caution as the structure of the resolved compound was not known and optical rotations were not reported.

In the following experiments resolution of the diastereoisomers of 5-methyl THF by fractional crystallisation from water/ethanol and by chromatography on a DEAE cellulose column was attempted. Before work on 5-methyl THF was undertaken, the optical rotations of several standards were measured.

(i) Optical Rotation Measurements of Standards.

Experimental.

Optical rotatory dispersion (O.R.D.) curves were plotted on a 'Polarmatic 62' spectropolarimeter over the range 588 nm. ⟶ 370 nm. Sample solutions were contained in a 1 cm. light path silica windowed cell, (vol. 0.6 ml.). The cell-chamber was thermostated at 30°C. After plotting the sample curve the cell was thoroughly washed out with solvent (10 rinses) then filled with solvent and a blank was run. The amount of folate in solution was calculated from $\Sigma mol = 28.5 \times 10^3$ at pH 13.

The specific rotation $[\alpha]$ of each sample was calculated from

$$[\alpha]_{\lambda}^{t^{o}} = \frac{100\alpha}{1xc}$$

Where:

α = measured angle of rotation

t^{o} = temperature

λ = wavelength

1 = length of light path (in decimetres)

c = concentration in g./100 ml.

TABLE XV

Optical Rotation Measurements.

Sample	Concentration mg./ml.	Solvent	pH	$[\alpha]_D^{30}$
L-glutamic acid	6.9	0.1M HCl	1.0	+ 16.4
"	7.0	Dist. H_2O	5.6	+ 6.6
"	7.0	Phosphate buffer	7.0	- 7.5
"	6.9	0.1M NaOH	13.0	+ 3.9
PAB-L-glutamic acid	4.8	0.1M HCl	1.0	- 16.9
"	4.9	Phosphate buffer	7.0	+ 12.4
"	5.1	0.1M NaOH	13.0	+ 4.2
Folic Acid	2.9	Phosphate buffer	7.0	+ 9.4
"	2.5	0.1M NaOH	13.0	+ 7.2
5-methyl-THF	5.8	Phosphate buffer	7.0	+ 24.1
"	6.3	0.1M NaOH	13.0	+ 11.2
5-n-butyl THF	2.8	0.1M NaOH	13.0	+ 10.1

(ii) Attempted Resolution of the Diastereoisomers of 5-Methyl THF by Column Chromatography on a DEAE Cellulose Column.

Method.

5-methyl THF was prepared in the usual manner. Fractions 90 - 115; 116 - 140; 141 - 166; 167 - 190 were lyophilised to give four samples (a), (b), (c) and (d)

Samples were examined by t.l.c., u.v. and ORD curves were plotted for each sample.

TABLE XVI

T.l.c. Data.

T.l.c. Solvent.	Sample	Rf.
(i)	5-methyl THF (reference)	0.87
"	" (a)	0.87
"	" (b)	0.87
"	" (c)	0.87
"	" (d)	0.87
"	Fl. impurity in all above samples	0.25
"	5-formyl THF	0.87
(ii)	5-methyl THF (reference)	0.65
"	" (a)	0.65
"	" (b)	0.65
"	" (c)	0.65
"	" (d)	0.65
"	Fl. impurity in above samples	0.20
"	5-formyl THF	0.55
(iii)	5-methyl THF (reference)	0.40
"	" (a)	0.40
"	" (b)	0.40
"	" (c)	0.40
"	" (d)	0.40
"	Fl. impurity in above samples	0.25
"	5-formyl THF	0.42

TABLE XVII

U.V. Data.

Sample	pH	λ max.(n m)	λ min.(n m)
(a)	1.0	270, 292	243, 282
(b)	"	270, 292	243, 282
(c)	"	270, 292	243, 282
(d)	"	270, 292	243, 282
(a)	7.0	290	248
(b)	"	290	248
(c)	"	290	248
(d)	"	290	248
(a)	13.0	288	248
(b)	"	288	247
(c)	"	288	247
(d)	"	288	247

TABLE XVIII

Optical Rotation Measurements.

Sample	Concentration mg./ml	$[\alpha]_D^{30}$
(a)	3.2	+ 13.2
(b)	3.3	+ 10.3
(c)	3.8	+ 6.4
(d)	4.6	+ 15.3
5-methyl THF ref	6.3	+ 7.3

(iii) Attempted Resolution of Diastereoisomers by Fractional Crystallisation.

Method.

Fraction (a) from above was recrystallised by dissolving (0.33 g.) in 6 ml. deaerated water (0.5% mecaptoethanol). The residue, fraction (i), was centrifuged and recrystallised. Ethanol (1 ml.) was added and the solution cooled to yield fraction (ii); a second addition of ethanol gave fraction (iii) and an excess (10 ml.) gave fraction (iv). Each sample was centrifuged, washed with ethanol (10 ml.) and dried under vacuum at room temperature and the optical rotation measured as above.

TABLE XIX

Optical Rotations of Fractions from Resolution by Fractional Crystallisation.

Sample	Concentration mg./ml.	$[\alpha]_D^{30}$
(i)	2.7	+ 11.9
(ii)	2.6	+ 6.3
(iii)	1.8	+ 14.6
(iv)	2.5	+ 8.6

Discussion.

It can be seen from Table XV that specific rotation varies greatly with pH. Although care was taken to ensure that all measurements on 'resolved' fractions were carried out at pH 13, the results are unsatisfactorily inaccurate. Impurities such as 5-methyl-5,6-DHF or 4a-OH-5-methyl THF may have contributed to this inaccuracy but the largest source of error appears to lie in the measuring technique. Measurements on the same sample of folate run consecutively gave an error of $\pm$ 35%. This can account for the variations in optical rotation of attempted resolved samples (Tables XVIII and XIX) but conversely, may have obscured any resolution which had taken place.

Inaccuracy in optical rotation measurements with folates seems universal, if we consider the literature values for the specific rotations of the two diastereisomers of 5,10-methylene THF resolved as mentioned above (see Table XX).

TABLE XX

Optical rotations of (+) and (-) 5,10-methylene THF.

+	-	Reference.
165°	82°	92
145°	97°	93
83°	106°	94

It would be reasonable to assume that such a large error is an accumulation of factors, the most important in this case being the sensitivity of the instrument to light absorbed in the cell. Folates absorb light strongly in the u.v. and visible region. This results in optical rotation values which are often lower than they should be. Rudiger[93] could not separate the diastereoisomers of 5-methyl THF by column chromatography but reports preparation of individual isomers from 5,10-methylene THF separated on a DEAE column. No values for the optical rotation of each isomer were given. Donaldson and Keresztesy[32] have claimed to separate the active and inactive isomers enzymatically and give $[\alpha]_D^{25} = + 12.9^o$ for the active isomer and $[\alpha]_D^{25} = + 4.0$ for the inactive isomer. Unfortunately the pH is not quoted and no details of the separation are given.

Clearly, the most accurate method of determining whether or not resolution was achieved is microbiological assay, (Only one diastereoisomer being biologically active). Unfortunately, this technique was not available during this investigation and so although there is no evidence that resolution was achieved the question must remain open.

CHAPTER III

Identification of Products of Oxidation of 5-methyl THF.

PART A. Preliminary Investigation.

PART B. Structure elucidation of Prefolic AB.

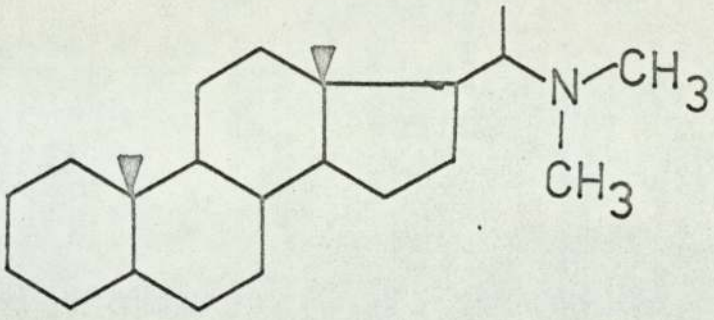
PART C. Investigation of the Formation of 8-Dehydro-4a-hydroxy-5-methyl THF.

PART A. Preliminary Investigation.

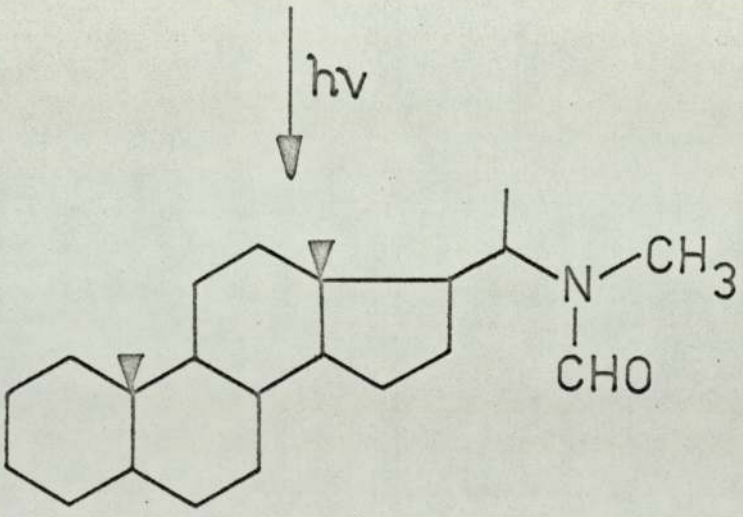
Introduction.

Chang[95] isolated citrovorum factor from horse liver in 1953. He reported that minor changes in the autolytic conditions significantly altered the yield of citrovorum factor activity. As very little free citrovorum activity was found to occur in fresh natural materials it was concluded that the isolated citrovorum factor was produced during the analytical procedure. The structure of the precursor was not discovered until almost ten years later, when Donaldson and Keresztesy[29-32] identified liver folate as 5-methyl THF. A similar situation arose in the study of blood folates. Toennies et al[96] reported large amounts of citrovorum factor present in blood. Later studies by Noronha and Aboobaker[35] showed that the major blood folate was 5-methyl THF. This first part of Chapter III describes a simple series of experiments to see if 5-formyl THF is produced as an oxidation product of 5-methyl THF and hence explain the observations of Chang and Toennies et al.

A reaction of this type has indeed been reported[97] for the $N(CH_3)$ group in the following amino steroid molecule: -



XXII



The reaction proceeded when the starting material under oxygen was irradiated in the presence of methylene blue photosensitizer. As it is possible that the pterin portion of 5-methyl THF could act as a photosensitizer, the first experiment was performed without added photosensitizer.

At pH 1, 5-formyl THF loses a molecule of water to yield 5,10-methenyl THF(IX)[98]. This species has a strong characteristic absorption at 356 nm. and, it was hoped, would be a convenient method of observing any 5-formyl THF produced in the oxidation of 5-methyl THF.

A short time-study was carried out (under

the conditions to be used for the oxidation experiment) for the reaction

$$\text{5-formyl THF} + H^+ \rightleftharpoons \text{5,10-methenyl THF}^+ + H_2O.$$

The oxidation of 5-methyl THF in the presence of riboflavin photosensitizer was also investigated. Beavon[56] has reported that folic acid is the major product under these conditions. His work was repeated.

Materials and Methods.

U.v. spectra were recorded on a Perkin-Elmer PE 137 or Unicam SP 700 spectrophotometer, Nuclear Magnetic resonance spectra on a Perkin-Elmer R.14 or Varian HA100D spectrometer. Thin layer chromatograms were run on cellulose powder MN300 F254 (Macherey, Nagel and Co., Duren, Germany). The following solvent systems were used.

(i) 0.1M phosphate buffer, pH 7.0

(ii) n-Propanol/water/0.88 s/g aqueous ammonia (200:100:1 v/v)

(iii) The organic phase of 1-butanol/acetic acid/water (4:1:5 v/v).

Samples were observed as absorbing or fluorescing spots when viewed in u.v. light (λ = 254 n m).

Methods and Results.

1. Time study for 5-formyl THF $\underset{\longleftarrow}{\overset{H^+}{\longrightarrow}}$ 5,10-methenyl THF^+ reaction.

5-Formyl THF (Ca salt) (10 mg) was dissolved in 0.1M ammonium acetate/0.5M phosphate buffer solution (10 ml) pH 7. An aliquot (0.2 ml) of this solution was diluted to 5 ml with 0.1M HCl and placed in a u.v. cell which was stoppered for the duration of the study.

Results

TABLE XXI

Time (mins)	Absorbance λ 287 n m	Absorbance λ 356 n m
5	0.99	0.35
8	0.92	0.43
11	0.87	0.49
14	0.83	0.54
17	0.78	0.59
20	0.75	0.63
23	0.72	0.66
26	0.69	0.69
29	0.67	0.71
48 hrs.	0.48	0.77

On the basis of these results, half an hour was allowed for the acidified 5-methyl THF oxidation solution to equilibriate before the u.v. spectrum was recorded.

2. Oxidation of 5-methyl THF in absence of photosensitizer.

Method.

5-Methyl THF (as Ba salt)(30 mg.) was purified by dissolving in 0.1M ammonium acetate (2 ml.) and passing through a G.15 sephadex column (2.5 mm x 30 cm). Elution was carried out with 0.1M ammonium acetate. The u.v. spectrum of each fraction (5 ml.) was recorded. Tubes 28-31 were pooled to yield a solution of 5-methyl THF (1 mg/ml) in 0.1M ammonium acetate. Enough solid sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate were added to give a solution of 0.5M phosphate buffer pH 7. The solution was stirred magnetically under air for 5 hrs. Two aliquots (0.2 ml) were taken from the solution every hour. After dilution to 5 ml. with 0.1M HCl and 0.1M phosphate buffer (pH 7) respectively, the u.v. spectra were recorded.

Observations.

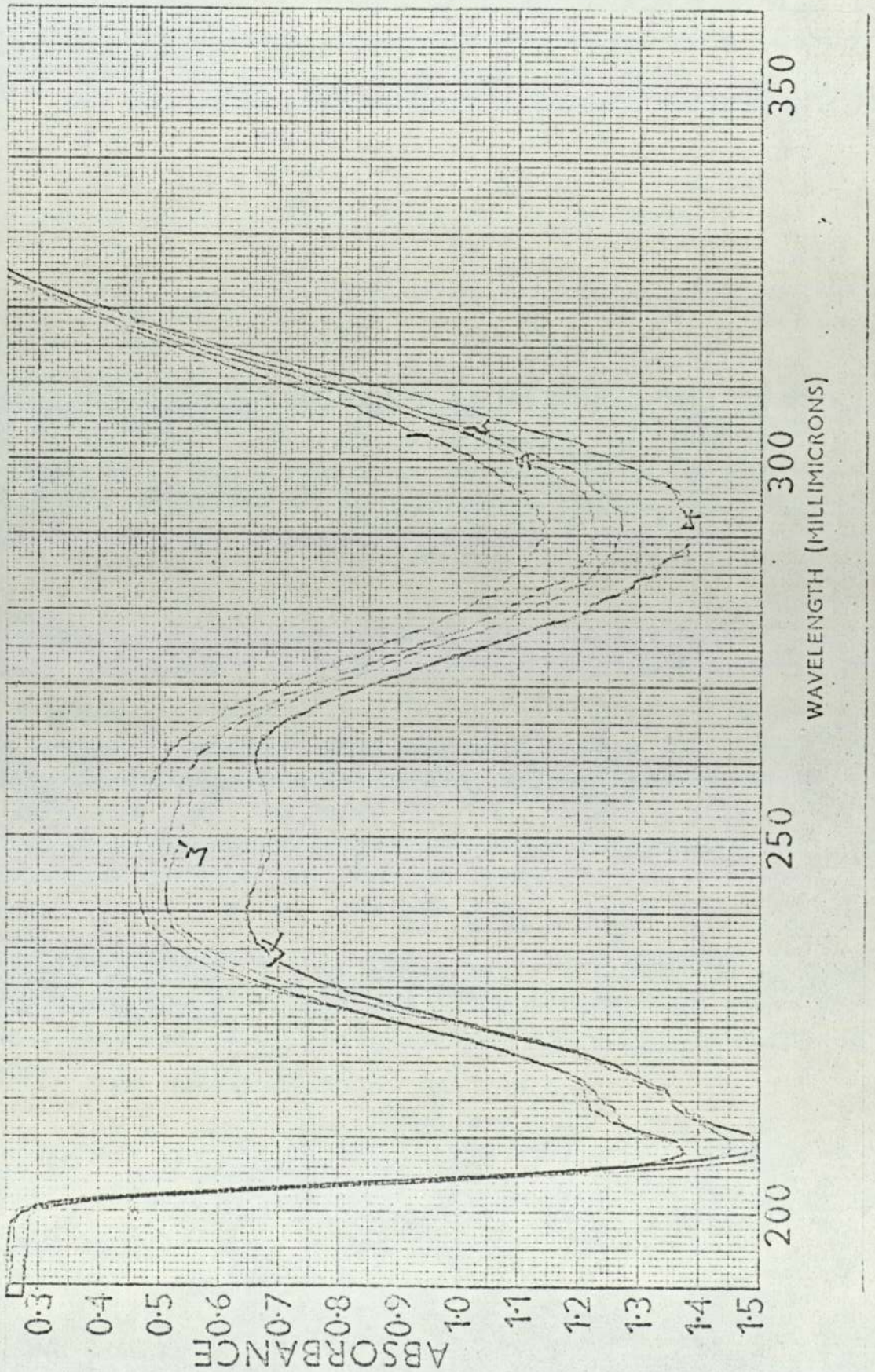
After 1 hour, a small peak at 250 nm appeared in the spectrum run at pH 7; this gradually increased over 5 hours and then remained constant. The ratio of $\Sigma\lambda_{250}:\Sigma\lambda_{290}$ was 1:1.7 (see u.v. spectra on following pages). This product has been reported by Donaldson and Keresztesy, who named it prefolic AB.[29]

Very little change in the spectrum run at pH 1 was observed over 5 hours. The absorption at 290 nm decreased but no increase in absorption at 356 nm was found.

SPECTRUM 5.

Oxidation of 5-methyl THF in 0.5M phosphate buffer pH 7. (0 - 2 hrs.)

u.v. solvent: 0.1M phosphate buffer pH 7.

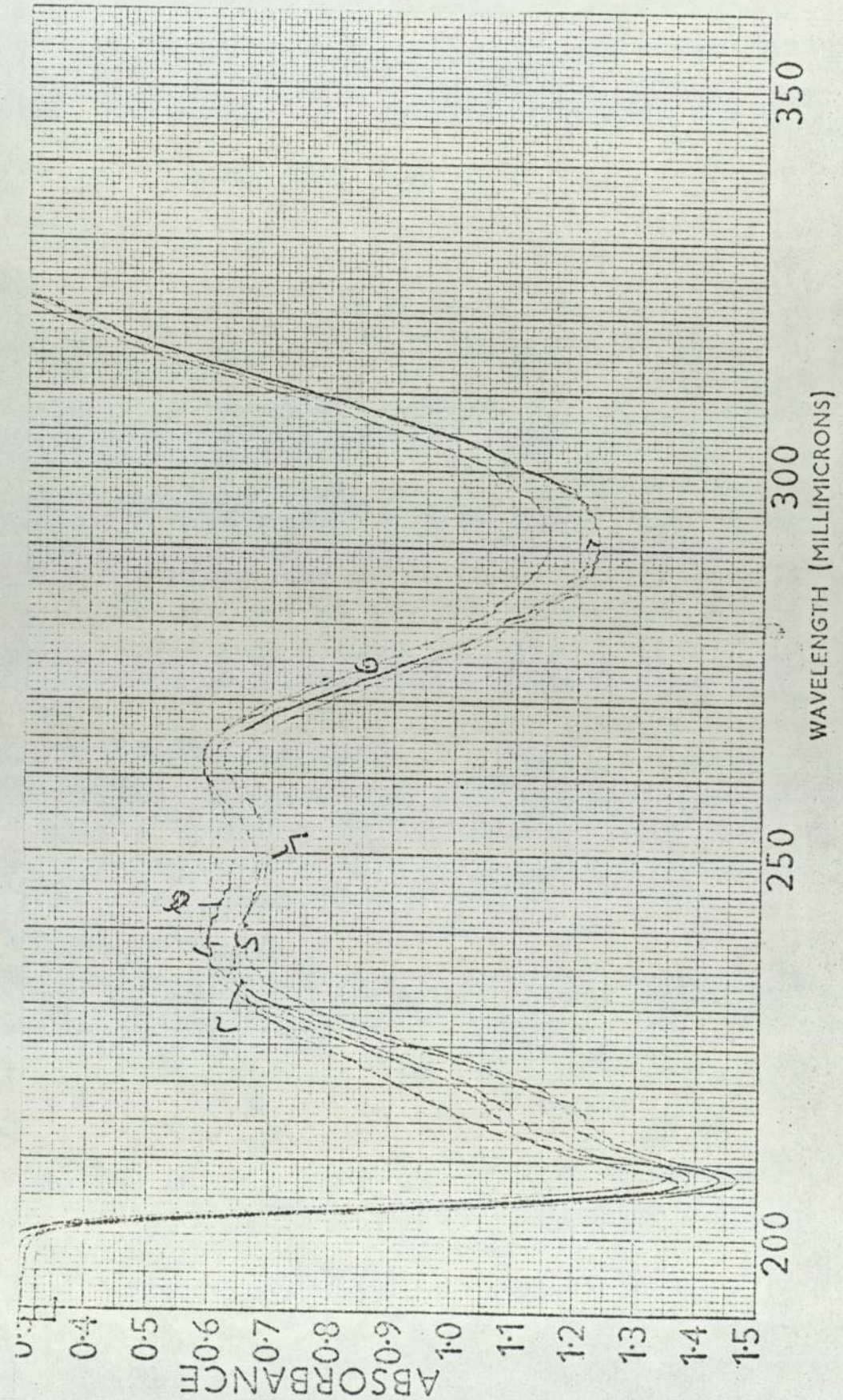


SPECTRUM 6.

Oxidation of 5-methyl THF in 0.5M phosphate buffer pH 7.

(2 - 5 hrs.)

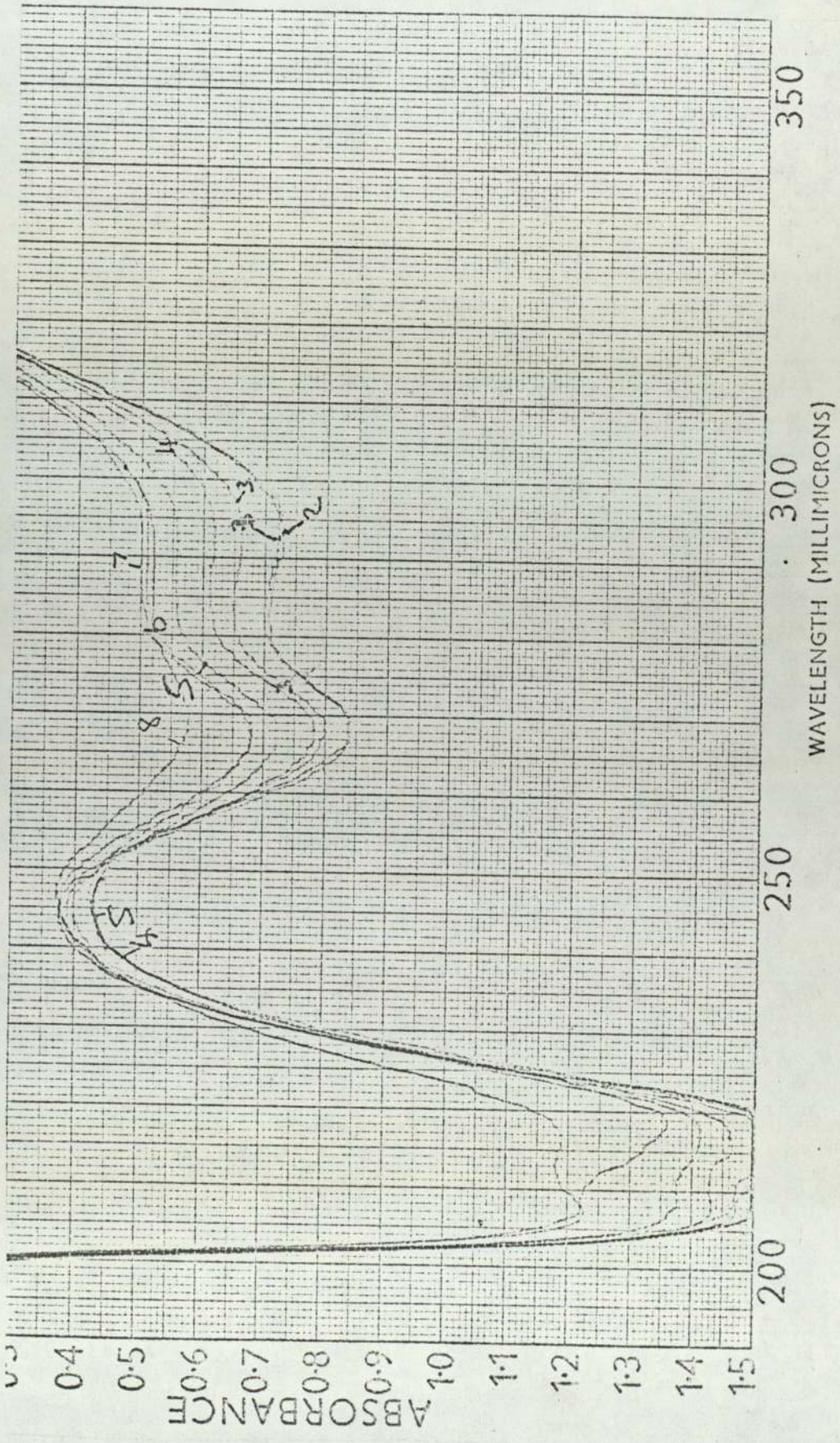
U.v. solvent: 0.1M phosphate buffer pH 7.



SPECTRUM 7.

Oxidation of 5-methyl THF in 0.5M phosphate buffer pH 7 (0 - 5 hrs.)

U.v. solvent: 0.1M HCl (pH 1).



3. Oxidation of 5-Methyl THF in presence of photosensitizer.

Method.

5-Methyl THF (Ba salt)(5 mg) was dissolved in each of the following solvent systems, contained in 5 ml round-bottomed flasks:

a)	Distilled water	1 ml.
b)	Distilled water + riboflavin (1 mg)	1 ml.
c)	Phosphate buffer 0.1M pH 7	1 ml.
d)	Phosphate buffer 0.1M pH 7 + riboflavin (1 mg)	1 ml.

After flushing for 1 minute with oxygen, the flasks were stoppered and shaken at room temperature under normal laboratory lighting for 18 hours. The residues after lyophilisation were subjected to t.l.c. in three solvent systems.

Results.

TABLE XXII

T.L.C. System	Ox. Solvent.	R_f	Inference.
(i)	-	0.85	5-methyl THF (std)
"	-	0.85	5-formyl THF (std)
"	a	0.95	5-methyl DHF ?
"	"	0.85	5-methyl THF
"	b	0.95	5-methyl DHF ?
"	"	0.85	5-methyl THF
"	"	0.75	Riboflavin
"	c	0.90	5-methyl DHF ?
"	d	0.90	5 methyl DHF ?
"	"	0.80	5 methyl THF
"	"	0.75	Riboflavin.
(ii)	-	0.55	5-methyl THF (std.)
"	-	0.42	5-formyl THF (std.)
"	a	0.55	5-methyl THF
"	"	0.35	5-methyl DHF ?
"	b	0.57	5-methyl THF
"	"	0.37	5-methyl DHF ?
"	"	0.05	Riboflavin.
"	c	0.55	5-methyl THF
"	"	0.35	5-methyl DHF ?
"	d	0.52	5-methyl THF
"	"	0.35	5-methyl DHF ?
"	"	0.1	Riboflavin.
(iii)	-	0.68	5-methyl THF (std.)
"	-	0.55	5-formyl THF
"	a	0.70	5-methyl THF
"	"	0.32	5-methyl DHF ?
"	b	0.70	5-methyl THF
"	"	0.30	5-methyl DHF ?
		0.00	Riboflavin.

4. Oxidation of 5-Methyl THF in the presence of riboflavin by the method of Beavon.[56]

5-Methyl THF (Ba salt) was dissolved in 5% (v/v aqueous ammonia (10 mg. ml^{-1}) containing 1% (v/v) 2-mercaptoethanol and riboflavin (10^{-3}M). This was solution (a). A similar solution, omitting 5-methyl THF, was also prepared. An aliquot (2 ml) of this solution was kept in the dark for 18 hours (Solution (c)), while a second aliquot (Solution (b)) was irradiated, along with solution (a), with a 100 W tungsten lamp (in an 'Anglepoise' reflector) 23 cm from the liquid surface. Solutions (2 ml) were contained in 10 ml beakers and placed on aluminium foil. Irradiation lasted 18 hours.

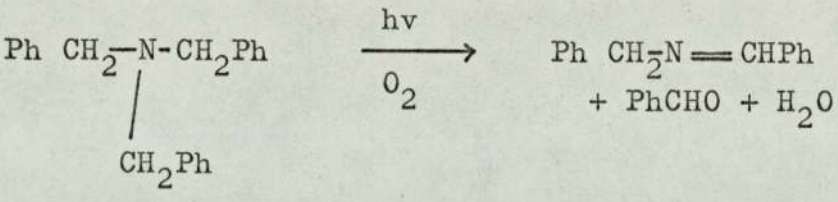
Results.

TABLE XXIII

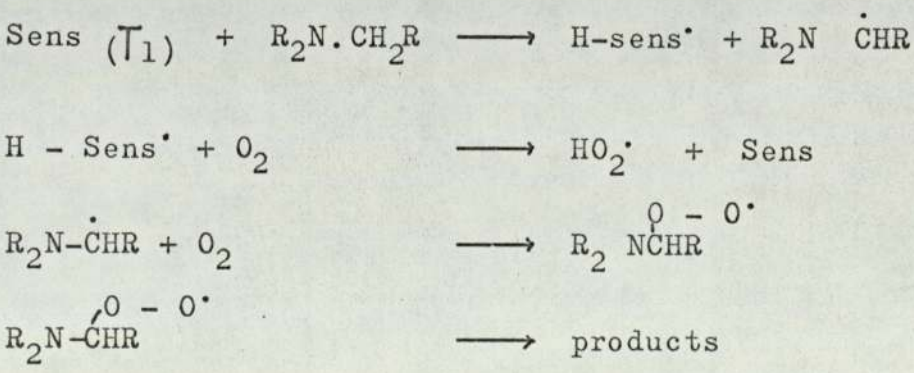
T.L.C. Solvent.	Solution	R_f	Inference
(i)	a	0.92	5-methyl-5,6-DHF
"	a	0.80	Riboflavin
"	std.	0.75	Riboflavin (std.)
"	std.	0.50	Folic acid (std.)
"	b	0.75	Riboflavin
"	c	0.75	Riboflavin
(ii)	a	0.30	5-methyl-5,6-DHF
"	a	0.00	Riboflavin
"	std.	0.00	Riboflavin (std.)
"	std.	0.22	Folic acid (std.)
"	b	0.00	Riboflavin
"	c	0.00	Riboflavin
(iii)	a	0.50	5-methyl-5,6-DHF
	a	0.05	Riboflavin
	std.	0.05	Riboflavin (std.)
	std.	0.00	Folic acid (std.)

Discussion.

No evidence for the formation of 5-formyl THF from 5-methyl THF, oxidised either with or without riboflavin, could be found. This result ties in with observations on the photosensitized oxidation of amines, published by Bartholomew and Davidson.[99-101] A typical reaction for tertiary amines is that of tribenzyl amine with benzophenone photosensitizer. The reaction can be expressed by:



They found that the oxidation proceeded via ground state oxygen (the efficiency of the reaction increased as oxygen concentration decreased) and postulated the following scheme: -



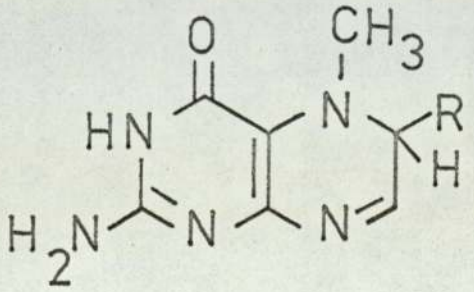
If a similar scheme is applied to 5-methyl THF, the expected products would be formaldehyde and 7,8-DHF not 5-formyl THF.

No evidence could be found to support Beavon's observation that the product of 5-methyl THF oxidation in the presence of photosensitizer is folic acid. As he only used one t.l.c. solvent (organic phase of butanol/ acetic acid (glacial)/water, 4:1:5) (solvent (iii)) and as the Rfs in this solvent of the two species involved, folic acid and riboflavin, are 0.00 and 0.05 respectively, his findings should be viewed with caution.

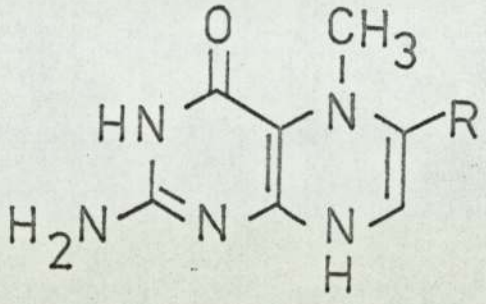
PART B. Structure Elucidation of Prefolic AB.

Introduction.

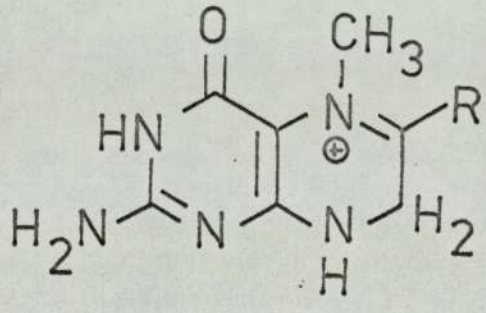
Donaldson and Keresztesy[29] observed that prefolic A, on standing overnight in 1% $Na_2 HPO_4$, resulted in a compound with a u.v. spectrum differing from the starting material. They called the product prefolic AB. It exhibits a characteristic absorbance at 250 nm, together with a longer wavelength absorbance at 290 nm. $\Sigma\lambda 250$: $\Sigma\lambda 290$, 1:1.5. Larrabee et. al.[36] showed that the product formed by aeration of 5-methyl THF at pH 8.7 required one mole of hydrogen for complete reduction to regenerate the starting material, and therefore concluded that the oxidation product had a 5-methyl dihydro structure. These findings were corroborated by Rohrbough,[102] but the exact structure of the dihydro product remained unknown. The three possible dihydro structures shown below were all considered by Donaldson and Keresztesy.[31]



XIV



(XV)



(XXIII)

These workers assayed the biological activity of the product after incubation with tetrahydrofolate reductase and transformylase by measuring the amount of resulting 5-formyl THF formed. A non-enzymatic reduction of the 7,8 or 5,8-dihydro structure would yield a mixture of

diastereoisomers of 5-methyl THF and this would be only 50% biologically active. However the 5,6-dihydro structure (XIV) retains the assymetric C (6) atom and on reduction (non-enzymatic) should be 100% active.

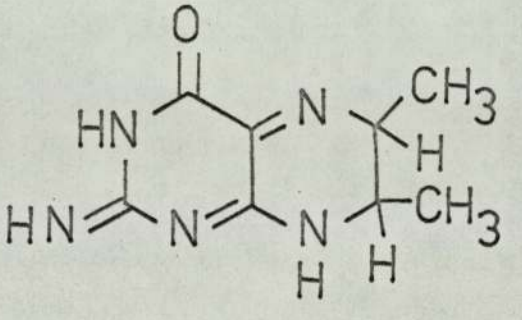
It was found that 5-methyl dihydrofolate was as active as 1,L-5-methyl THF, provided that one of the following reducing agents, ascorbic acid, reduced glutathione, cysteine, homocysteine, or mercaptoethanol was added to the incubation medium. Without a reducing agent, 5-methyl dihydrofolate was inactive. All these reagents are capable of reducing 5-methyl dihydrofolate to 1,L-5-methyl THF, therefore the assymetric C (6) atom is retained in the dihydro compound.

The possibility that the C (9) - N (10) bond was involved in the oxidation of 5-methyl THF was discounted by the observation that 5-methyl-10-formyl THF could be oxidised to a dihydro level which behaved chemically and spectrophotometrically like 5-methyl DHF. Both compounds showed new ultra violet absorption in the region 245 nm (pH 7) and both were stable to alkali but labile to acid. In addition, 5-methyl-10-formyl-tetrahydrofolate was rapidly oxidised by 2,6-dichloro-phenolindophenol[31].

Consequently, 5-methyl-5,6-DHF (XIV) was proposed as the oxidation product of 5-methyl THF. Further evidence was obtained for this structure by Scrimgeour and Vitols,[103] who showed that 5-methyl THF labelled with tritium at C (6) retained the label on

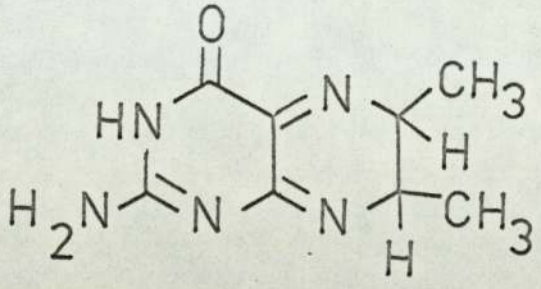
oxidation to the dihydro level, whereas loss of label occurred when the starting material was tritiated at C (7).

Kaufman[104] proposed that a 5,6-dihydro structure was the intermediate in the oxidation of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine to the corresponding 7,8-dihydropteridine. Later, Hemmerich[105] proposed that the intermediate was a quinonoid dihydro type.



XXIV

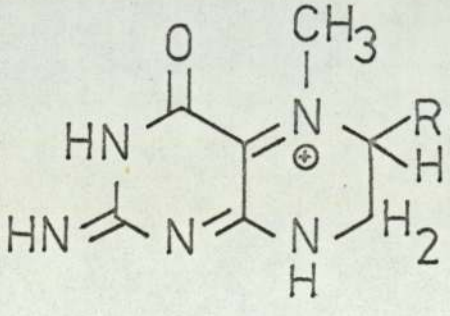
p-quinonoid.



(XXV)

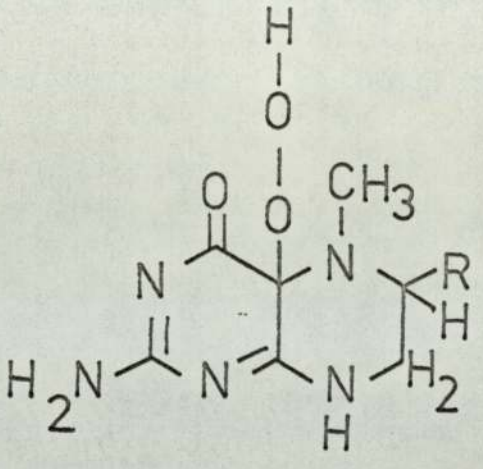
o-quinonoid.

It can be seen that a charged species (XXVI) based on one of the above structures would also explain the observations of Donaldson and Keresztesy:



(XXVI)

So too the 4a-hydroperoxide structure (XXVII).



XXVII

(An 8a-hydroperoxide structure has been proposed as an intermediate in the oxidation of many tetrahydropterins by Mager and Berends[106-110] (see Chapter VI).)

In order to decide which of the three possible structures was correct, prefolic AB was synthesised and subjected to n.m.r., e.s.r., u.v., t.l.c. and microbiological assay.

Preparation of Prefolic AB.

1. Method (a) (described by Gapski, Whiteley and Huennekens[111]).

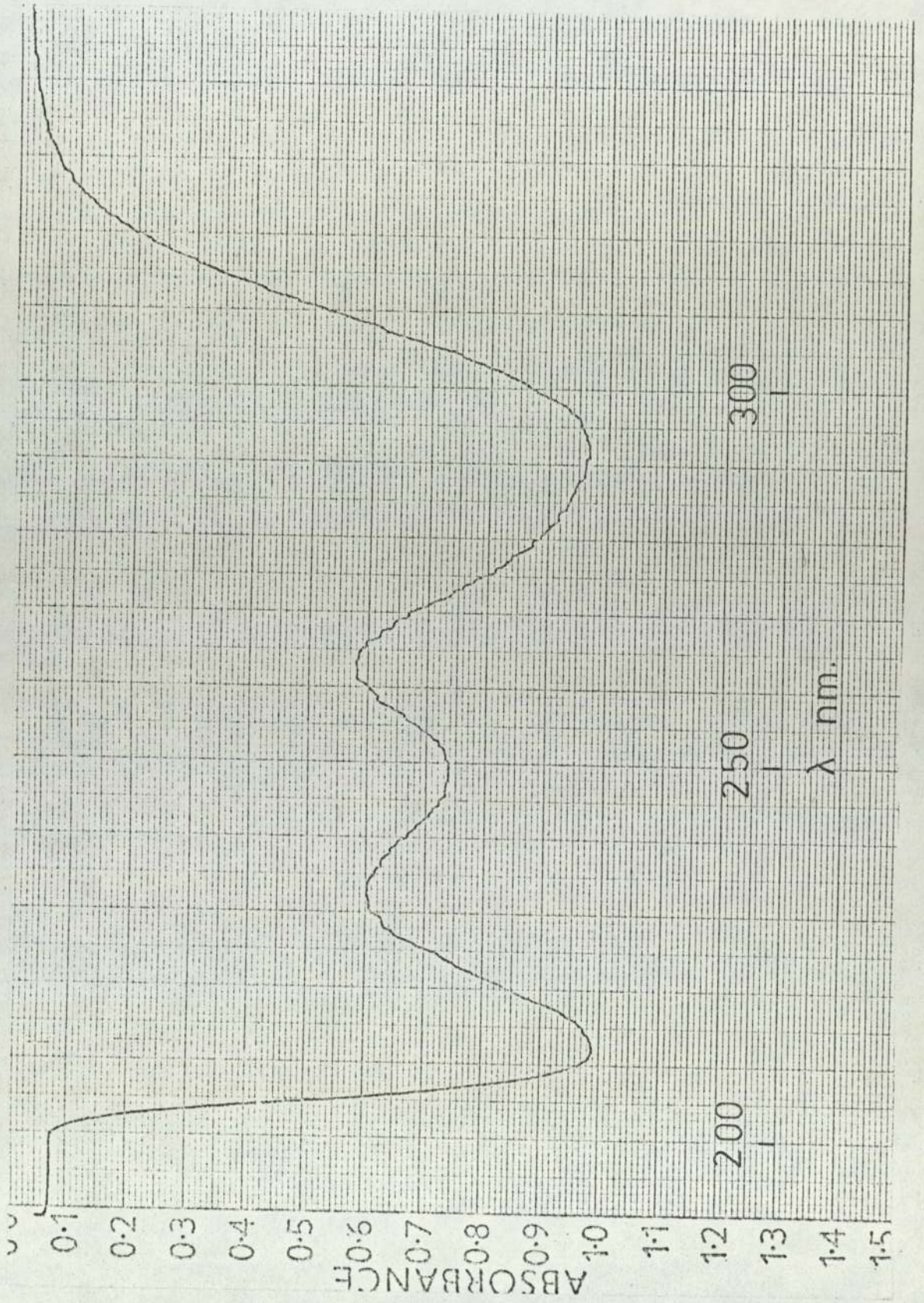
5-Methyl THF (50 mg) was dissolved in 10 ml. 0.5M phosphate buffer (pH 5.6). Potassium ferricyanide (95 mg) was added and the mixture was stirred at room temperature under nitrogen for 30 minutes. The solution was diluted to 100 ml. with cold water and chromatographed on a 1.8 x 33 cm. column of DEAE cellulose. Elution was initiated with 250 ml. 0.1M ammonium acetate pH 7 with a concentration gradient (0.1M - 0.5M, 750 ml. each) of the same salt. The first 500 ml. of eluate was discarded, then 12 ml. fractions were collected. The u.v. spectrum of each fraction was recorded in the range 220 - 350 nm. Prefolic AB was found in tubes 18 - 24. These fractions were freeze-dried.

When the u.v. spectrum of the dried product was recorded at pH 7, the characteristic absorption of 5-methyl-5,6-DHF at 250 nm had disappeared (see spectra on the following two pages). Gupta and Huennekens[112] have reported that the product is very labile in air, due to trace amounts of acetate in the lyophilised solid. Their attempts at recrystallisation failed. A second attempt at preparing prefolic AB by this method also failed. Again the product rearranged or decomposed during lyophilisation.

SPECTRUM 8

5-Methyl-5,6-DHF in solution before lyophilisation, prepared by the method of Gapski et al.[111]

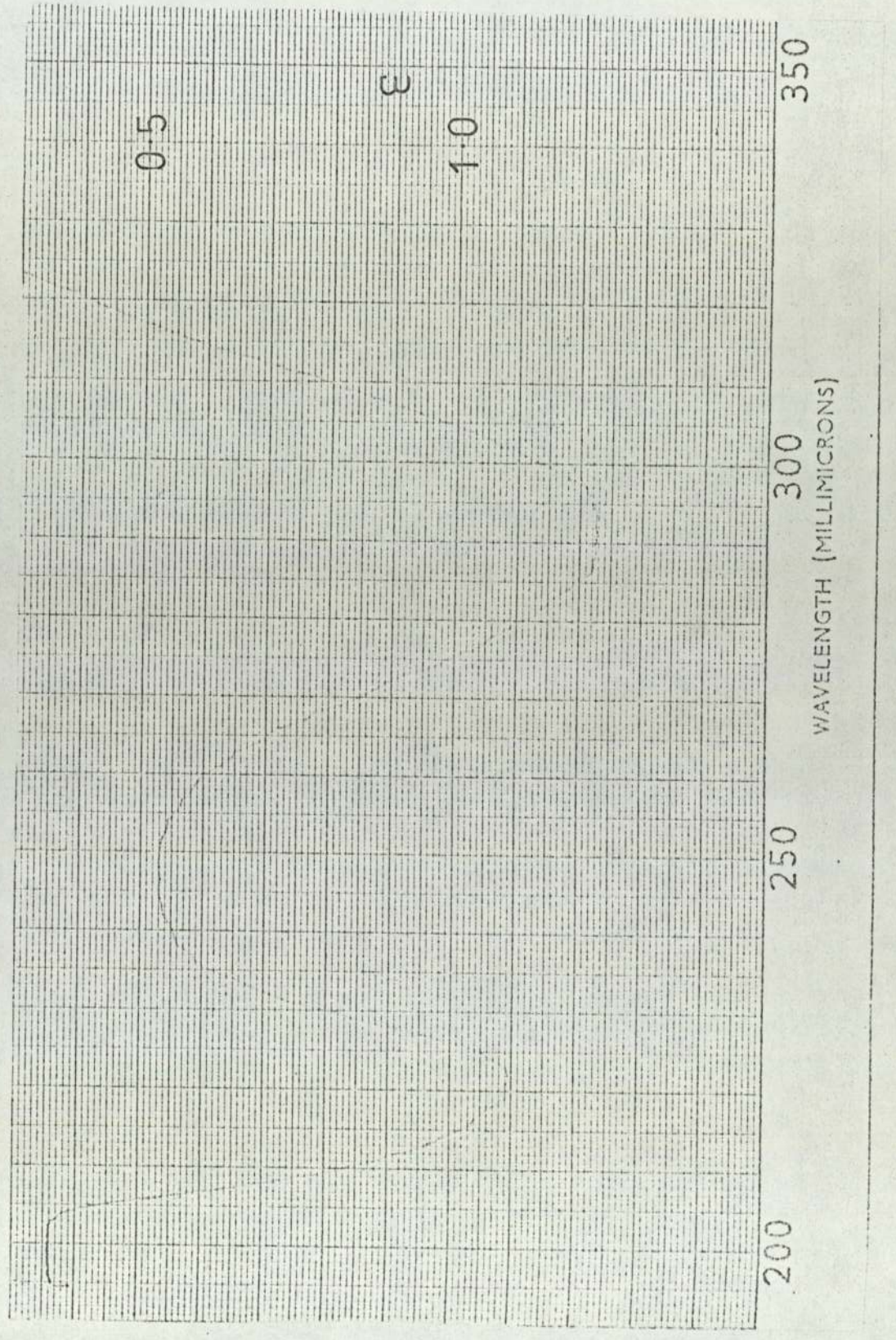
U.v. solvent: 0.1M phosphate buffer pH 7.



SPECTRUM 9

5-Methyl-5,6-DHF after lyophilisation, prepared by the method of Gapski et al[111].

U.v. solvent: 0.1M phosphate buffer pH 7.



2. Method (b). (Simple oxidation in phosphate buffer).

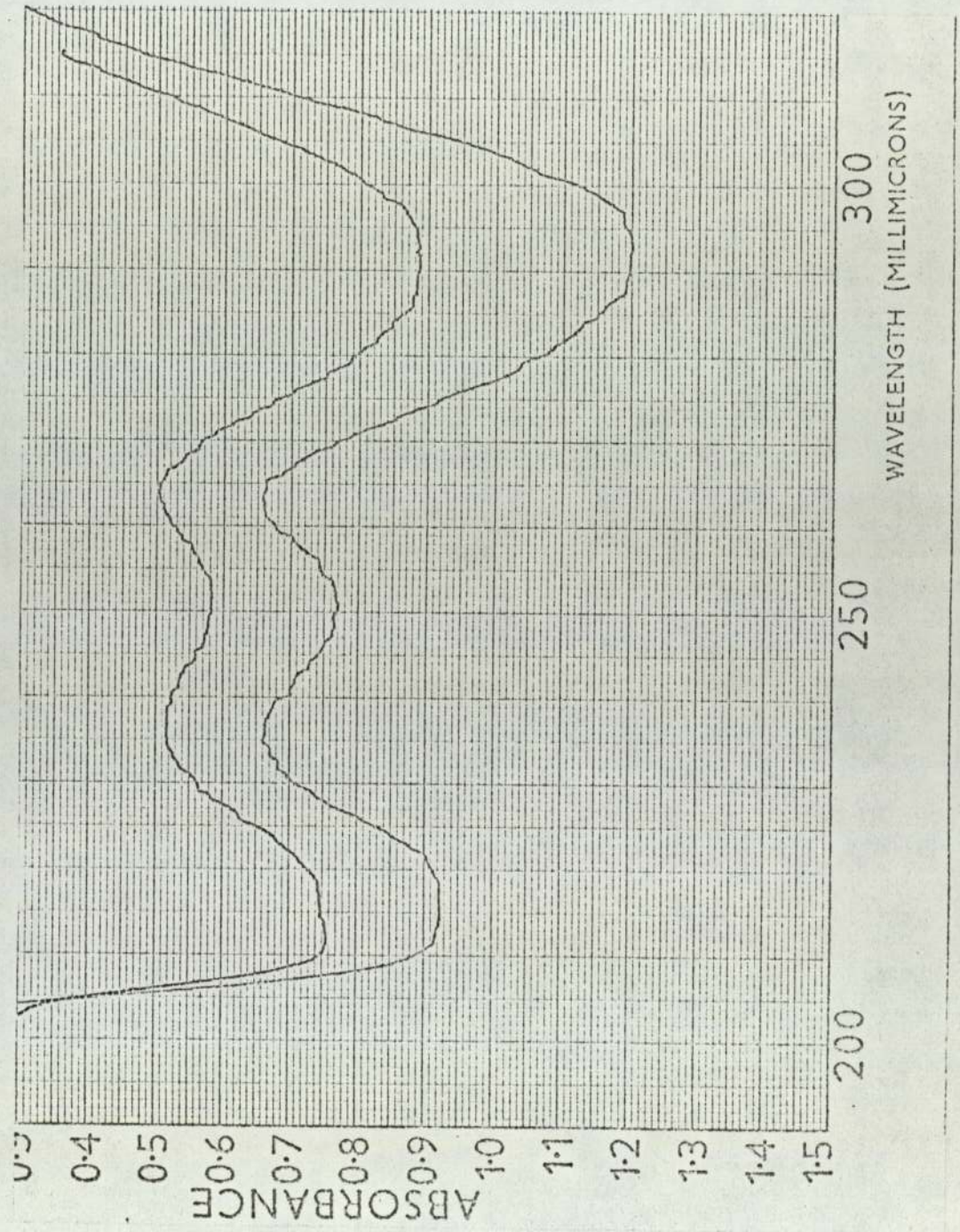
5-Methyl THF (Ba Salt) (50 mg) was stirred in 0.1M disodium hydrogen orthophosphate solution pH 8.7 under pure oxygen. The oxidation was followed manometrically (see Chapter IV). When one mole of oxygen had been taken up the solution was immediately lyophilised. The u.v. spectrum of the product is given on the next page and shows the characteristic absorption of Prefolic AB at 250 nm. Phosphate-free samples of Prefolic AB were prepared by replacing 0.1M disodium hydrogen orthophosphate solution with 10^{-4}M copper sulphate solution pH 5.6. Calculated for $C_{20}\ H_{21}\ N_7\ O_6$ Ba. $4H_2O$, C 35.18, H 4.50, N 14.32 found C 35.32, H 4.26, N 13.94%. A detailed look at the products of the oxidation is described later, in which it was found that 5-methyl-5,6-DHF accounted for almost 90% of products when oxidised in 10^{-4}M $CuSO_4$ for 1.5 hrs.

Viscontini[113] has reported that 6,7-diphenyl-5-methyl-5,6-dihydropterin rearranges at low pH to 6,7-diphenyl-5-methyl-5,8-dihydropterin. T.l.c. and microbiological assay were performed on acidified samples of prefolic AB, to see if there was any evidence for a similar rearrangement in this case. The results are shown in Tables XXIV and XXV.

SPECTRUM 10.

5-Methyl-5,6-DHF after lyophilisation prepared by method b in Method Section.

U.v. solvent: 0.1M phosphate buffer pH 7.



T.l.c. data for Prefolic AB. TABLE XXIV.

T.l.c. Solvent	Method of Prepn.	R_f	Inference
(i)	a	0.97	Prefolic AB?
"	"	0.85	5-methyl THF (std.)
"	"	0.55	Folic acid (std.)
(ii)	"	0.40	Prefolic AB?
"	"	0.45	5-Methyl THF (std)
"	"	0.00	Folic acid (std)
(iii)	"	0.22	Prefolic AB?
"	"	0.47	5-methyl THF (std)
"	"	0.00	Folic acid (std)
(i)	b	0.92	Prefolic AB
"	"	0.85	5-methyl THF (std)
"	"	0.95	Prefolic AB (acidified)
(ii)	"	0.30	Prefolic AB
"	"	0.45	5-methyl THF (std)
"	"	0.67	Prefolic AB (acidified)
(iii)	"	0.30	Prefolic AB
"	"	0.41	5-methyl THF (std)
"	"	0.35	Prefolic AB (acidified)

TABLE XXV.

Microbiological Assay Results .

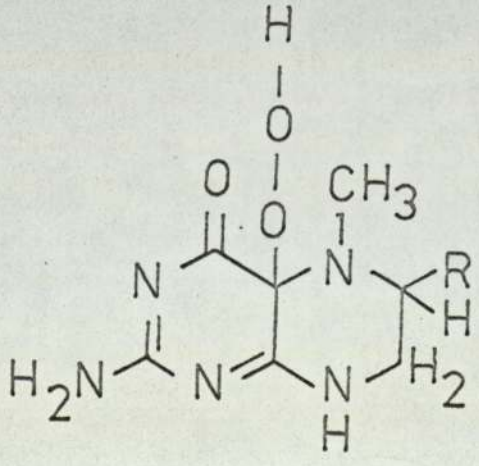
Response of L. casei.

Sample	Prep.	Assay Method	Activity
5-methyl THF		Autoclave + ascorbate	100%
Prefolic AB	a	" " "	4%
Prefolic AB	b	" " "	94%
Prefolic AB	b	" - ascorbate	5%
Prefolic AB	b	Aseptic - ascorbate	5%
Prefolic AB (acidified)	b	Autoclave + ascorbate	3%

The lack of biological activity of prefolic AB after acidification is the most striking evidence in favour of rearrangement having occurred. (As samples are diluted thousands of times with phosphate buffer before being assayed, the small amount of acid present in the initial sample would not alter the final pH of the medium and hence would not account for the lack of activity). Further evidence for this rearrangement and its importance is discussed shortly.

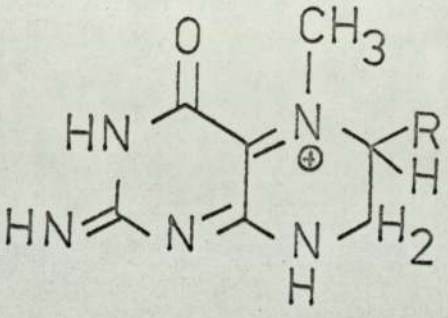
Discussion.

Although the evidence in favour of a 5,6-dihydro structure appears substantial, a 4a-hydroperoxide structure (XXVII),

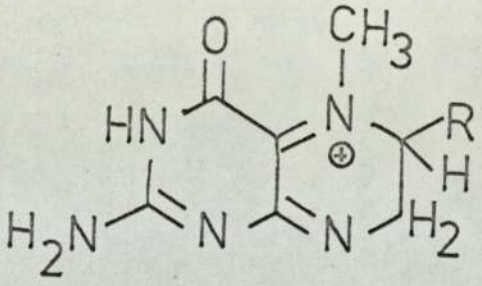


(XXVII)

or a charged quinoid structure[80] (XXVIII) or (XXIX)



(XXVIII)



(XXIX)

would explain the retention of the proton at C_6 and the ease of reduction of the intermediate. This ease of reduction of 5-methyl-5,6-DHF, coupled with its acid catalysed rearrangement, can throw some light on the problem. Being easily reduced, 5-methyl-5,6-DHF is a fairly powerful oxidising agent and will react positively with two of the common peroxide test reagents, ferrous ammonium thiocyanate solution, and NN dimethyl-p-phenylenediamine hydrochloride (the former turns from orange to blood red, the latter from pale pink to cherry red.) Below pH 4.5, this positive

reaction is lost. Hence it would seem that the acid catalysed rearrangement product does not give a positive reaction. A charged quinonoid structure would not be expected to rearrange in acid and so the positive result would still be expected at low pH. On acidification, a 4a - hydroperoxide structure might be expected to eliminate hydrogen peroxide, which would still give a positive reaction or alternatively, but less likely, rearrange to give 8-dehydro-4a-hydroxy-5-methyl THF (XVI) (see Part C of this Chapter). Comparison of n.m.r. spectra (p. 108) of the two species shows that this has not happened.

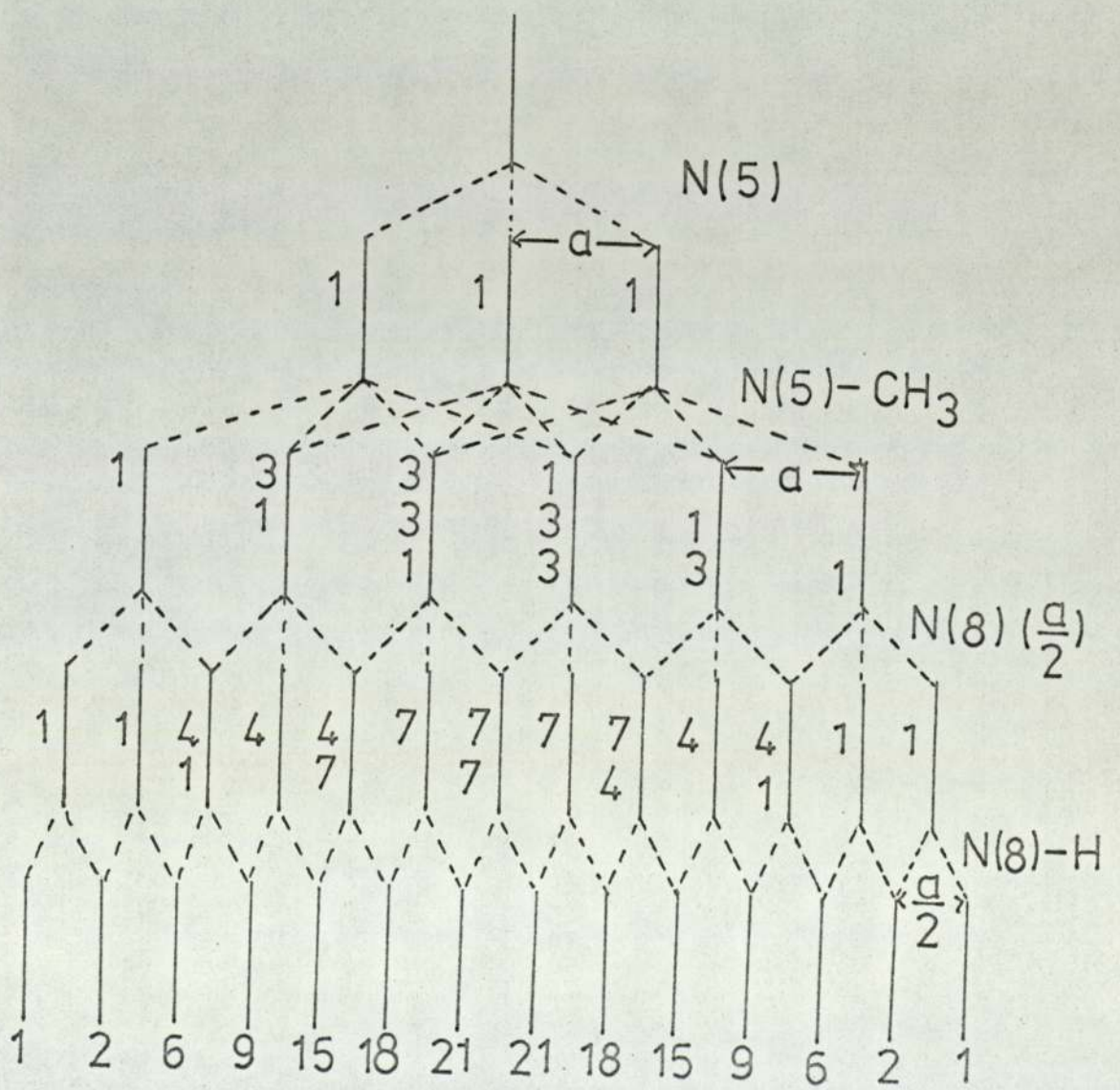
The n.m.r. spectrum for the acid catalysed rearrangement product of prefolic AB in TFA shows, τ 7.6 (2H,m), τ 7.2 (2H,d), τ 6.5 ($\frac{1}{2}$H,S), τ 5.8 (3H,S), τ 5.1 (1H,S), τ 5.0 (2H,b.m), τ 2.2 (2H,d,J8Hz) τ 2.0 (2H,d,J8Hz). The spectrum for 8-dehydro-4a-hydroxy-5-methyl THF shows, τ 7.5 (2H,m), τ 7.26 (2H,d) τ 6.6 (3H,S) τ 6.0 (2H,d), τ 5.8 (1H,b), τ 5.6 (1H,d,Jgem 14Hz), τ 5.2 (1H,d,Jgem 14 Hz), τ 5.0 (1H,b), τ 3.0 (2H, d,J8Hz) τ 2.1 (2H,d,J8Hz).

These species are obviously non-identical. The only reasonable explanation of the observations is that of a 5,6-dihydro structure rearranging in acid to a 5,8-dihydro structure. This accords well with Viscontini's work[113].

M.O. theory[114] predicts that the 5,8-dihydro structure is easily oxidised but difficult to reduce, whereas the reverse is true for the 5,6-dihydro structure. Reconsideration of the microbiological assay data presented

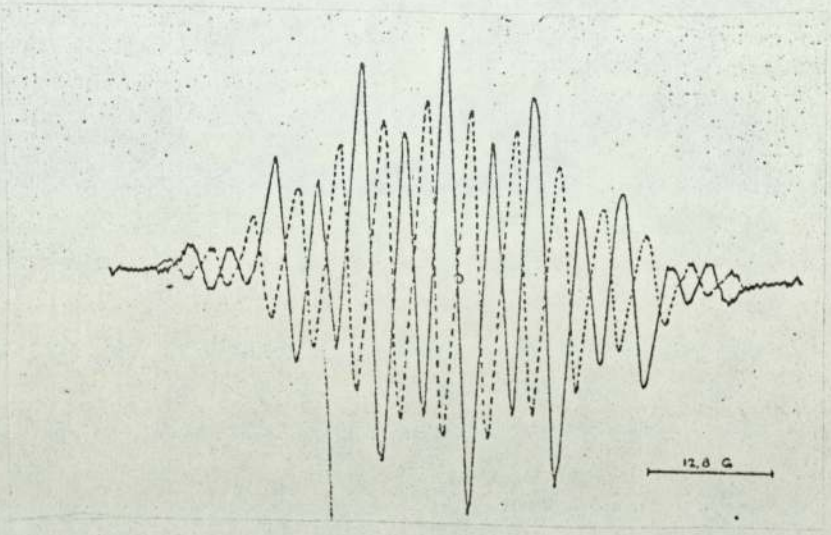
on page 100 shows that ascorbate is capable of reducing prefolic AB back to the tetrahydro level, but is incapable of reducing the acidified sample of prefolic AB. This indicates that prefolic AB is a 5,6-dihydro structure, whereas the acid catalysed decomposition product is a 5,8-dihydro structure.

Ehrenberg et al[115] have found that 5-methyl-6,7-diphenyl-5,6-dihydropterin is oxidised by hydrogen peroxide in trifluoroacetic acid, forming a 5,8-dihydro pterin radical stable enough for detection and structural elucidation by electron spin resonance hyperfine analysis. The splitting pattern obtained for the 5,8-dihydro structure is shown below:



This gives rise to the spectrum shown on the following page. The spectrum obtained in T.F.A. for the free radical obtained from the oxidation product of 5-methyl THF is given on page 107. This spectrum is of poor quality but does give some useful information on the product of oxidation. The large signal in the middle of the spectrum is due to solid radical[116]. Although it is extremely difficult to say exactly how many lines are present in this spectrum, it is safe to say that there are more than 7, which is the number of lines present in the spectrum of the tetrahydro starting material[115](p.107). The line intensities for the left hand side of the observed spectrum are given in Table (XXVI) (p.113), together with those reported for the 5,8-dihydro structure[115]. Further evidence for the 5,8-dihydro structure being the acid catalysed rearrangement product of prefolic AB is obtained from its n.m.r. spectrum, which shows the N(5)- CH_3 singlet pulled down field from τ 6.7 in the tetrahydro case to τ 5.8 for the product. The C(9) H_2 signal is also pulled downfield from τ 5.9 to τ 5.0, and the C (7) H_2 signal has disappeared from this area of the spectrum and reappeared superimposed on the aromatic proton signals at τ 2.1 (5H). The spectrum also shows a small singlet at τ 6.7, probably due to unoxidised 5-methyl THF.

Viscontini also observed that the 5-methyl-5,8-dihydro structure demethylates much more rapidly than the 5-methyl tetrahydro structure yielding a 7,8-dihydro product. Twenty-three hours after recording the n.m.r. spectrum (No. 14) a second spectrum (No. 16) was recorded on the same sample.

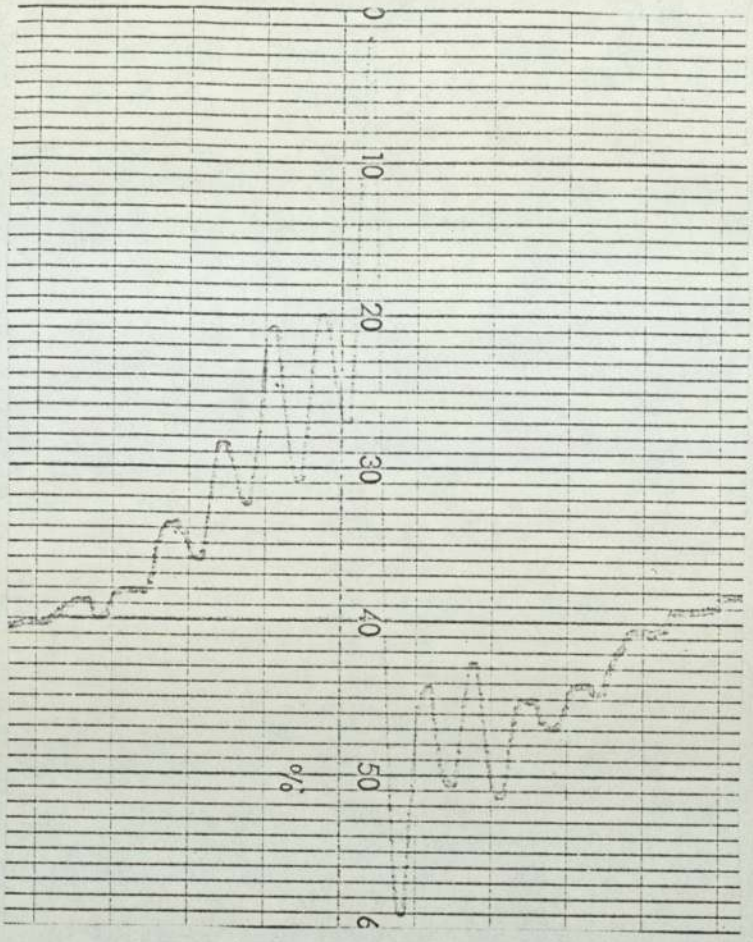


SPECTRUM 11

E.s.r. spectrum obtained by Ehrenberg et al.[115] for 5-methyl-6,7-diphenyl-5,8-dihydropterin radical.

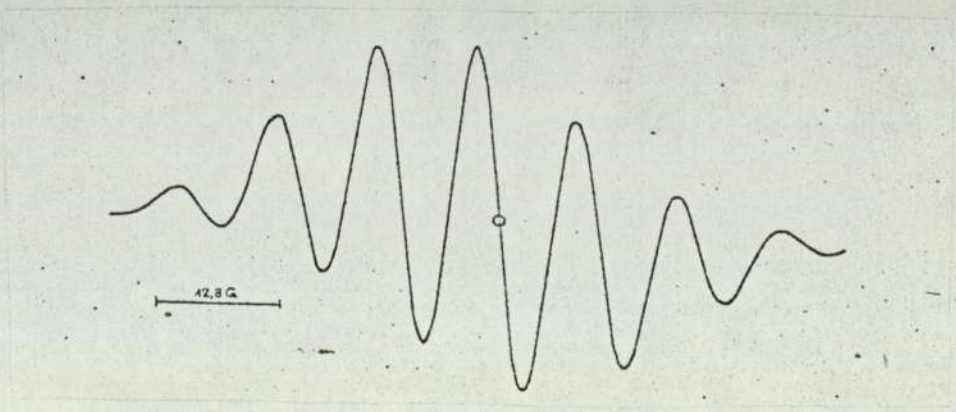
Solvents: - - - - - - - - - - - - - CF_3 COOH

——————————— CF_3 COOD



SPECTRUM 12

E.s.r. spectrum of 5-methyl-5,8-DHF radical in CF_3 COOH.

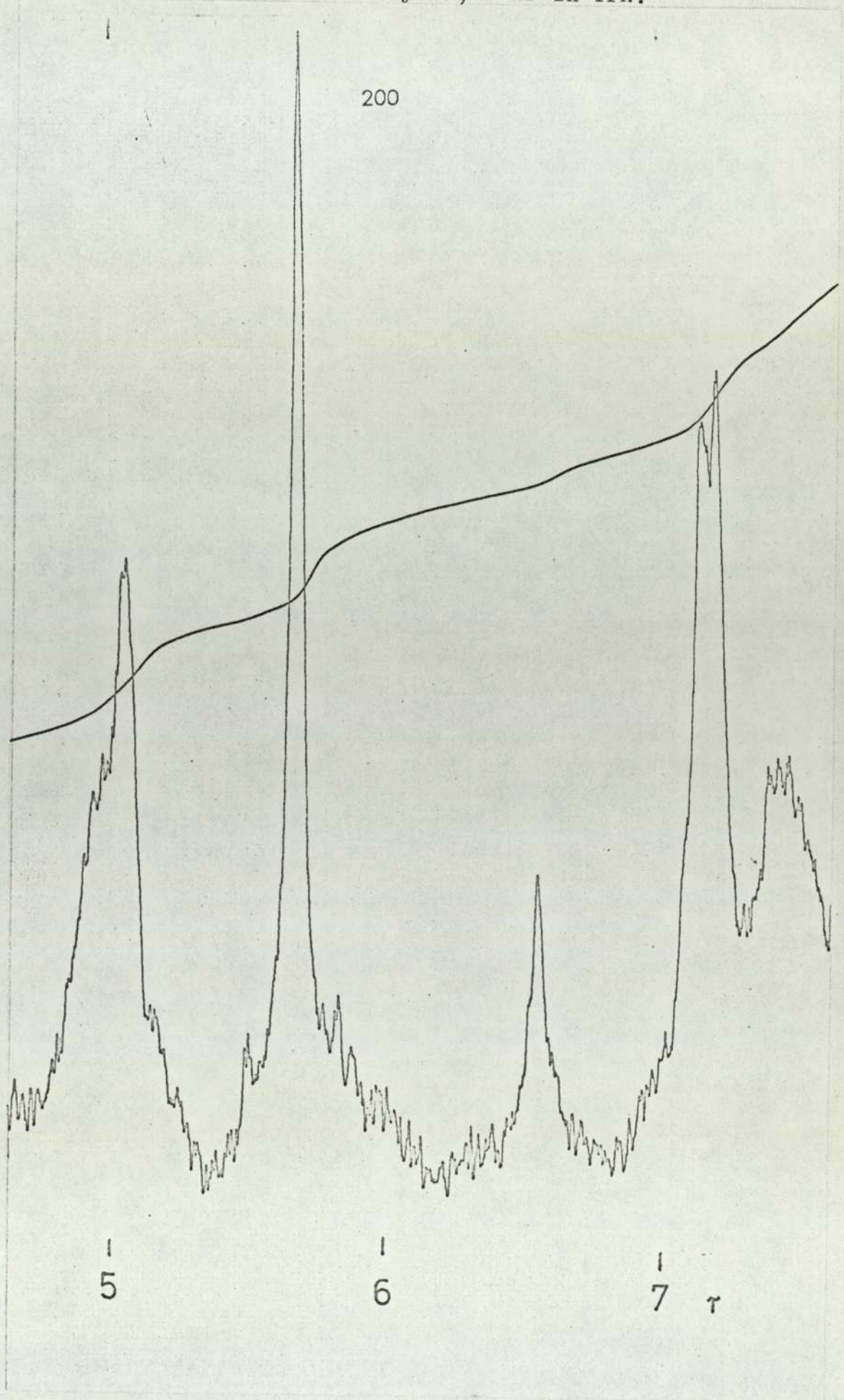


SPECTRUM 13

E.s.r. spectrum of 5-methyl-6,7-diphenyl-5,6,7,8-tetrahydropterin radical in CF_3 COOH.[115]

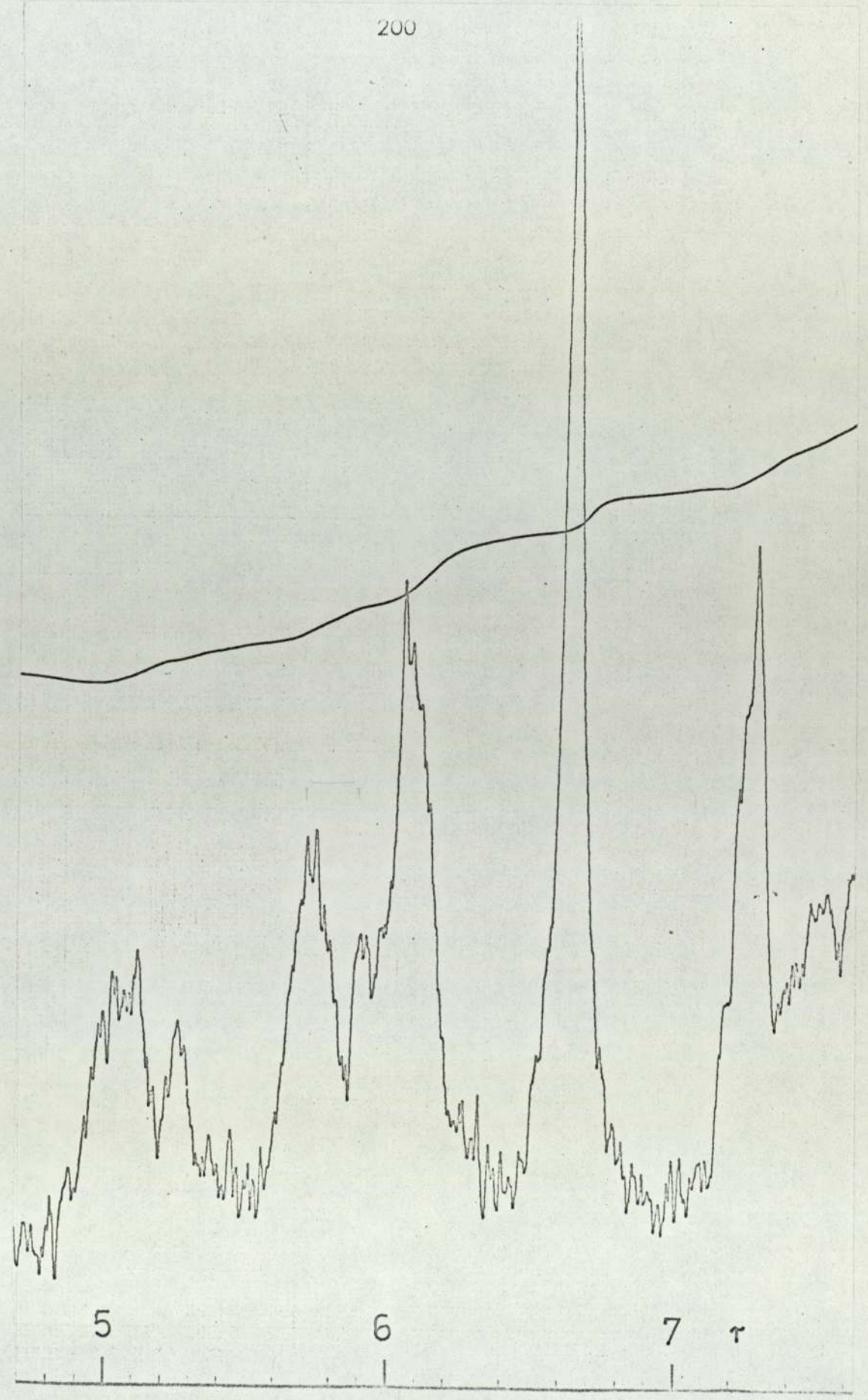
SPECTRUM 14

^{1}H n.m.r. spectrum of 5-methyl-5,8-DHF in TFA.



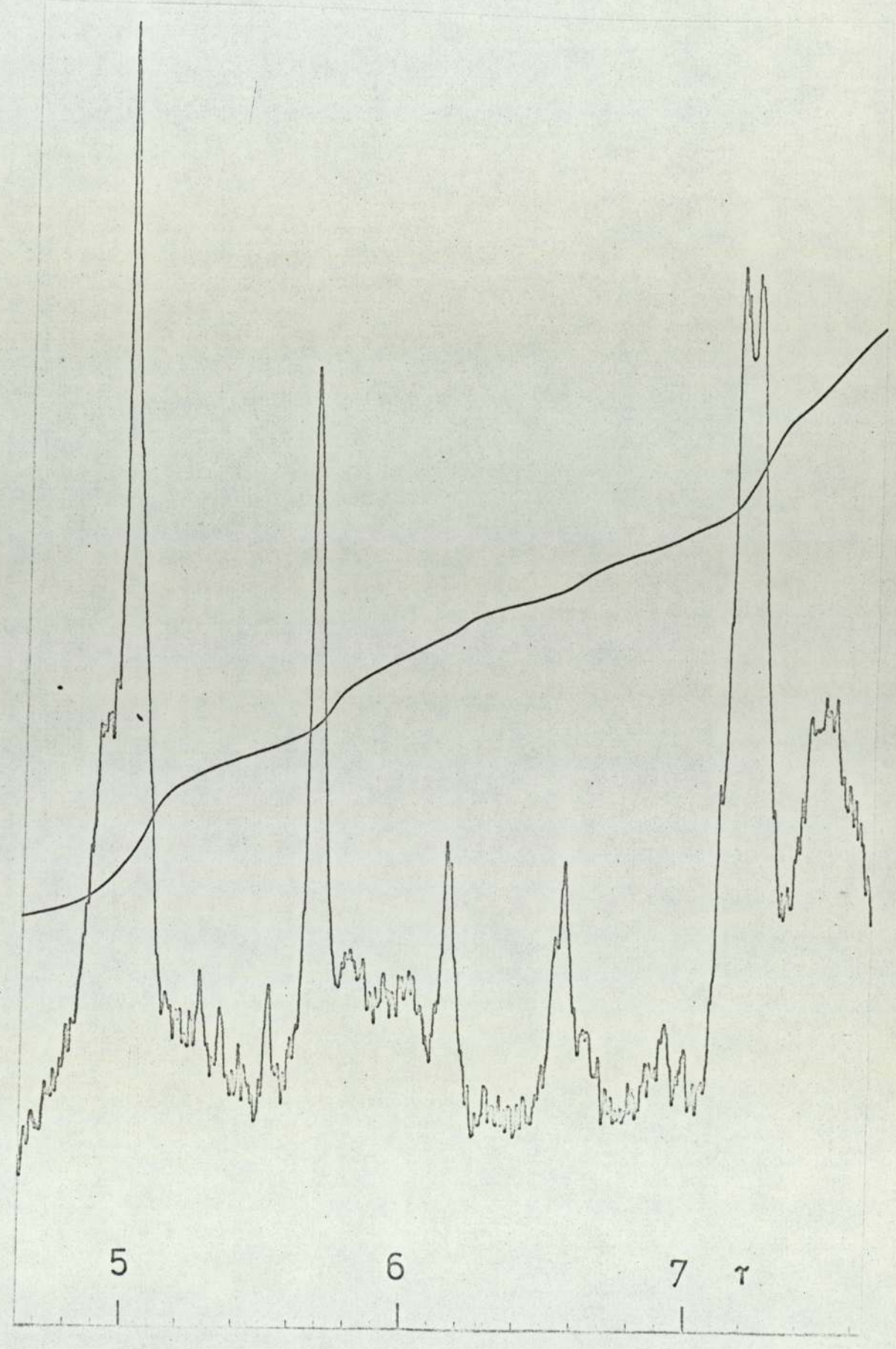
SPECTRUM 15

^{1}H n.m.r. spectrum of 4a-hydroxy-5-methyl THF in TFA.



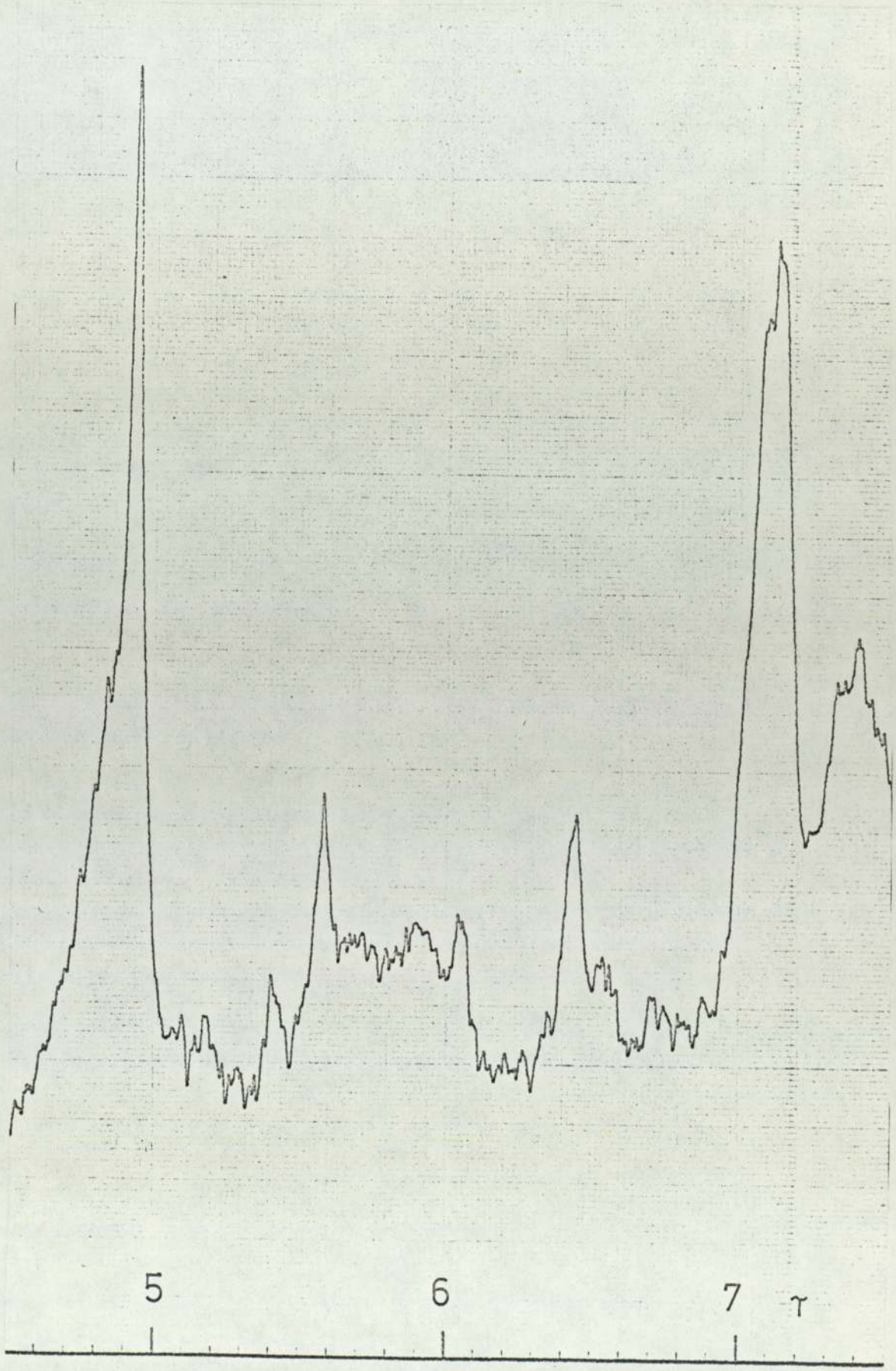
SPECTRUM 16

^1H n.m.r. spectrum of 5-methyl-5,8-DHF after 23 hrs. in TFA.



SPECTRUM 17

^{1}H n.m.r. spectrum of 5-methyl-5,8-DHF after 5 days in TFA.



It can be seen that the N (5) CH_3 peak is markedly diminished(τ 5.8 (1H)). A third spectrum (No. 17) was recorded after 5 days. The N (5) CH_3 signal here is still further diminished and the strongest peaks remaining in this portion of the spectrum can be attributed to the glutamate protons τ 7.5 (2H), τ 7.25 (2H), C-9 H_2 and α C-H τ 5.1 (3H). These observations are similar to those of Viscontini. Further evidence for demethylation is obtained from the e.s.r. spectrum recorded one week after recording that for the freshly made sample. As can be seen (Spectrum No. 18), the number of lines has decreased to 5, which cannot be explained either by a tetrahydro structure, (7 lines), 5,8-dihydro structure (14 lines) or 5,6-dihydro structure (7 lines at least). This spectral pattern would be expected for a 7,8-dihydro structure IF the coupling coefficients for N(5) and N(8) were equal and if no other coupling occurred. The splitting pattern is given below.

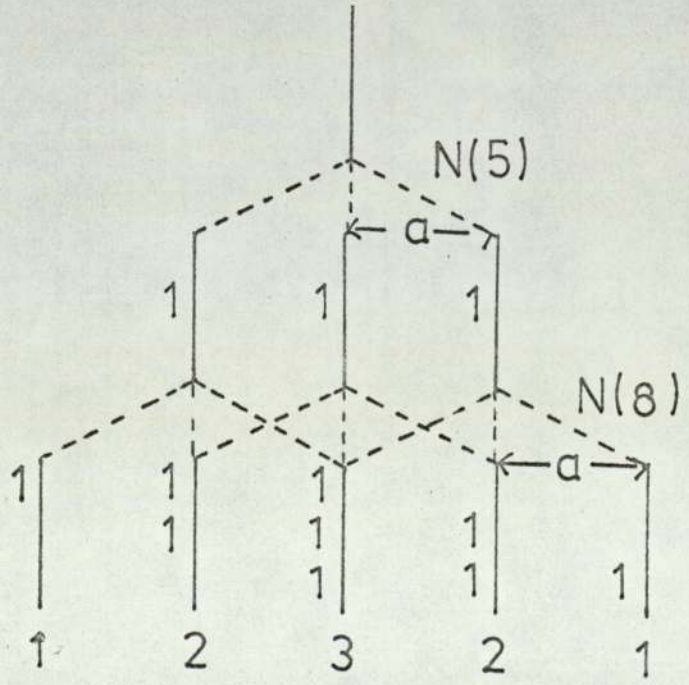


TABLE XXVI

Results of E.S.R. studies.

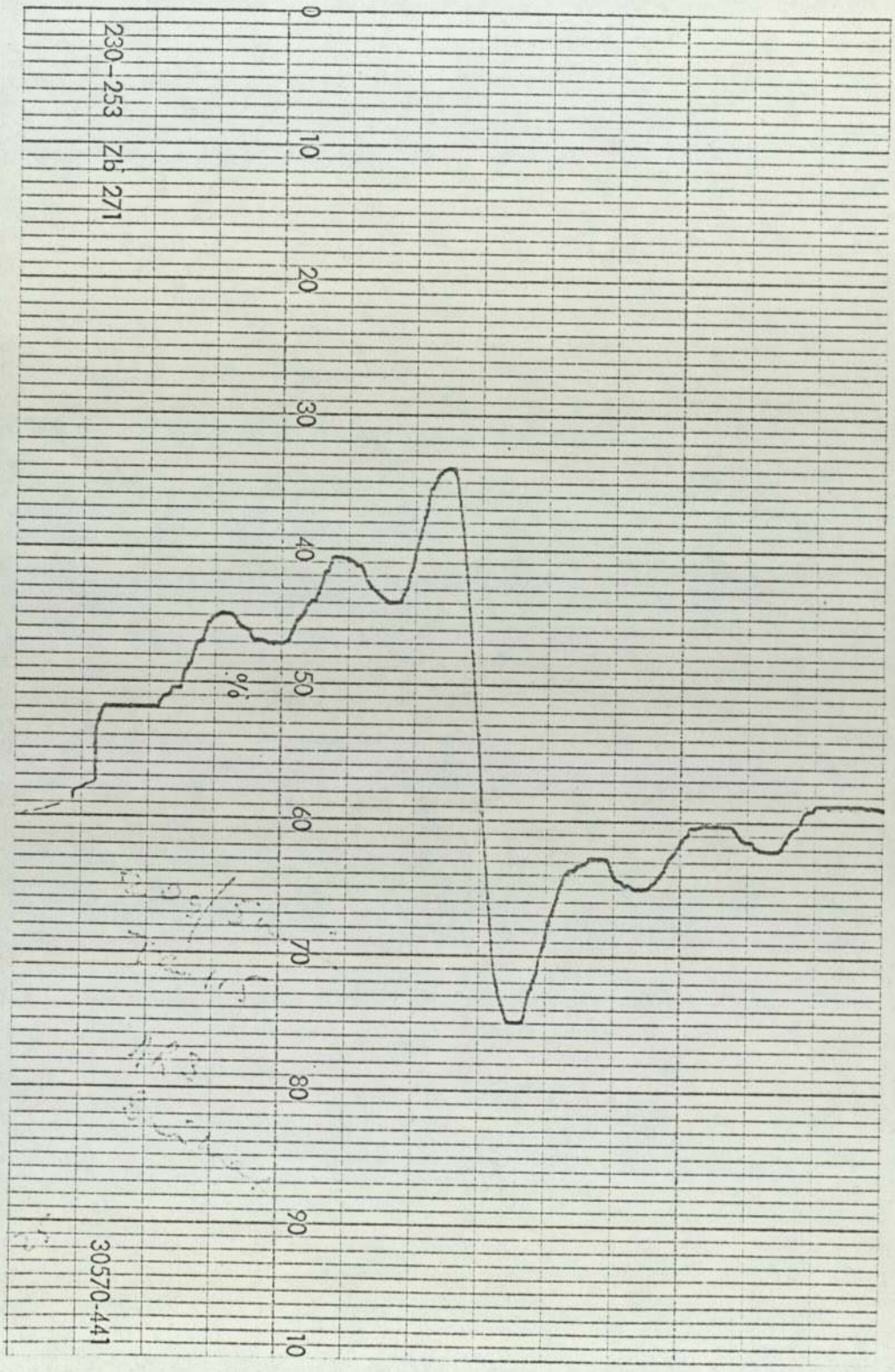
Line intensities for 5-methyl-5,8-DHF

Reported by Ehrenberg et al[115] compared with those observed in this study.

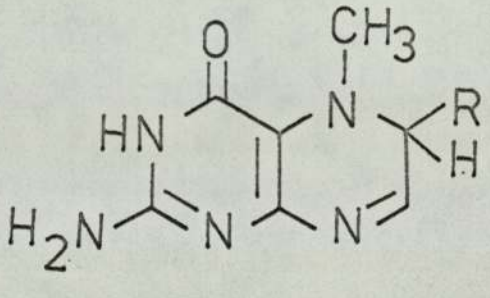
	Relative line intensity
Literature values	1, 2, 6, 9, 15, 18, 21
Observed values	1, 2, 6, 12, 19, 20, 38

SPECTRUM 18

E.s.r. spectrum of 5-methyl-5,8-DHF after 7 days in TFA.



The expected line intensities are 1,2,3; those observed are 1,2,5.3 (again solid radical is present). Unfortunately there is no definite evidence for the given spectrum being that of 7,8-DHF; however the preceding evidence does indicate that a similar situation to that reported by Viscontini[115] has been observed and that the structure of prefolic AB is 5-methyl-5,6-DHF (XIV).

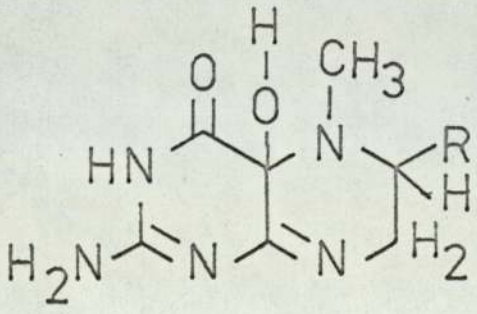


(XIV)

PART C. Investigation of the Formation of 8-Dehydro-4a-hydroxy-5-methyl THF.

Introduction.

A second oxidation product of 5-methyl THF was reported by Gupta and Huennekens[65]. Treatment of 5-methyl THF with H_2O_2 at pH 8 gave 80% 5-methyl-5,6-DHF and 20% of the second material in the products. On lowering the reaction pH to 6 and prolonging the reaction time, they increased the yield of the second product at the expense of 5-methyl-5,6-DHF[111]. Using the same conditions, 5-methyl-5,6-DHF can be converted to this product, suggesting the course of the overall reaction to be 5-methyl THF ⟶ 5-methyl-5,6-DHF ⟶ second oxidation product. Using tritium and ^{14}C labelling at C (6) and N (5)-CH_3 respectively, they showed that the (5) methyl group and the proton at C_6 were retained in the product and by using $H_2{}^{18}O_2$ demonstrated the incorporation of an extra atom of oxygen. N.m.r., u.v., and elemental analysis finally suggested the structure



(XVI)

8-dehydro-4a-hydroxy-5 methyl THF.

This product is interesting because although produced from 5-methyl-5,6-DHF and H_2O_2 by what is ostensibly an oxidation reaction, the oxidation state of the final product remains unchanged.

The remainder of this chapter investigates the formation of 8-dehydro-4a-hydroxy-5-methyl THF.

METHODS.

A. Preparation of 8-Dehydro-4a-hydroxy-5-methyl THF.

This was prepared by a modification of the method described by Gapski et al[111].

5-Me THF (500 mg) were dissolved in a mixture of 0.2M ammonium acetate (20 ml) and 20 vol. H_2O_2 (20 ml). It was not necessary to adjust the pH to pH 6. After stirring for 1 hr. the solution was lyophilised and chromatographed on a 2.5 x 32 cm. column of A 25 DEAE Sephadex. Elution was initiated with 200 ml. of distilled water followed by 0.1M ammonium acetate. The first 2 litres of eluent were discarded. Fractions of 15 ml. were then collected. A full u.v. spectrum was recorded on every fifth tube and those tubes with an absorption maximum at 280 nm (tubes 45 - 110) were pooled and lyophilised.

The nmr spectrum of the product showed:
τ 7.5 (2H,m), τ 7.26 (2H,d), τ 6.6 (3H,s), τ 6.0 (2H,d) τ 5.8 (1H,b), τ 5.6 (1H,d, Jgem 14Hz), τ 5.2 (1H,d,Jgem 14 Hz), τ 5.0 (1H,b), τ 3.0 (2H,d, J8Hz), τ 2.1 (2H,d,J8Hz). Reported by Gapski et al.,τ7.5 (2H,m), τ 7.26 (2H,d) τ 6.63 (3H,s), τ 6.05 (2H,d), τ 5.9 (1H,m), τ 5.7 (1H,d) τ 5.2 (1H,d), τ 5.06 (1H,b), τ 3.04 (2H,d), τ 2.2 (2H,d).

B. Oxidation of 5-Methyl THF.

(i) 5-Methyl THF (Ba Salt) (40 mg.) was stirred in distilled water (50 ml.) under oxygen for 15 hours. The residue after lyophilisation was dissolved in 0.1M ammonium acetate, pH 7 (1.5 ml.) and placed on a DEAE Sephadex Column (10 x 80 mm). Elution was carried out with 0.1M Ammonium acetate pH 7. The first 120 ml. of eluate were discarded; 15 ml. fractions were then collected automatically. The u.v. spectrum of each fraction was recorded.

(ii) As above, with the modification that the oxidation step was carried out in 10^{-4}M Cu SO_4 for 1 hour.

(iii) 5-Methyl THF was oxidised in 10^{-4}M Cu SO_4 under O_2 for 40 minutes, then the reaction vessel was thoroughly flushed with nitrogen for 15 minutes and the reaction mixture incubated under nitrogen for 14 hours.

(iv) 5-Methyl THF was oxidised in distilled water (50 ml.) containing catalase* (1,200 Keil units/g.:2.5 mg) under oxygen for 15 hours.

---

* This enzyme avidly destroys H_2O_2 according to the equation:

$$2\ H_2O_2 \longrightarrow O_2 + 2H_2O$$

The strength of the enzyme is measured in terms of Keil units where one unit is the amount of catalase necessary to decompose one gramme of 30% hydrogen peroxide in ten minutes at 25°C.

RESULTS AND DISCUSSION.

The elution profiles for the four methods of oxidation are given, together with those for the starting material and the preparation of 8-dehydro-4a-hydroxy-5-methyl THF. Five compounds are present in the starting material. Four of them are minor; peaks (a) (b) correspond to the fluorescent impurities described earlier in Chapter II, peak (c) corresponds to 8-dehydro-4a-hydroxy-5-methyl THF and (d) to 5 methyl-5,6-DHF. Both of the latter compounds are produced by partial oxidation of 5-methyl THF. As no antioxidants were used in the final stages of preparation of this sample, they are to be expected. The elution profile for the preparation of 8-dehydro-4a-hydroxy-5-methyl THF shows that little 5-methyl-5,6-DHF is produced under the conditions of the preparation. 5-Methyl-5,6-DHF and 8-dehydro-4a-hydroxy-5-methyl THF accounted for 99% of the starting material judged by areas under the elution peaks (using Σ_{280}mol = 17.8×10^3 for 8-dehydro-4a-hydroxy-5-methyl THF, Σ_{290}mol = 30.8×10^3 for 5-methyl-5,6 DHF). The ratio of these products varied according to the reaction time. Longer reaction times ($\simeq$ 14 hrs) favoured 8-dehydro-4a-hydroxy-5-methyl THF production (see Table XXVII). This agrees with the observation of Gapski et al.[111]

DIAGRAM IV.

Elution profile of 5-methyl THF.

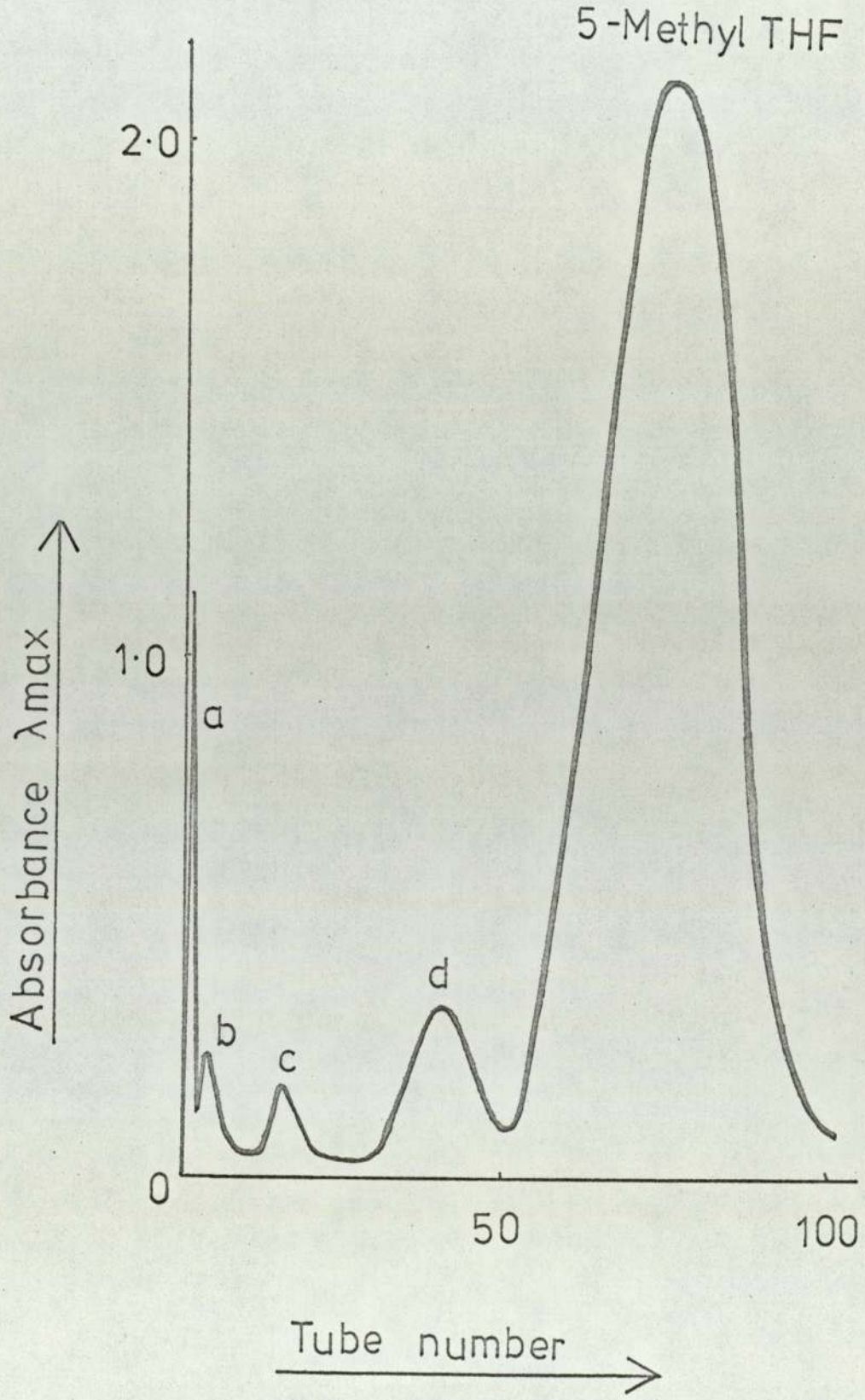


DIAGRAM V.

Elution profile for the preparation of 8-dehydro-4a-hydroxy-5-methyl THF.

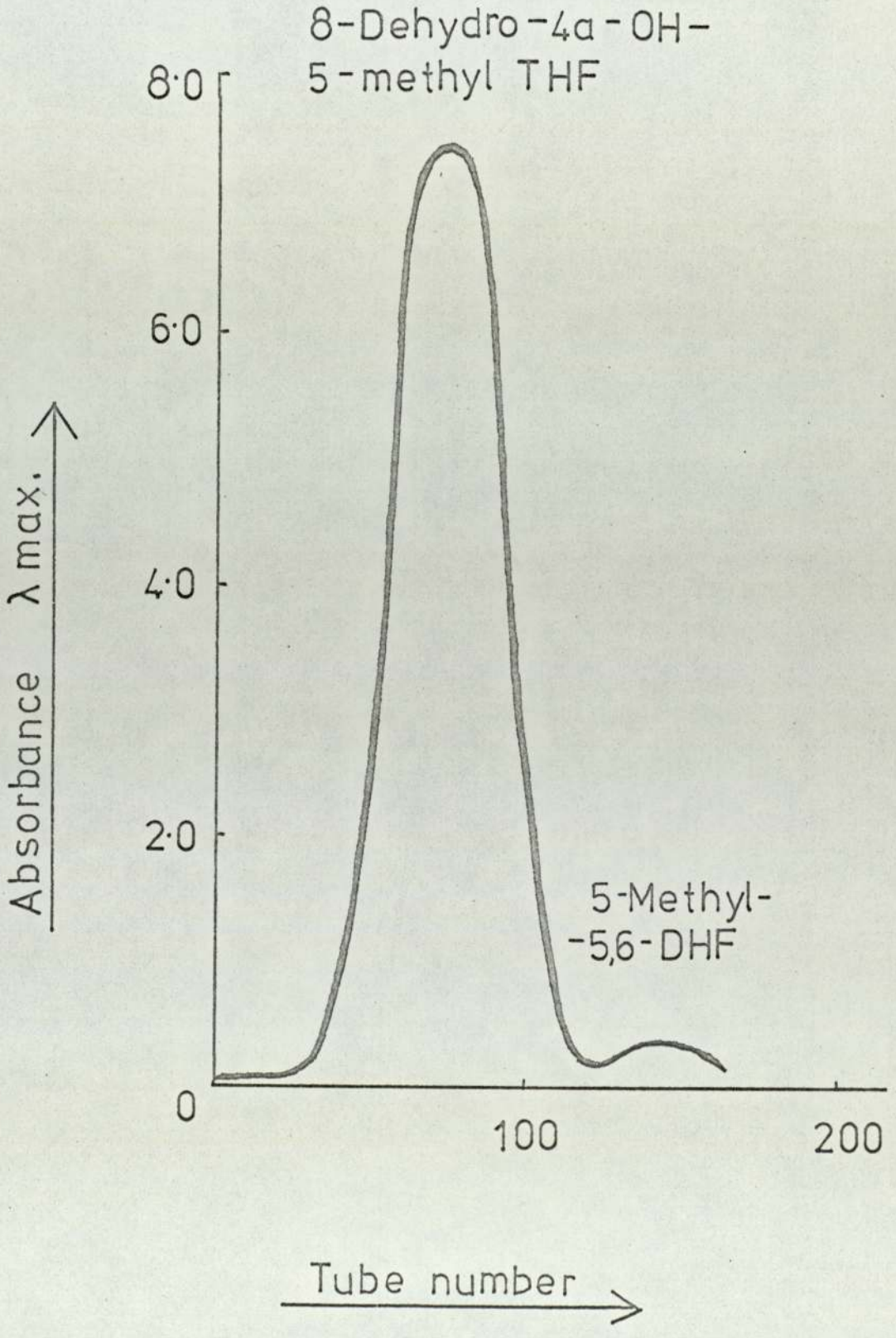


DIAGRAM VI.

Elution profiles for oxidation products of 5-methyl THF.

Oxidation methods (ii) and (iii)

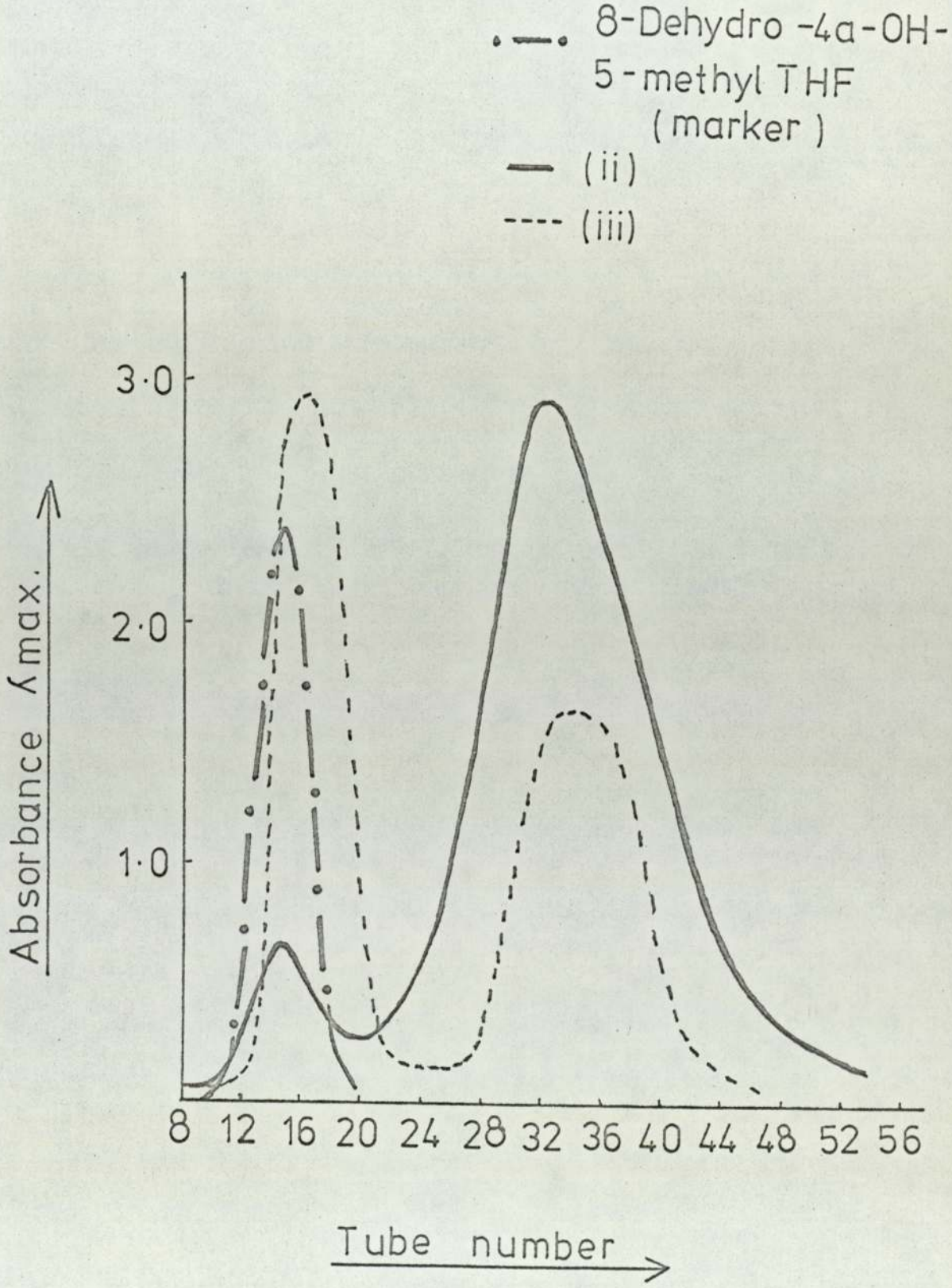


DIAGRAM VII.

Elution profiles for oxidation products of 5-methyl THF. Oxidation methods (i) and (iv).

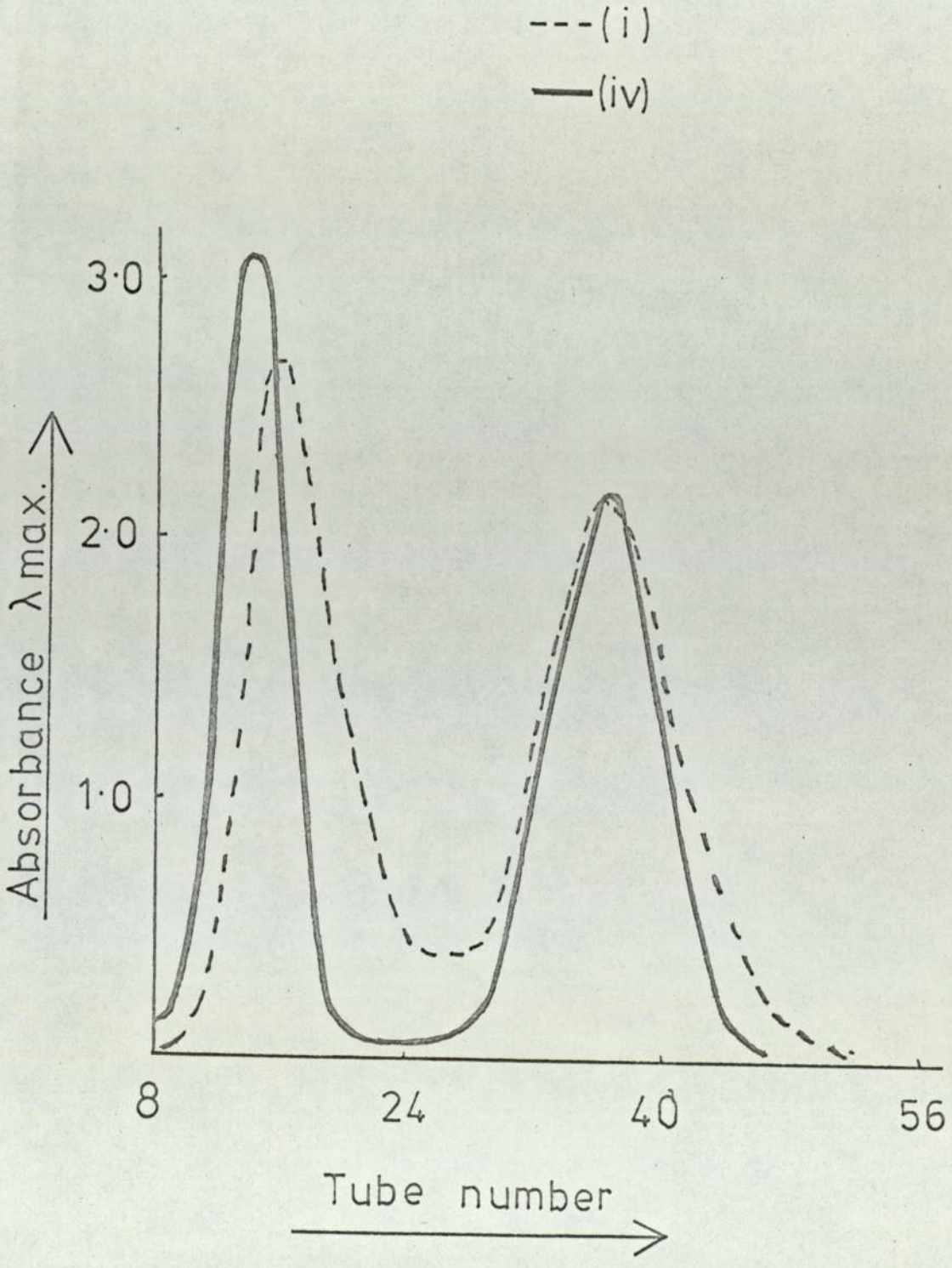
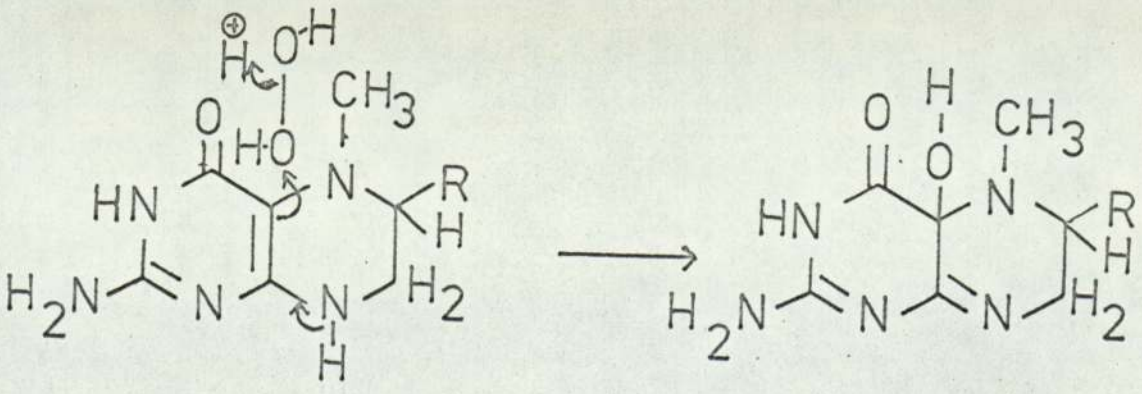


TABLE XXVII.

Product Analysis.

Method of oxidation	% 8-dehydro-4a-hydroxy-5-methyl THF.	% 5-methyl-5,6-DHF
i	59.9	40.1
ii	13.8	84.2
iii	47.3	52.7
iv	54.0	46.0

Huennekens has demonstrated by $H_2{}^{18}O_2$ studies that the reaction between 5-methyl THF and H_2O_2 must be concerted - if the alcohol were produced by combination of hydroxyl and alkyl radicals, the label would be 'scrambled' and very little incorporation would be observed.[111] The reaction pathway can therefore be described as follows:



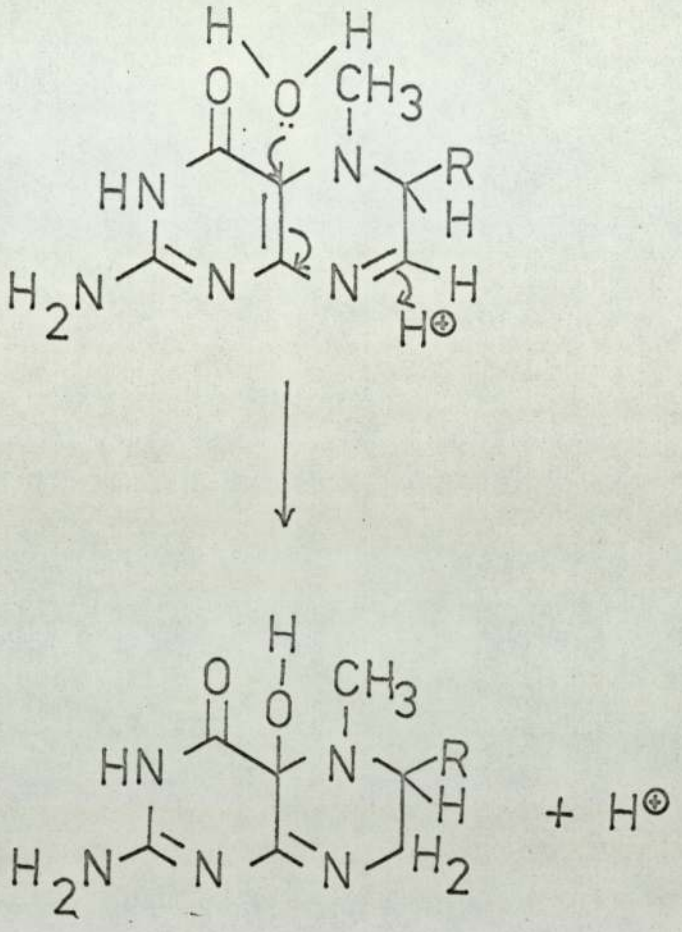
Evidence for this is given by the observation that at lower pHs (< pH 7) the yield of alcohol increases.* Although no $H_2{}^{18}O_2$ studies were performed with 5-methyl-5, 6-DHF, it has been reported that the 4a-alcohol is similarly produced.[111] The results in the above table show that catalase does not markedly affect the amount of 8-dehydro-4a-hydroxy-5-methyl THF produced, moreover if a solution of 5-methyl-5,6-DHF is incubated under N_2 for 14 hours, a large amount of 8-dehydro-4a-hydroxy-5-methyl THF is produced. The electron density at 4a calculated for 5-methyl-5,6-DHF was 1.02: this very slight excess electron density would not be expected to favour electrophilic attack.

A feasible explanation for the formation of 8-dehydro-4a-hydroxy-5-methyl THF from 5-methyl-5,6-DHF is simply the addition of water to 5-methyl-5,6-DHF, as in the scheme shown overleaf (Scheme IV).

---

* Electrophilic attack by H_2O_2 at C_{4a} is also favoured on the grounds of electron density calculations. These were carried out from the MO theory data amassed by Pearson[114]. Electron density at C_{4a} was calculated to be 1.10 for 5-methyl THF.

SCHEME IV.



Again, evidence for this is supplied by the low pH used for the reaction. Gapski, Whiteley and Huennekens[111] carried out the reaction at pH 4.8 and obtained a yield of 60% after 1 hr. In the experiments described above, the yield obtained was 60% after 14 hrs. at pH 5.6. Theoretical evidence is also available. Using Pearson's data[114], the π bond order of the $N_{(8)}$- $C_{(7)}$ bond was calculated to be 1.20. This high π bond order would favour protonation at the required position. Obviously protonation is necessary to bring about saturation at C_7 and also nucleophilic attack at C_{4a}.

CHAPTER IV.

Kinetics of Oxidation of 5-Methyl THF.

Introduction.

Most of the work carried out on the kinetics of autoxidation of tetrahydropteridines has concentrated on the oxidised intermediate. Mager and Berends[110] have used a simple manometric technique and observed the qualitative pH dependence on rate, but an extensive quantitative study on rates was not carried out. Blair and Pearson obtained interesting results from the study of oxidation of tetrahydrobiopterin[54] and tetrahydrofolic acid[55]. The first stable product in each case has a 7,8-dihydro structure. As the major, initial, stable product in the case of 5-methyl THF is a 5,6-dihydro structure, an attempt was made to observe any differences in the rate or general oxidative behaviour of 5-methyl THF and the above two species. In view of the biological importance of 5-methyl THF, it is surprising that no quantitative data is available on its rate of oxidation.

Rates were measured by a simple manometric technique to obtain the order of reaction with respect to 5-methyl THF and oxygen. Buffer catalysis has been demonstrated for the rearrangement of the quinonoid dihydro intermediate[117], but the effect of buffer on the initial step of the oxidation has not been measured. Stocks-Wilson[118] has reported acetate and phosphate buffer catalysis in the oxidation of tetrahydrofolic acid at pH 7 and found that phosphate was a better catalyst

than acetate. This observation was studied in the case of 5-methyl THF using two acetate and two phosphate buffers of varying concentration.

The effect of pH on the rate of oxidation of THB and THF has been studied in detail by Pearson[54,55], who showed that the rate was reduced on going from pH 6 ⟶ pH 1 and enhanced on going from pH 8 ⟶ pH 12. A similar study was performed on 5-methyl THF.

The effect of photosensitizers; riboflavin, benzoquinone, specific radical scavangers; ethanol or methanol for OH· radicals, and general radical scavangers; phenol, quinol, 8-hydroxyquinoline-5-sulphonic acid were also studied manometrically. As reported elsewhere,[119] copper was found to catalyse such autoxidation reactions, so the buffer systems used in these studies were assayed for copper impurity by atomic absorption spectroscopy. The effect of removing transition metal ion impurities with EDTA was also studied.

The rates of oxidation of sodium ascorbate (a widely used antioxidant), quinol, and 5-formyl THF were also measured manometrically.

Table (XXXVI) gives the estimated half-lives of 5-methyl THF in phosphate buffer and distilled water at 25°C under various partial pressures of oxygen. From this, some idea of the biological stability of 5-methyl THF may be gained. (pO_2 in blood is approximately 104 mm. Hg.).

The factor for converting manometer reading to

moles of gas consumed was calculated from the following expression: -

$$PV = nRT \qquad \text{(i)}$$

where P = pressure
V = volume
n = number of moles of gas
R = universal gas constant
T = temperature

at constant volume and temperature we get: -

$$\Delta n = \Delta P \times \frac{V}{RT} \qquad \text{(ii)}$$

The manometer was filled with Brady's solution, density 1.033. The relationship between ΔP and change in height on the manometer is given by: -

$$\Delta P = \frac{\Delta h \times 1.033}{13.6 \times 760} \qquad \text{(iii)}$$

where ΔP = change in pressure
Δh = change in height on the manometer
13.6 = density of Hg
1.033 = " " Brady's solution
760 = mm. of Hg at normal pressure.

expression (ii) now becomes:

$$\Delta n = \frac{\Delta h \times 1.033}{13.6 \times 760} \times \frac{V}{RT} \qquad \text{(iv)}$$

V (the volume of the apparatus) was measured by completely filling the apparatus including the manometer

connection with water at room temperature. The water was then poured carefully into a measuring cylinder. The volume of reactant solution (normally 50 ml) was then subtracted from this and the resulting figure used in the above expression. This method was found to be sufficiently accurate. Subsequent measurement of rates of oxidation under identical conditions gave results which varied by up to 10%. The accuracy of volume measured in the above way would be greater than this.

Expression (iv) can be simplified to: -

$$\Delta n = \Delta h \times A$$

where A is a constant for a given set of apparatus. Values of A for the two sets of apparatus used throughout these studies were 0.5582×10^{-5} and 0.4948×10^{-5} respectively.

A diagram of the reaction vessel is given on page 134.

The enolate concentration at a given pH is simply calculated in the following way

From,

$$\text{5-Methyl THF} \rightleftharpoons \text{Enolate}^- + H^+$$

we get,

$$K = \frac{[\text{Enolate}^-][H^+]}{[\text{5-Methyl THF}]} \qquad \text{(i)}$$

where K is the equilibrium constant calculable from the pKa.

The total amount of 5-methyl THF (ionised and unionised at C(4)) is given by

$$[\text{5-Methyl THF}]_{\text{Total}} = [\text{5-Methyl THF}]_{\text{Unionised (at C(4))}} + [\text{Enolate}^-]_{\text{(at C(4))}} \qquad \text{(ii)}$$

The concentration of unionised species at any given time being given by,

$$[\text{5-Methyl THF}]_{\text{unionised (at C(4))}} = \frac{[H^+][\text{Enolate}^-]}{K} \qquad \text{(iii)}$$

substituting (iii) in (ii)

$$[\text{5 - Methyl THF}]_{\text{Total}} = [\text{Enolate}^-] + \frac{[\text{Enolate}^-][H^+]}{K}$$

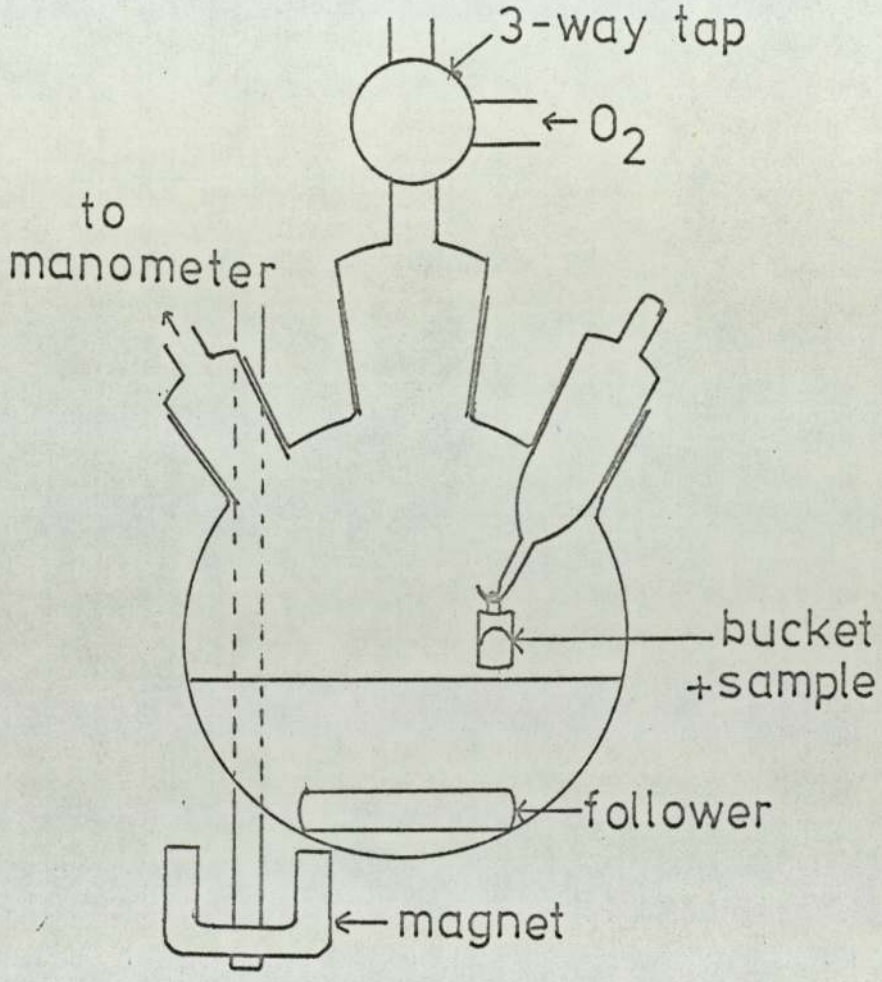
$$[\text{5 - Methyl THF}]_{\text{Total}} = [\text{Enolate}^-]\ 1 + \frac{[H^+]}{K}$$

$$\therefore\ [\text{Enolate}^-] = \frac{[\text{5-Methyl THF}]_{\text{total}}}{1 + \frac{[H^+]}{K}}$$

$[\text{5 - Methyl THF}]_{\text{total}}$ is the initial concentration of 5-Methyl THF used in the run - in this case, 9.3×10^{-4}M. The estimated pKa for the 3,4-amide group of 5-methyl THF was found to be 10.9 (see Chapter II), therefore Ka = 1.122×10^{-11}.

DIAGRAM VIII

Diagram of reaction vessel used in manometric kinetic studies.



Materials and Methods.

U.v. spectra were recorded on a Perkin-Elmer PE 137 or Unicam SP 700 spectrophotometer, Nuclear Magnetic resonance spectra on a Perkin-Elmer R 14 or Varian HA100D spectrometer. Thin layer chromatograms were run on cellulose powder MN300 F254 (Macherey, Nagel and Co., Duren, Germany). The following solvent systems were used:-

(i) 0.1M phosphate buffer, pH 7.0

(ii) n-Propanol/water/0.88 s/g aqueous ammonia (200:100:1 v/v)

(iii) The organic phase of 1-butanol/acetic acid/water (4:1:5 v/v).

Samples were observed as absorbing or fluorescing spots when viewed in u.v. light (λ = 254 n m).

METHODS.

Oxidations were carried out in a 100 ml. three-necked flask. The manometer connection, a three-way tap, and the sample bucket hook fitted into each neck respectively. All connections were of ground glass and were cleaned and regreased with vaseline after each run. The volume of solution was 50 ml. Equilibration with oxygen was achieved by thoroughly flushing the vessel and manometer connecting tube with oxygen for 15 minutes while vigorously stirring the solution magnetically. Still flushing the apparatus with oxygen, the weighed sample, contained in a small glass bucket, was placed in the flask and suspended above the solution by the hook. The manometer was joined to the connecting tube by a lightly greased ground glass joint. As this join was being made the three-way tap was opened to atmosphere, (this prevents oxygen pressure blowing away the manometer fluid). The tap was then immediately closed to atmosphere and oxygen. Twenty minutes were allowed for the apparatus to attain thermal equilibrium before commencing runs. To start a run, the hook was rotated, the bucket and sample entered the solution and the stop-clock was started. Manometer readings were taken at constant volume every 2 or 5 minutes depending on the speed of reaction. The reaction vessel was adjacent to a two-necked 100 ml. flask containing 50 ml. of solution. One neck was fitted with a two-way tap; the other was connected to a manometer. This registered

fluctuations in atmospheric pressure and temperature in water bath in which the vessels were immersed.

(i) Order of reaction with respect to 5-Methyl THF.

Reactions were done in 0.1M phosphate buffer pH 7 under an atmosphere of pure oxygen. The concentration of 5-Methyl THF was varied between 1.87×10^{-4}M and 9.33×10^{-4}M.

(ii) Order of reaction with respect to oxygen partial pressure.

The concentration of 5-Methyl THF used in each run was 4.37×10^{-4}M; again, the solvent was 0.1M phosphate buffer pH 7. The extremes of oxygen partial pressure were air ($pO_2 = 0.2$) and pure oxygen ($pO_2 = 1.0$). A nitrogen/oxygen mixture ($pO_2 = 0.566$) was prepared in a gas burrette. The apparatus was flushed four times with this mixture before the thermal equilibration stage.
The concentration of 5-methyl THF in the remaining studies was 9.3×10^{-4}M.

(iii) Effect of buffer on the rate of oxidation.

Rates were measured in the following buffers:

Sodium phosphate	pH 7 0.1M, 0.5M, 1M, 1.5M, 2 M.
Ammonium phosphate	pH 7 0.1M, 1M.
Ammonium acetate	pH 7 0.1M, 0.33M, 0.7M, 1.0M, 2M.
Sodium acetate	pH 7 0.1M, 1M.
Sodium hydroxide	0.1M,0.35M,0.75M,1.0M,2.0M,5.0M.

(iv) Effect of pH on the rate of oxidation.

The pH of distilled water was adjusted with dilute HCl or NaOH over the range pH 1 - 13. Concentrations of HCl used were 1M and 1×10^{-3}M. Concentrations of NaOH were, 6×10^{-4}M, 8×10^{-4}M, 2×10^{-3}M, 4×10^{-3}M, 5×10^{-3}M, 6×10^{-3}M, 8×10^{-3}M, 1×10^{-2}M, 1.5×10^{-2}M, 2×10^{-2}M, 5×10^{-2}M, 1×10^{-1}M. The pH was measured after each run. Two runs were done for each concentration of solvent.

(v) Effect of Additives.

Oxidations in distilled water were performed with each of the following metal ions, (10^{-4}M), Cu^{II}, Cr^{III}, Ag^{I} also $Fe^{III}(CN)_6$, Fe^{III} and Ag^{I} in 0.1M ammonium phosphate, pH 7 1M, ammonium acetate pH 7.2 and .1M phosphate pH 7, respectively.

The effect on the rate of oxidation of 5-methyl THF (25 mg) in .1M phos. pH 7 (50 ml.) of riboflavin, benzoquinone, phenol, 8-hydroxy quinoline-5-sulphonic acid, quinol, EDTA, (all 2×10^{-3}M), ethanol 2%, methanol 2% and potassium chloride 0.9M also ethanol 2% and EDTA (2×10^{-3}M) in 1M ammonium acetate were studied.

(vi) Effect of Light.

The reaction vessel was painted black and covered with aluminium foil. Phosphate buffer .1M pH 7 and 10^{-4}M $CuSO_4$ in water were used as solvents.

(vii) Effect of Temperature.

Runs were carried out in 0.1M phosphate buffer pH 7 (50 ml) with 25 mg of 5-methyl THF at the following temperatures, 25°C, 27°C and 34°C.

(viii) Investigation of the reation between Benzoquinone and 5-Methyl THF.

Benzoquinone (2 mg) was added to 2 ml of 5-methyl THF solution (5 mg as Ba Salt in 2 ml distilled water). The resulting solution (A) was immediately chromatographed in 2 solvents.

Results of Kinetic Studies.

A: i) Order with respect to 5-methyl THF concentration.

ii) Order with respect to oxygen concentration.

iii) Estimation of Ea.

TABLE XXVIII.

5-Methyl THF concentration (mg/50 ml.)	O_2 partial pressure	Temperature °C.	Rate x 10^7 moles O_2/min.
6.0	1.0	25	1.0
7.5	"	"	0.6 (5)
13.3	"	"	1.6
17.9	"	"	1.8
23.3	"	"	2.6
30.3	"	"	3.7
14.0	0.2	25	0.3
"	0.2	"	0.3
"	0.5 (6)	"	0.8 (5)
"	1.0	"	1.6
25.0	1.0	25	2.1
"	"	27	2.5
"	"	34	3.7

All in 0.1M phosphate buffer pH 7.

These results are represented graphically on the following three pages. As can be seen (Diagrams 1X and X), the rate of oxidation has first order dependence on 5-methyl THF concentration and O_2 partial pressure. This has also been found to be the case with THF and THB[54, 55, 119], however, a substantial difference in the rate of oxidation between THF or THB and 5-methyl THF was observed, the latter being oxidised 10 times more slowly under identical conditions.

The overall activation energy for 5-methyl THF oxidation (Diagram XI) was found to be 57 kJ/mole. As the overall activation energies for THF and THB were found to lie in the range 55-58 KJ/mole, it would appear that the retardation in the case of 5-methyl THF is caused by steric hindrance. This would be expected if the site for oxygen attack is position 4a (see Chapter VI).

As oxygen solubility is temperature dependent, the rate at 34°C was corrected according to data from Umbreit et al[120].

DIAGRAM IX

Order of reaction w.r.t. [5-methyl THF].

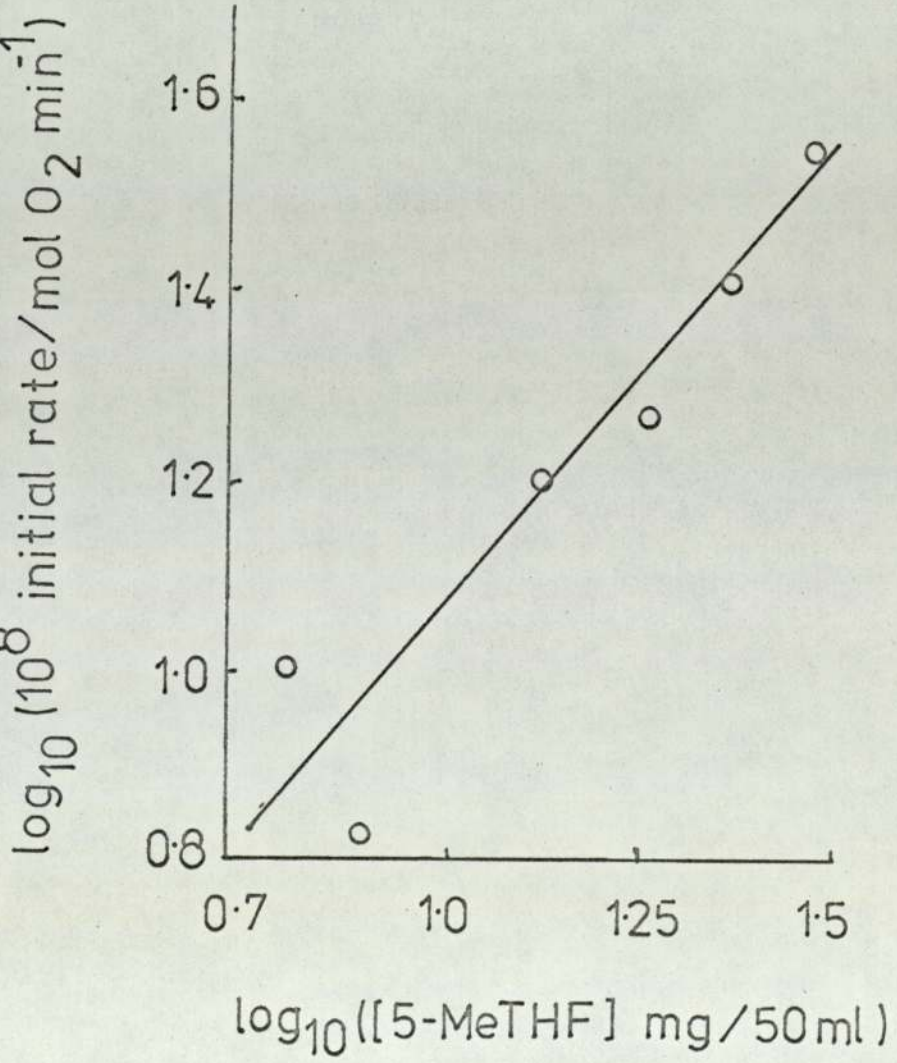


DIAGRAM X.

Order of reaction w.r.t. [O_2].

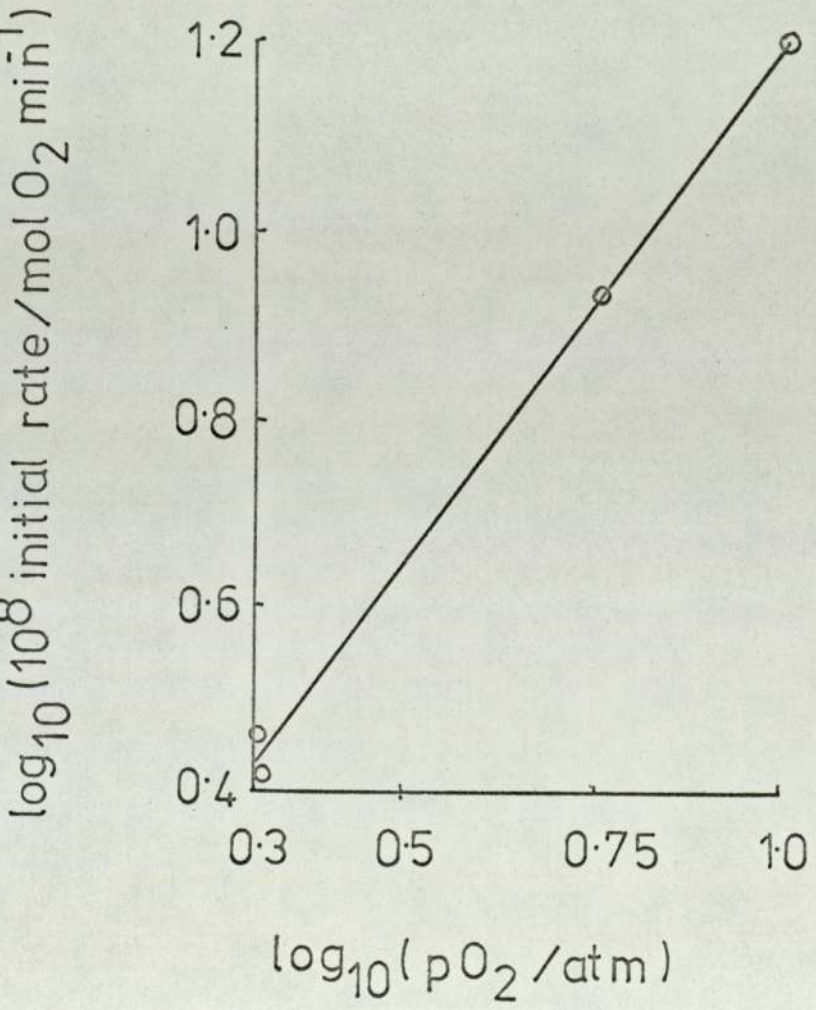
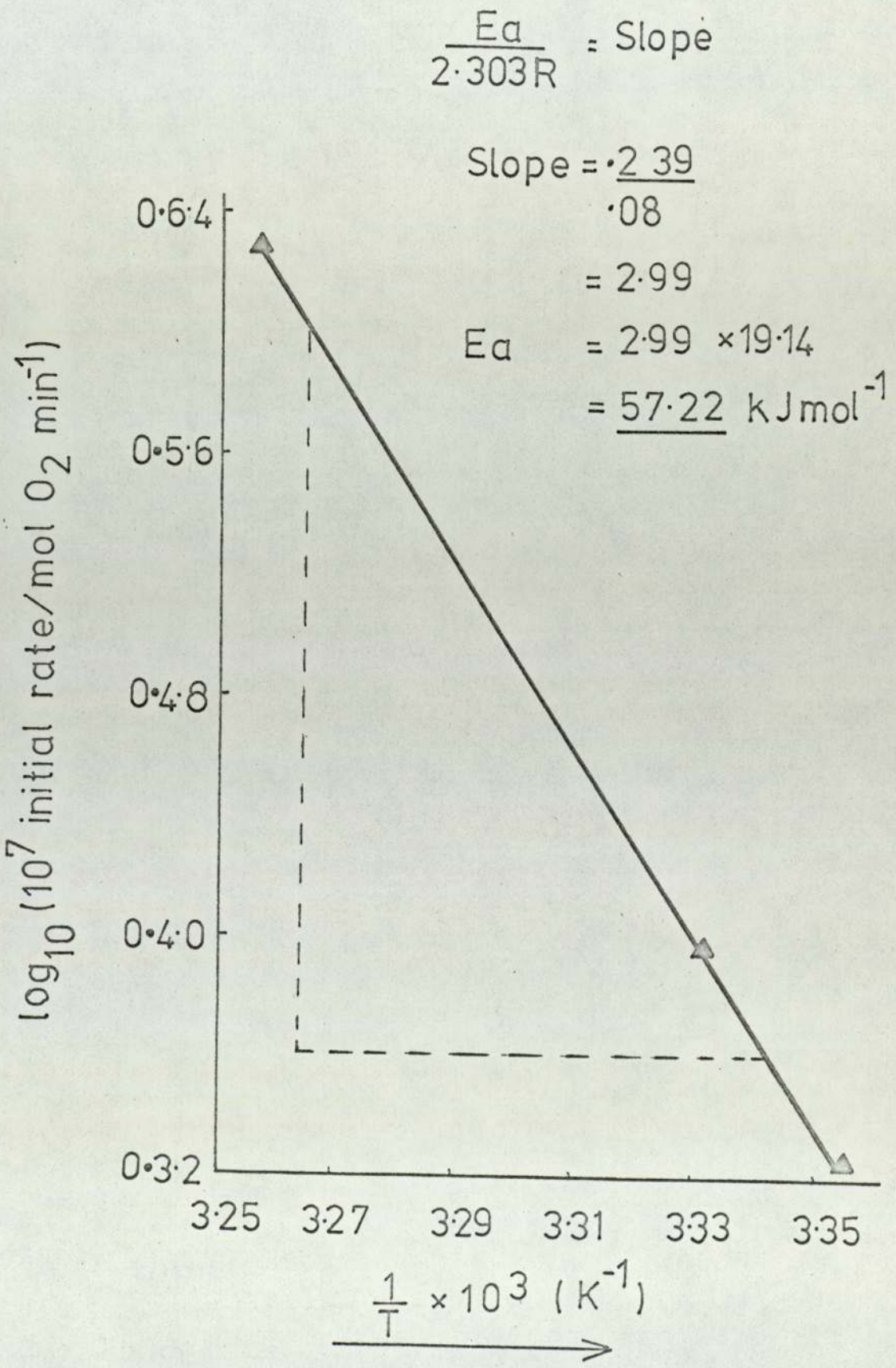


DIAGRAM XI.

Temperature dependence of O_2 uptake rates.



B: Effect of Buffer Concentration.

Temp 25°C; pO_2, 1.0.

TABLE XXIX

Buffer	Concentration M	Rate x 10^7 mol O_2/min
Sodium phosphate	0.1	2.7
" "	"	2.6
" "	1.0	5.3
" "	"	5.5
" "	2.0	4.0
" acetate	0.1	1.0
" "	1.0	1.2
Ammonium "	0.1	2.0
" "	0.33	3.8
" "	0.7	4.1
" "	1.0	4.4
" "	2.0	3.0
" "	5.0	3.5
" phosphate	0.1	1.6
" "	1.0	2.2
Sodium hydroxide	0.1	4.4
" "	1.0	5.4
" "	2.0	5.9
" "	5.0	4.1
" "	"	4.0

A graph of these results is given on the next page. The striking thing about these results is that they offer no evidence for catalysis by phosphate or acetate, yet the rate of reaction invariably increases with buffer concentration up to 2M after which, in the case of two of the buffers, it begins to decrease.

The latter can be simply explained by decrease in oxygen solubility with increasing buffer concentration. Although no figures are available for the specific buffers used above, values are available for ammonium chloride, sodium chloride, and sodium hydroxide[121]. These are presented in Table (XXX). An obvious trend is apparent and the very great difference in solubility over the concentration range used in these experiments is enough to explain the decrease in rate at higher concentrations of buffer. Opposing this trend in the lower concentrations of buffer is the observed acceleration of rate with buffer concentration. As will be seen in Part D, this increase is best explained by the presence of Cu^{II} ion impurities.

It should be noted that although results are acceptably reproducible for samples of a given batch of 5-methyl THF with the same batch of buffer, reproducibility between different batches of materials is not as good, since variations of up to $\pm$ 30% occur. Therefore when comparisons are made between various buffers and additives, samples from the same batches of starting materials must be used.

This seemingly great inaccuracy could also result from the presence of differing amounts of Cu^{II} ion impurities in different batches of materials.

DIAGRAM XII

Effect of buffer concentration on rate of O_2 uptake.

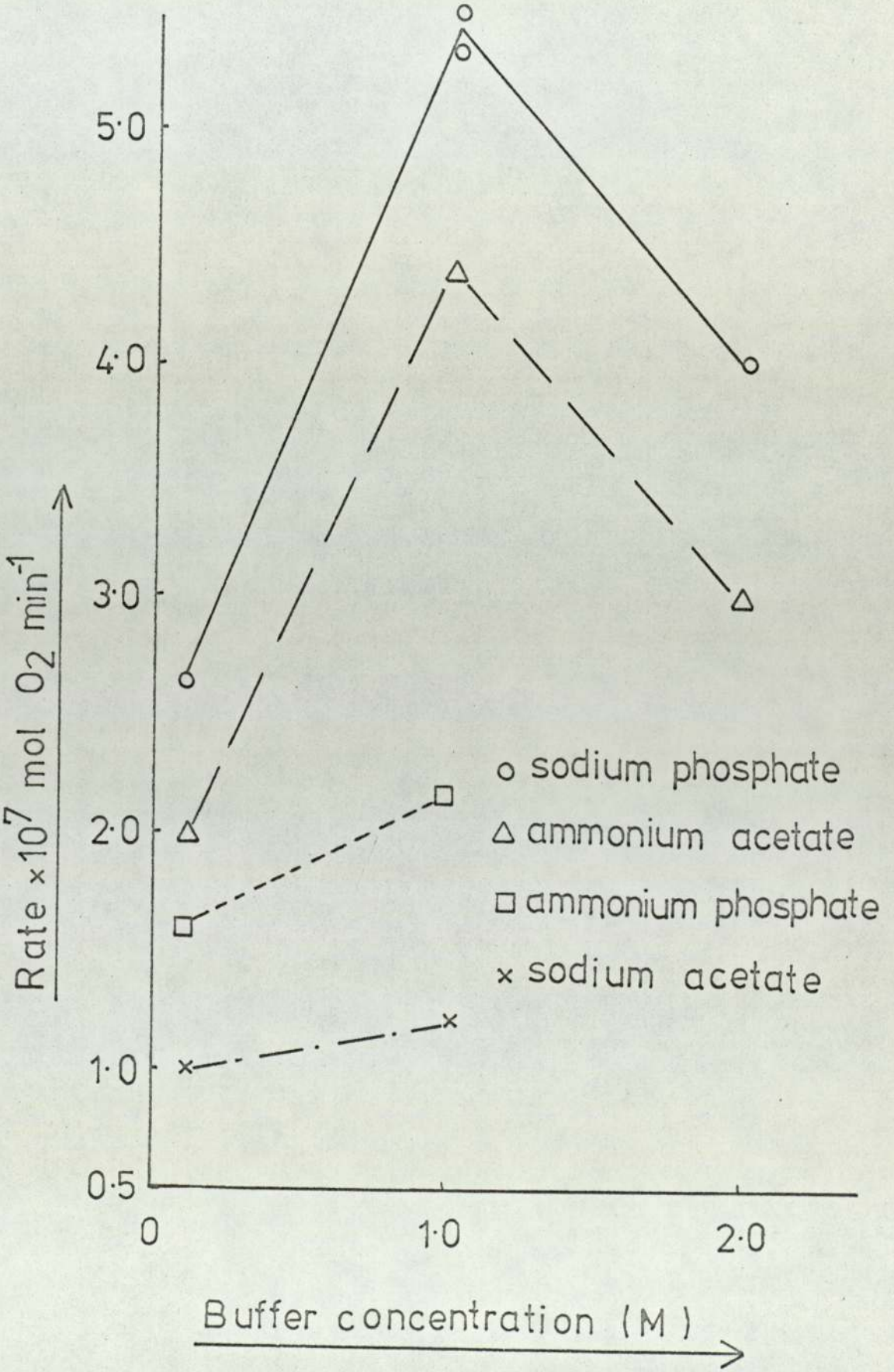


TABLE XXX

Variation of O_2 Solubility with Buffer Concentration[121]

Temp. 25°C.

Buffer	Conc. M	Solubility cc./litre
Dist. H_2O	-	5.78
NaCl	0.125	5.52
"	0.25	5.30
"	0.50	4.92
"	1.0	4.20
"	2.0	3.05
"	4.0	1.62
NH_4Cl	0.125	2.31
"	0.25	1.16
"	1.0	0.07
NaOH	0.5	5.16
"	1.0	4.14
"	2.0	2.73

c: Effect of pH

TABLE XXXI

Temp. 25°C; pO_2, 1.0.

Solvent	pH	Rate x 10^7 moles O_2/min.
HCl 10^{-1}M	1.2	0.2
HCl 10^{-1}M	1.2	0.2
HCl 10^{-3}M	4.0	0.9
Dist. H_2O	5.6	1.3
Dist. H_2O	5.6	1.4
NaOH 10^{-4}M	8.6	1.3
NaOH 10^{-4}M	8.5	1.2
NaOH 10^{-4}M	8.7	1.4
NaOH 6 x 10^{-4}M	9.3	1.4 (5)
NaOH 8 x 10^{-4}M	9.4	1.4 (6)
NaOH 6 x 10^{-3}M	9.7	1.5 (5)
NaOH 6 x 10^{-3}M	9.7	1.6
NaOH 2 x 10^{-3}M	9.9	1.7
NaOH 8 x 10^{-3}M	10.3	2.0
" 8 x 10^{-3}M	10.3	2.1
" 10^{-2}M	10.8 (5)	2.6 (5)
" 10^{-2}M	10.8 (5)	3.2
" 10^{-2}M	10.8 (5)	2.9
" 1.5 x 10^{-2}M	11.4	3.5
" 1.5 x 10^{-2}	11.4	3.3
" 2 x 10^{-2}	11.7	3.6
" 2 x 10^{-2}M	11.7	3.9
" 3 x 10^{-2}M	12.0 (5)	4.1
" 5 x 10^{-2}M	12.2	4.2
" 5 x 10^{-2}M	12.2	4.3
" 10^{-1}M	12.5	4.2
" 10^{-1}M	12.5	4.4

pKa = 10.9

TABLE XXXII

Calculation of Corrected Rate and % Ionisation of 3,4-amide group.

pH	% ionisation	Obs. Rate x 10^7	Corr. Rate mol O_2/min.
9.3	0.2	1.4 (5)	0.13
9.4	0.3	1.4 (6)	0.15
9.9	0.8	1.7	0.47
10.3	18.3	2.0	0.89
10.3	18.3	2.1	0.99
10.8 (5)	44.3	2.6 (5)	1.89
10.8 (5)	44.3	2.9	2.15
10.8 (5)	44.3	3.2	2.44
11.7	85.0	3.6	3.39 (6)
11.7	85.0	3.9	3.69 (6)
12.0 (5)	92.6	4.1	4.0
12.5	97.5	4.2	4.17
12.5	97.5	4.4	4.37

The reaction rate decreased markedly from pH 5.6 to 1.2: at the latter pH, the rate was too slow to be measured accurately by a simple manometric technique. The decrease in rate is explained by protonation of N(5) inhibiting electron removal[86, 122, 123]. A large increase in rate was obtained on going from pH 9.0 to 12.5 (see Diagrams XIII and XIV). This cannot be explained solely in terms of copper impurity. The concentration of copper in 0.1M phosphate buffer pH 7 is 8.8×10^{-7}M and the rate of oxidation in this system 2.6×10^{-7} mol /min, however the concentration of copper impurity in 0.1M sodium hydroxide is 6.0×10^{-7}M and the rate in this system is 4.4×10^{-7} mol /min. Pearson[54,55] attributed a similar increase in rate over this pH range to the increase in formation of enolate anion, which in turn makes electron removal easier. It is possible to determine the rate of oxidation of the enolate form by subtracting the rate of the neutral species from the observed rate in the higher pH range. Five rates of reaction for the neutral species have been observed between pH 5.6 and 8.7; the average of these rates is 1.3 (2) $\times 10^{-7}$ mol /min. This value was used to correct rates observed for runs carried at pH 9.3 and above to give a corrected rate of reaction of the enolate species. The log-log plot for the concentration of enolate (% ionisation) against corrected rate is shown in Diagram XV, and is a reasonable straight line (slope 0.97), showing that between pH 9.7 - 12.5 the reaction has first order dependence on enolate anion concentration. This

observation was also made by Pearson[54,55] and has important consequences when considering the mechanism of oxidation (see Chapter VI).

Consultation of Table (XXX) will show that the variation in oxygen solubility from distilled water to 0.5M sodium hydroxide is 10%, therefore for 0.1M sodium hydroxide it will be $\simeq$ 2% and can be ignored.

DIAGRAM XIII

Rate of oxygen uptake at four pH values.

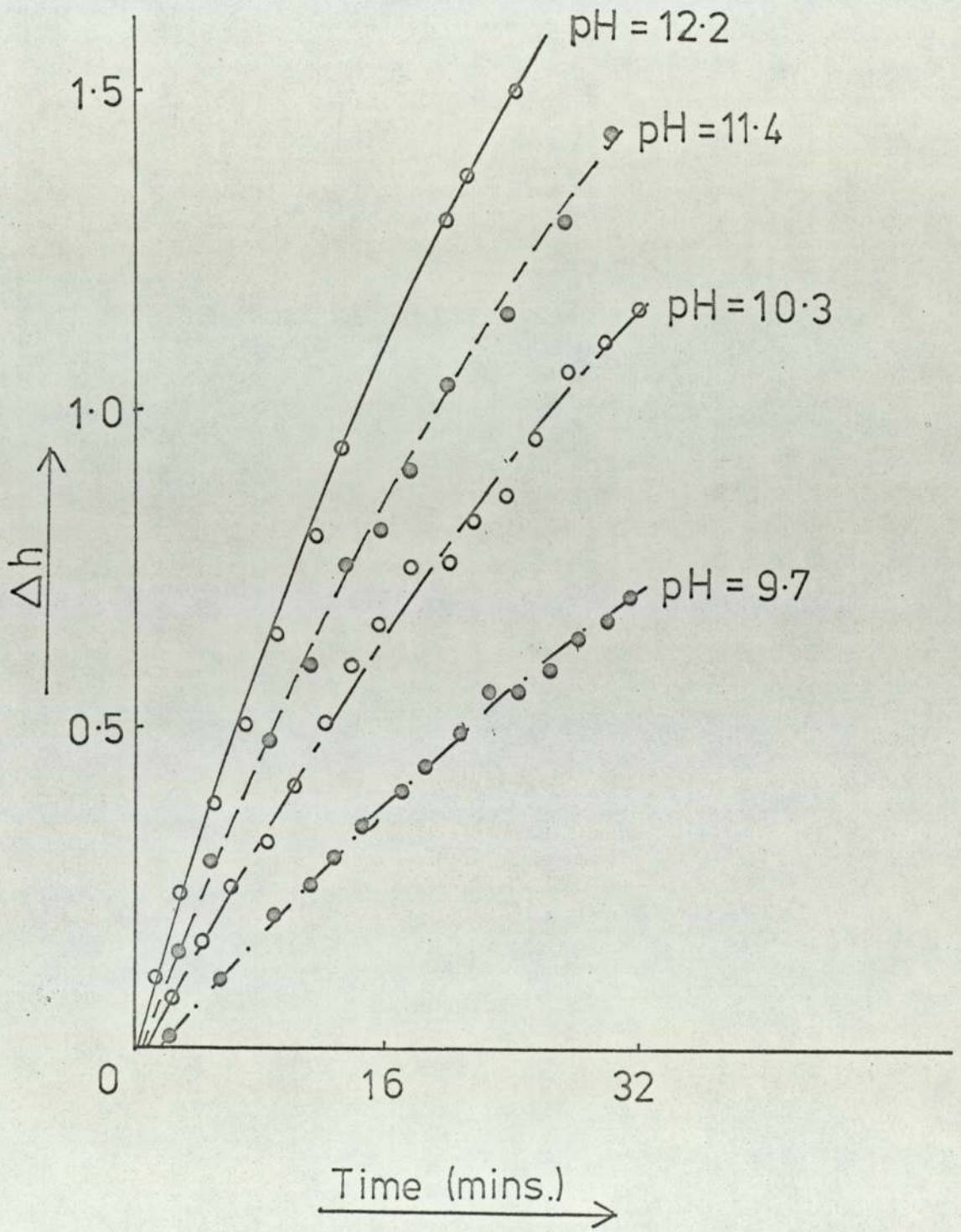


DIAGRAM XIV

pH dependence of O_2 uptake rates.

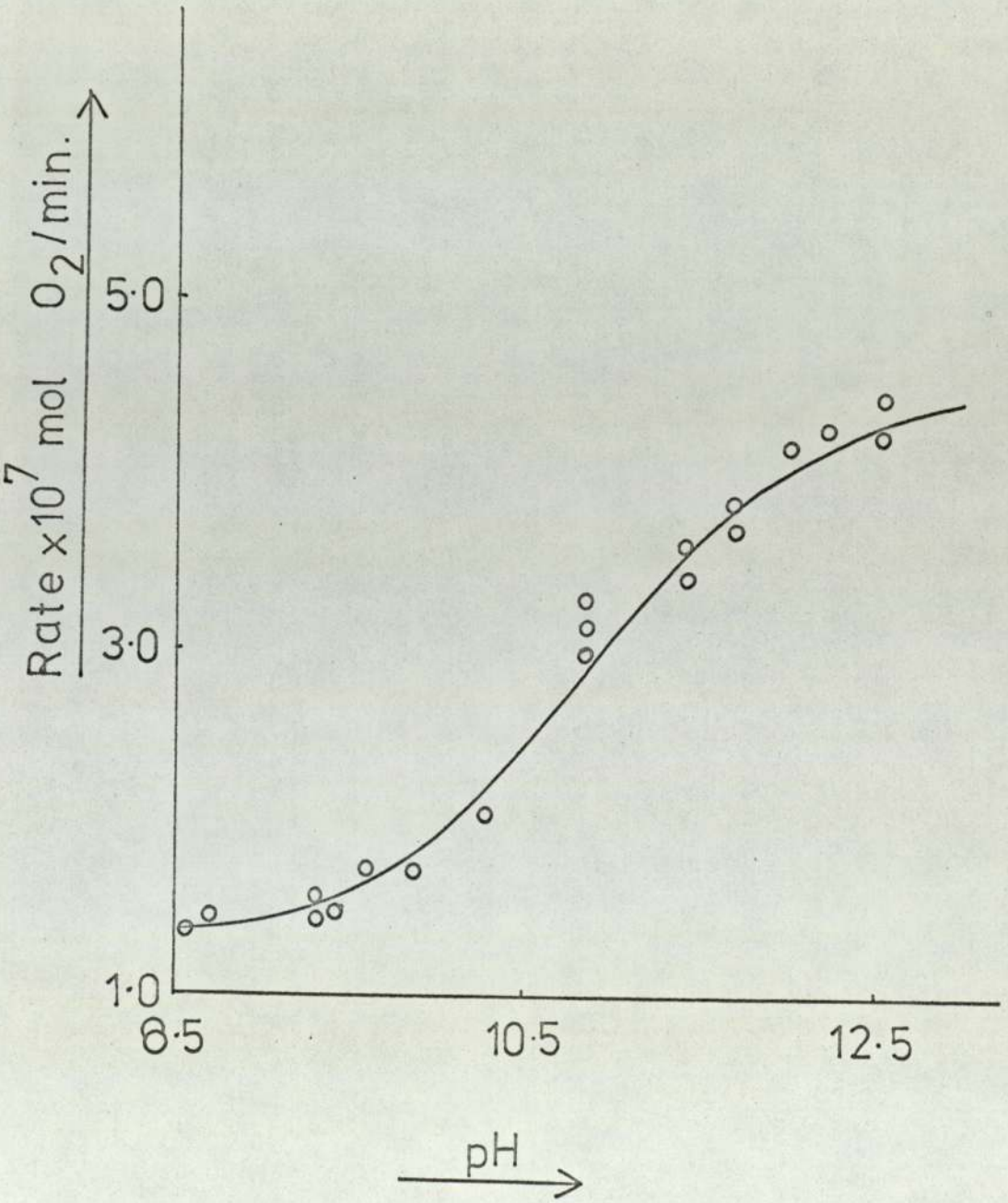
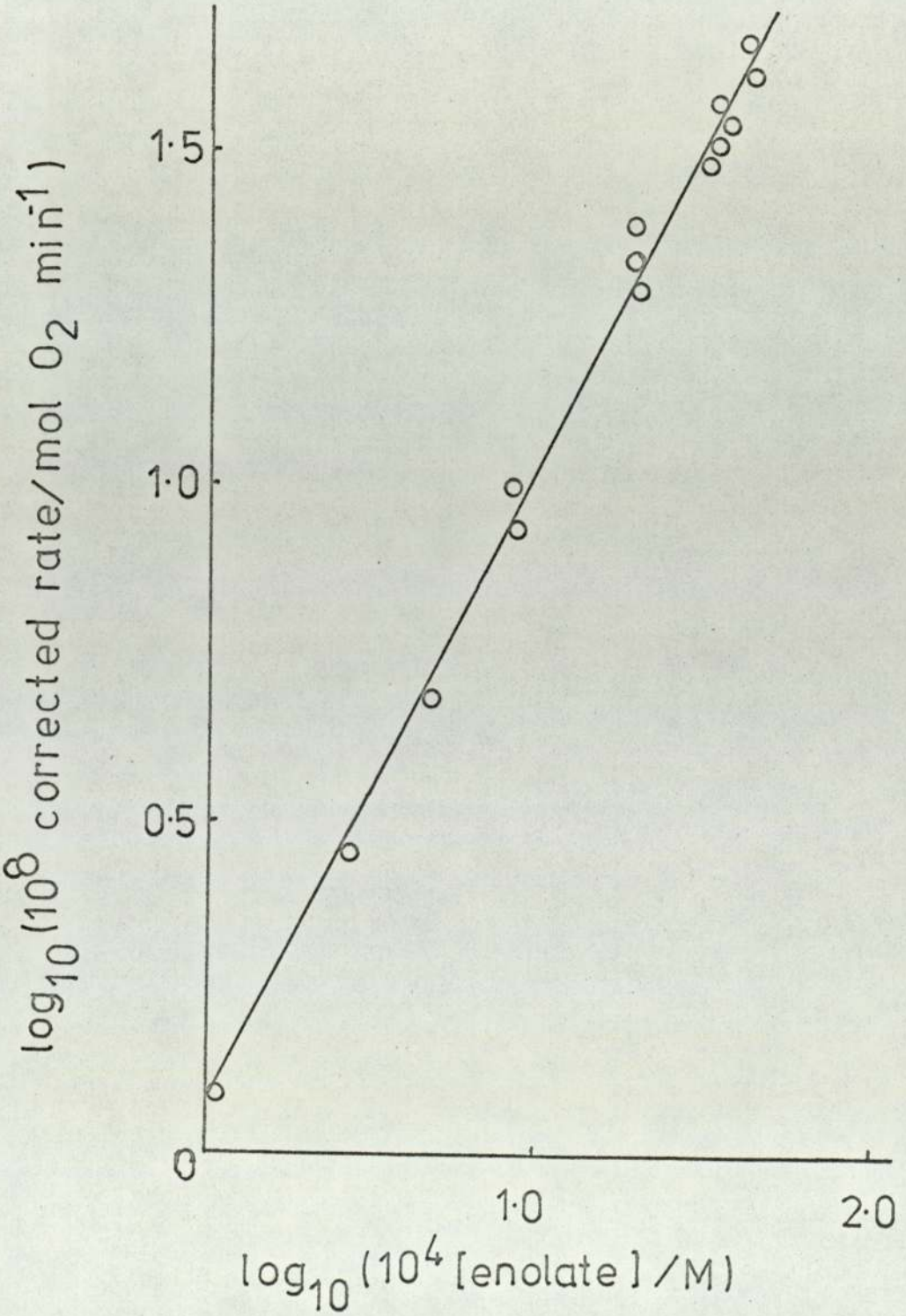


DIAGRAM XV

Order of reaction w.r.t. [enolate].



D: Effect of Additives.

TABLE XXXIII

Temp. 25°C; pO_2 1.0; pH 7.

Buffer	Buffer Conc. (M)	Additive	Additive Conc.	Rate x 10^7 mol O_2/min.
Sodium phosphate	0.1	None	-	2.6
" "	"	Riboflavin	2 mM	2.7
" "	"	"	"	2.7
" "	"	Phenol	"	1.5
" "	"	"	"	1.4
" "	"	8-hydroxy-quinoline-5-sulphonic acid	10^{-4} M	2.5
" "	"	"	2 mM	2.4
" "	"	Benzoquinone	"	0.1
" "	"	Quinol	"	7.0
" "	"	EDTA	"	2.0
" "	"	Methanol	2%	2.2
" "	"	Ethanol	"	2.6
" "	"	Blackened vessel		1.8
Ammonium acetate	1.0	None	-	4.4
" "	"	$FeCl_3$	10^{-4}M	4.1
" "	"	EDTA	2mM	2.7
" "	"	Ethanol	2%	4.4
" phosphate	0.1	None	-	1.6
" "	1.0	"	-	2.2
" "	"	"	-	2.1
" "	0.1	$Fe^{III}(CN)_6$	10^{-4}M	1.3
Distilled water		None		1.3
" "		$CrCl_3$	10^{-4}M	1.5
" "		$CuSO_4$	10^{-4}M	20.0
" "		$CuSO_4$ Blackened vessel	10^{-4}M	19.0
Sodium hydroxide	0.1	None	-	4.0
" "	"	KCl	0.9M	3.9
" "	1.0	None		5.4

(all with 0.93×10^{-3}M 5-MeTHF)

The largest observed effect on rate of oxygen consumption of any additive was that of copper. Other transition metal ions Fe^{III} and Cr^{III} had no marked effect. This suggests that Cu^{II} oxidises 5-methyl THF and the Cu^{I} so formed is quickly oxidised by O_2 back to Cu^{II}. Some evidence for this is provided by the ability of 5-methyl THF to reduce Fehling's solution. Ammonium acetate and sodium phosphate were found by atomic absorption spectroscopy to contain 500 times more copper impurity than sodium acetate or ammonium phosphate (see Table XXXIV) thus explaining the discrepancy in rates between these buffers.

Riboflavin did not accelerate the reaction rate, but the rate did decrease slightly in a blackened vessel. Nevertheless the reaction does proceed fairly readily in a blackened vessel, therefore it is likely that the reaction proceeds via ground state oxygen.

When benzoquinone was used in an attempt to photosensitise the reaction, there was no observable oxygen uptake. A possible explanation of this observation could be that benzoquinone itself oxidised the 5-methyl THF to give quinol and oxidised folate. The former could not oxidise because of the equilibrium set up between benzoquinone and quinol. Evidence for this is presented in Section E of this chapter.

When the ionic strength of the reaction medium was altered with potassium chloride, no change in the rate of oxidation was observed. Similarly, hydroxyl radical scavengers ethanol and methanol had no effect, but phenol, a general radical scavanger, decreased the rate by 50%.

TABLE XXXIV.

Concentration of copper ion impurities in various buffers.

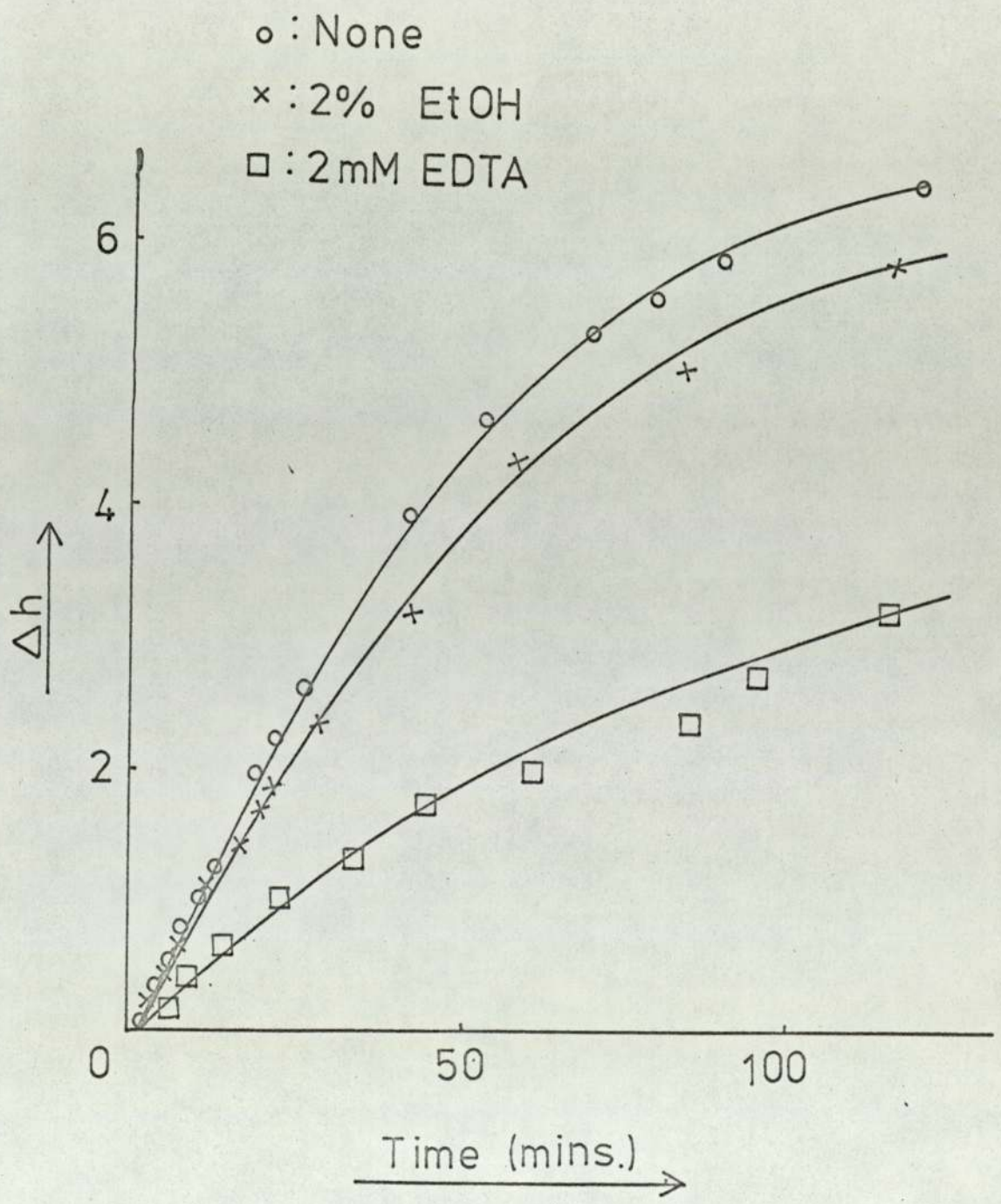
Buffer	Concentration of Cu^{II} x 10^{7}M.
Ammonium Acetate 1M	5.6
Sodium Acetate 1M	0.01
Sodium Phosphate 1M	88.0
Ammonium Phosphate 1M	0.03
Sodium hydroxide 1M	60.0

A similar result to the latter might have been expected on addition of 8-hydroxyquinoline-5-sulphonic acid, but this additive had no effect. Pearson[119] has reported that this scavenger does behave as an inhibitor in the oxidation of THB and THF. The lack of effect with 5-methyl THF could be due to the inability of such a bulky molecule to get close enough to the site of highest spin density to pull out the free electron. Here again, it would seem as though the N5(CH_3) group is sterically hindering the transfer of an electron and supports Pearson's calculation that C(4a) has the highest spin density.

8-Hydroxyquinoline-5-sulphonic acid has been reported as an inhibitor in the oxidation tetrahydropterins due to its chelation of transition metal ion impurities. EDTA reduced the rate by 25% in 0.1M sodium phosphate and 40% in 1M ammonium acetate (see Diagram XVI) showing that the effect of copper can be controlled with this chelating agent.

DIAGRAM XVI.

Effect of inhibitors on O_2 uptake rate.



E: Rates of Oxidation for Samples other than 5-Methyl THF.

TABLE XXXV.

Sample	Conc. mg/50 ml.	Solvent (0.1M)	Additive	Rate x 10^{-7} mol /min
5-formyl THF	20	NaPhos.	-	0.1
"	40	"	-	0.1
"	100	"	-	0.1
"	40	NaOH	-	0.2
Sodium ascorbate	50	NaPhos.	-	28.6
Quinol	11	"	-	2.2
"	"	"	Benzoquinone 2 mM	0.1

Estimation of half-life of 5-Methyl THF under various oxygen partial pressures.

TABLE XXXVI

Temp. 25°C.

Solvent	O_2 partial pressure mm-Hg	t $\frac{1}{2}$ (hrs)
0.1M phosphate buffer	104	10.9
"	172	8.0
"	430	2.8
"	760	1.5
Distilled water	104	20.4
"	172	15.0
"	430	5.2
"	760	2.8

As reported elsewhere, 5-formyl THF was found to be fairly stable to oxidation[124]. The rate was too slow to be measured manometrically, nor could any change in the u.v. spectrum be observed after 19 hours oxidation in 0.1M phosphate buffer with pure oxygen.

This stability is not unexpected as an electron withdrawing group would stabilise the reduced pteridine ring to oxidation. It is interesting that the opposite is true for the unreduced parent compound, where the ring nitrogens deplete the layer of π-electrons, thus decreasing the aromatic stabilisation. In the latter case, electron liberating groups (amino and hydroxy) help to compensate for this effect.

Sodium ascorbate was found to oxidise more rapidly than 5-methyl THF. Half-life values in 0.1M phosphate buffer under oxygen for the two respectively are 56 and 90 minutes. This antioxidant could not completely inhibit oxidation of 5-methyl THF in solution over long periods of time; however, as long as some ascorbate is present in solution, any 5-methyl-5,6-DHF produced would be reduced back to the tetrahydro state.

In order to explain the inhibitory effect of benzoquinone on the oxidation of 5-methyl THF, experiments were performed to see, firstly, whether 5-methyl THF is oxidised to 5-methyl-5,6-DHF by this oxidant and, secondly, whether benzoquinone inhibits the oxidation of quinol when they are in equal concentrations. As would be the case when 2 mM benzoquinone is added to 9.3×10^{-4}M 5-methyl THF

to form benzoquinone, quinol and 5-methyl-5,6-DHF (all approximately 1mM) . Unfortunately benzoquinone exhibits a strong absorption at 248 nm which masks the peak due to 5-methyl-5,6-DHF at 250 nm. Consequently, a spectrophotometric method proved fruitless, so t.l.c. was adopted. In order to detect any 5-methyl-5,6-DHF, formed chromatograms were sprayed with N N-dimethyl-p-phenylene diamine hydrochloride (1% aqueous solution). As mentioned earlier, this reagent reacts with 5-methyl-5,6-DHF to give a dark pink colouration.

TABLE XXXVII.

Investigation of Oxidation of 5-Methyl THF with Benzoquinone

T.l.c. Results.

T.l.c. solvent	Rf.	Sample	Reaction with NN dimethyl-p-phenylene diamine hydrochloride.
(i)	0.95	Solution (A)	+
"	0.80	5-methyl THF std.	-
"	0.30	Benzoquinone std.	+
(ii)	0.90	Benzoquinone std.	+
"	0.30	Solution (A)	+
"	0.20	5-methyl THF std	-

The results in Table XXXVII show that 5-methyl THF reacts with benzoquinone to give a product which oxidises NN-dimethyl-p-phenylene diamine hydrochloride. This indicates that the product is 5-methyl-5, 6-DHF.

The effect of benzoquinone on the oxidation of quinol can be seen from Table XXXV. The inhibitory effect of benzoquinone on the oxidation of quinol supports the earlier postulate that when benzoquinone (2 mM) is added to 5-methyl THF (0.93 mM) 5-methyl THF, quinol, and benzoquinone are all produced in equal concentrations and this mixture is stable to oxidation, therefore no oxygen uptake is observed.

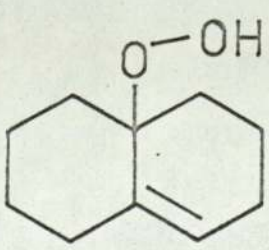
CHAPTER V.

Attempted observation and isolation of alkyl hydroperoxides.

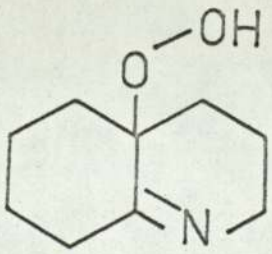
Introduction.

Many people have demonstrated the production of hydrogen peroxide during the autoxidation of tetrahydropteridines. Stocks-Wilson[118] was able to distil H_2O_2 from a THF aerial oxidation mixture. Mager and Berends[110] have measured the amount of H_2O_2/organic peroxides present when tetrahydropteridines are oxidised by pure oxygen and have shown that 1 mole of H_2O_2/organic peroxide is produced for every mole of oxygen consumed. Unfortunately, they were not able to distinguish between an organic peroxide and hydrogen peroxide. Studies by Pearson[119] carried out at - 78°C on THF and tetrahydrobiopterin have also shown the formation of H_2O_2. Using N N-dimethyl-p-phenylene diamine hydrochloride solution (1%) as a spray reagent for t.l.c.s he showed, using H_2O_2 standard, that the only observable peroxide produced during these low temperature studies was hydrogen peroxide.

In the experiments described in this chapter, hydrogen peroxide was found in the cold trap after lyophilisation of aerial oxidation mixtures by testing with ferrous ammonium thiocyanate and N N-dimethyl-p-phenylene diamine hydrochloride solution. Tetiary hydroperoxides with the following structures have been reported[125].



(XXX)
(mp 60°C)



(XXXI)
(mp 100°C)

It was hoped that the corresponding 4a-hydroperoxide for 5-methyl THF might be isolated by column or thin layer chromatography or observed kinetically. Much has been said about 8-dehydro-4a-hydroxy-5-methyl THF. By analogy with this compound a 4a-hydroperoxide, if formed in the autoxidation of 5-methyl THF, should be stable enough to be isolated. If isolation cannot be achieved, it should be possible to observe any slightly stable oxygenated intermediate produced during the autoxidation of 5-methyl THF by comparing the rate of oxygen uptake and rate of product formation. The former is dealt with in the last chapter, the latter can be done spectrophotometrically by measuring the rate of appearance of the peak at 250 nm at pH 7. The concentration of 5-methyl-5,6-DHF is given by:

$$CB = \frac{x\,(\Sigma^{A} - \Sigma^{A'})}{z(\Sigma^{B'} - \Sigma^{A'})}$$

where CB = concentration of 5-methyl-5,6-DHF

x = initial concentration of 5-methyl THF.

z = ratio of $\Sigma_{\lambda 290}$: $\Sigma_{\lambda 250}$

$\Sigma^{A} = 31.8 \times 10^{3}$ $\Sigma mol_{\lambda 290}$ for 5-methyl THF

$\Sigma^{A'} = 9.1 \times 10^{3}$ $\Sigma mol_{\lambda 250}$ for 5-methyl THF

$\Sigma^{B'} = 21.1 \times 10^{3}$ $\Sigma mol_{\lambda 250}$ for 5-methyl-5,6 DHF

The solvent systems used in the spectrophotometric technique were 1M ammonium phosphate pH 7 and 10^{-4}M $CuSO_4$ pH 5.6. The latter was chosen to build up the radical cation concentration and hopefully the concentration of

intermediate. Vanderschmitt and Scrimgeour[126] have observed rapid electron transfer from 5-methyl THF to Cu^{2+} to give a purple free radical product. This rapid electron transfer was tested using Fehlings solution, it was found that 5-methyl THF did reduce the test solution giving a buff precipitate. The product after lyophilisation from the spectrophotometric run in $CuSO_4$ solution was examined by t.l.c. The following solvent systems were used.

(i) 0.1M phosphate buffer, pH 7.0

(ii) n-Propanol/water/0.88 s/g aqueous ammonia (200:100:1 v/v)

(iii) The organic phase of 1-butanol/acetic acid/water (4:1:5 v/v).

Samples were observed as absorbing or fluorescing spots when viewed in u.v. light (λ = 254 n m).

Experimental

(1) Qualitative isolation

(a) Small scale.

5-Methyl THF (10 mg) in distilled water (10 ml) was shaken under air for 18 hrs. The reaction mixture was then found to react positively (orange → blood red) with ferrous ammonium thiocyanate; a fresh solution of 5-methyl THF decolourised the reagent). The residue after lyophilisation also gave a positive test with the same reagent. This solid was dissolved in distilled water (0.5 ml) and chromatographed on an 8 cm^3 column of G25 sephadex with H_2O solvent. Fractions (2 ml) were collected by hand and tested with the above reagent. Tubes 4 and 5 reacted positively. The experiment was repeated and again the contents of tube 4 gave a positive test. This fraction was lyophilised and u.v. and i.r. spectra recorded.

(b) Large Scale.

5-Me-THF (250 mg Ba salt) in distilled water (250 ml) was stirred under air for 18 hrs. at room temperature. The reaction mixture was lyophilised, dissolved in distilled water (20 ml) and chromatographed on a 3.8 x 45 cm. column of G15 sephadex, eluted with distilled water. The u.v. spectrum of each fraction (12 ml) was recorded, and each was tested with ferrous ammonium thiocyanate solution. Tube No. v.$\Sigma_{\lambda max}$ was plotted and the following fractions were pooled and lyophilised:

1: tubes 13 - 15, 2: 16 - 20, 3: 21 - 25, 4: 26 - 32, 5: 40 - 54. T.l.c.s in 3 solvents and u.v. spectra in 3 solvents were run on each sample.

(2) Spectrophotometric Rate Determination.

5-Methyl THF (35 mg) was stirred into 50 ml of 1M ammonium phosphate, previously equilibrated with oxygen. An aliquot (.1 ml) was taken immediately and diluted to 5 ml. with 0.1M phosphate buffer pH 7. The u.v. spectrum was then quickly recorded in the range 240 - 1300 nm. Aliquots were removed every 10 minutes and the u.v. spectrum of each was recorded. A constant stream of oxygen was passed over the solution, stirred magnetically at 25°C. After 3 hours, spectra were recorded every 20 minutes. The reaction was followed for 7 hours in this manner.

When 10^{-4}M $CuSO_4$ solution was used, spectra were recorded every 2 minutes for 30 minutes; an infinity reading was taken after 45 minutes. The oxidised solution of the latter run was lyophilised and the product looked at on t.l.c.

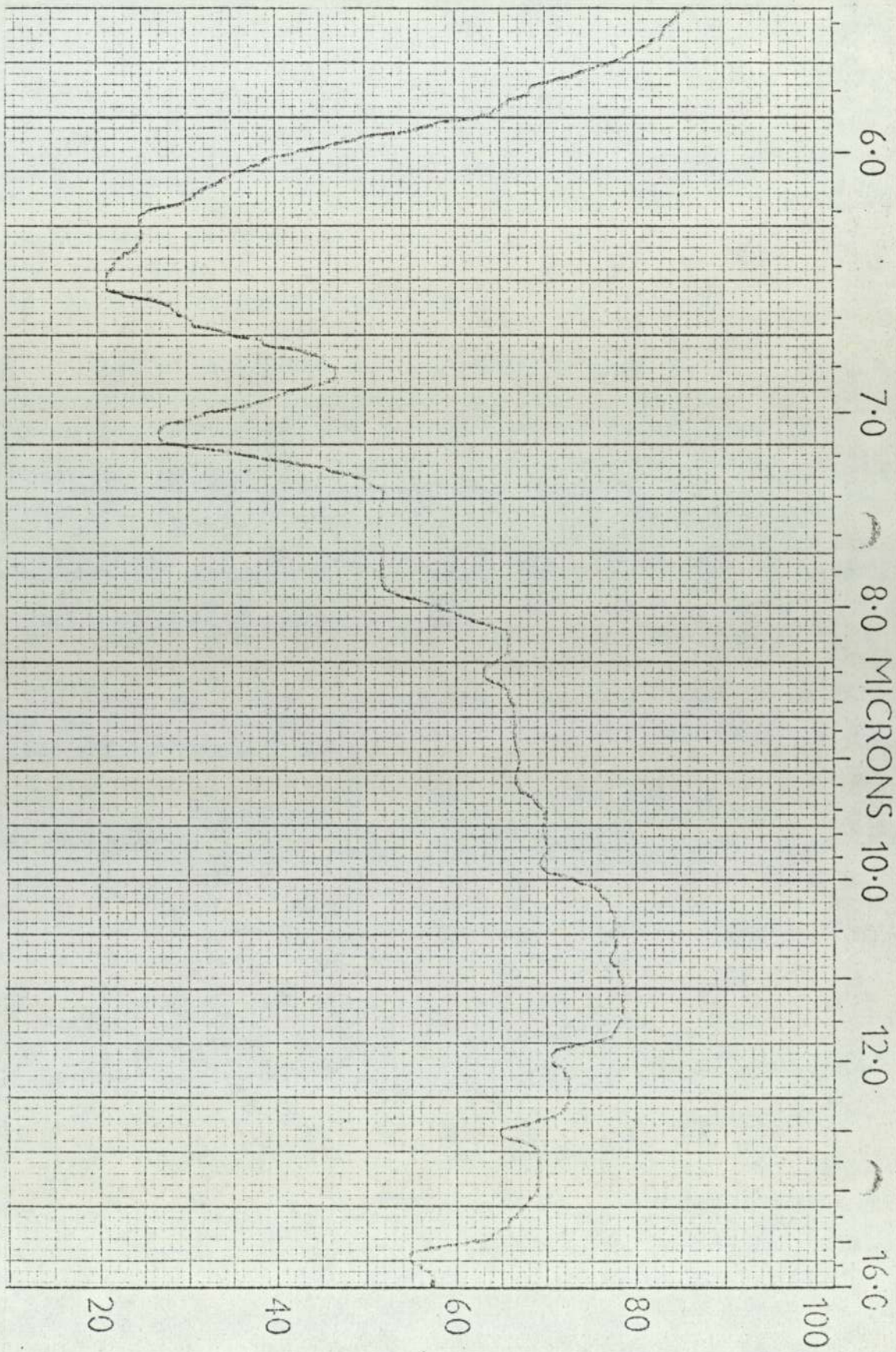
Results and Discussion.

(i) Small scale investigation.

The i.r. spectrum of the product (fraction 4 after lyophilisation) does not differ markedly from that of the starting material (See spectra (19) and (20)). A weak absorbance at ($\simeq$11μ) for O-O-stretch might have been expected if the product was a hydroperoxide. The u.v. spectrum of the product (See spectrum (21)) indicates the product to be a mixture of 5-methyl THF and 5-methyl-5,6-DHF. T.l.c. supported this.

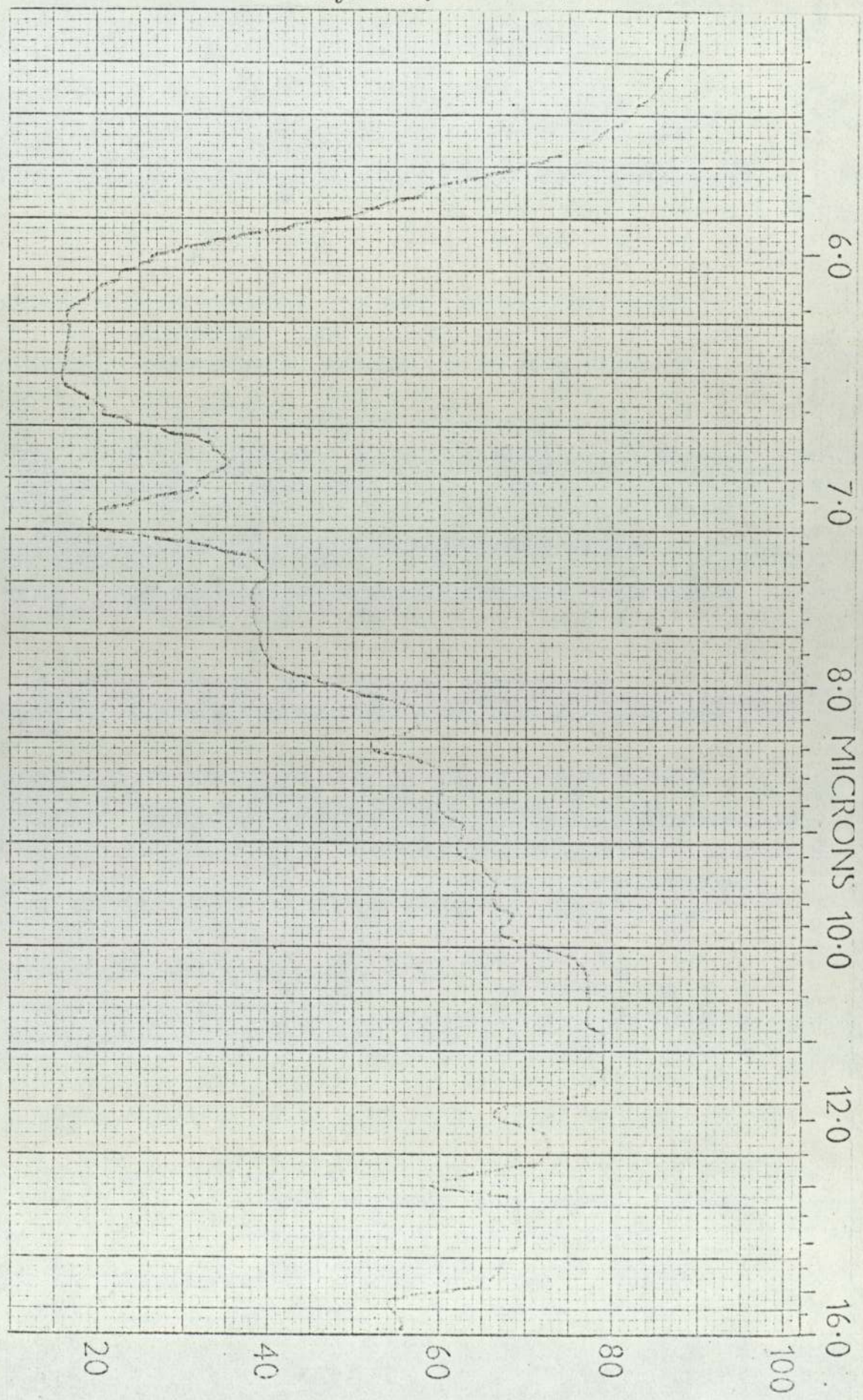
Spectrum 19

I.r. spectrum of suspected folate hydroperoxide.



Spectrum 20

I.r. spectrum of 5-methyl THF.



Spectrum 21

U.v. spectrum of fraction No. 4 (suspected folate hydroperoxide).

Solvent: 0.1M phosphate buffer pH 7.

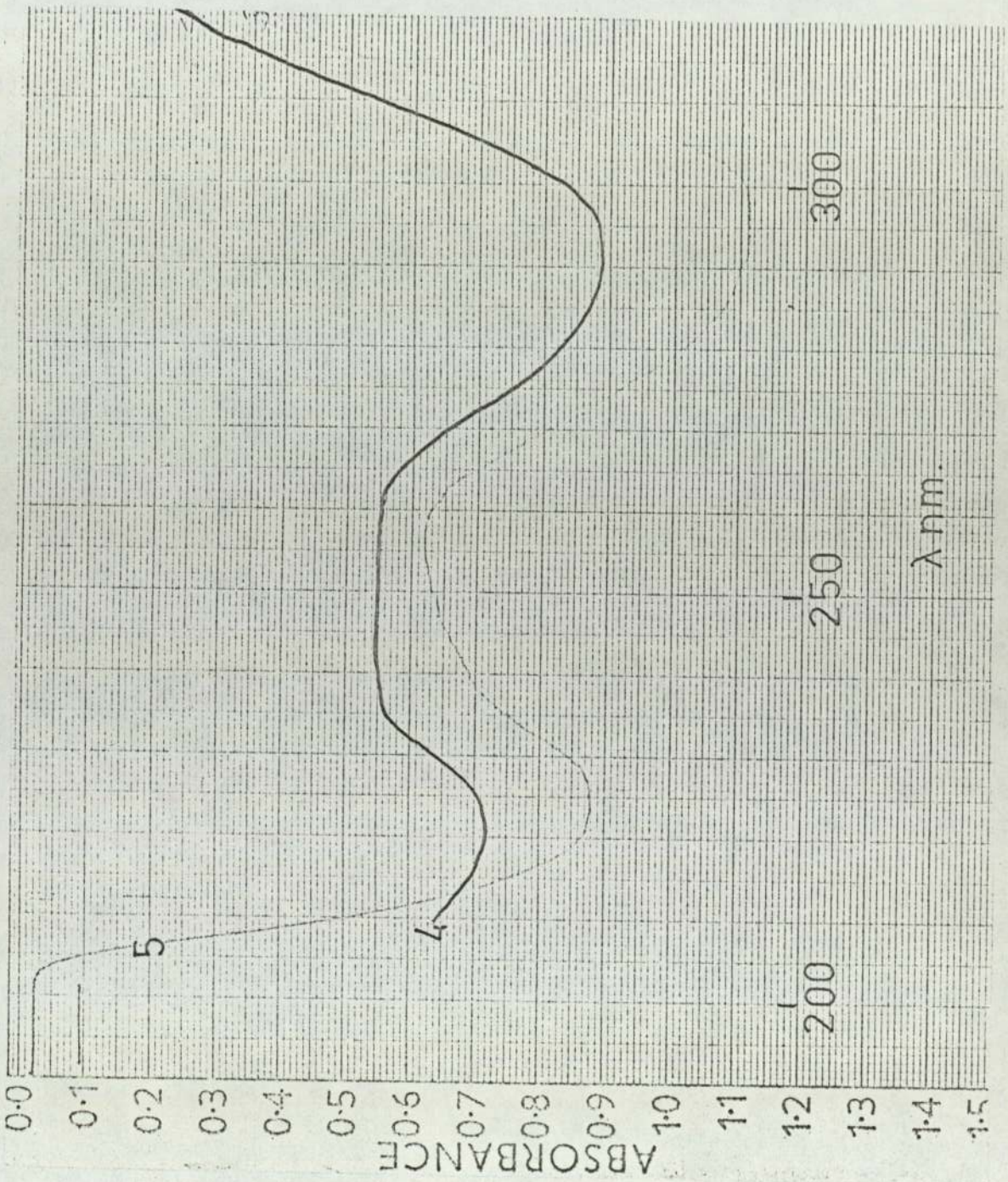


TABLE XXXVIII

(ii) T.L.C. Data for large scale investigation.

T.L.C. solvent.	Peak Number	Result peroxide test.	Rf	Inference
(i)	1	-	0.95	4a-alcohol
"	2	+	0.95	5-methyl-5,6,-DHF
"	3	-	0.95	5-methyl THF
"	4	-	0.55	Fl. imp. in starting
"	5	-	0.30	material
"	std.	+	0.95	5-methyl-5,6-DHF
"	"	-	0.91	
"	"	-	0.55	5-methyl THF
"	"	-	0.30	
(ii)	1	-	0.41	4a-alcohol
"	2	+	0.35	5-methyl-5,6-DHF
"	3	-	0.60	5-methyl THF
"	4	-	0.50	Fl. imp. in starting
"	5	-	0.30	material
"	std.	+	0.34	5-methyl-5,6-DHF
"	"	-	0.60	5-methyl THF
"	"	-	0.50	
(iii)*	1	-	0.25	4a-alcohol
"	2	-	0.30	5-methyl-5,6-DHF(decomp)
"	3	-	0.41	5-methyl THF
"	4	-	0.70	Fl. impurities in
"	5	-	0.30	starting material
"	std.	-	0.30	5-methyl-5,6-DHF(decomp)
"	"	-	0.41	5-methyl THF

* No positive tests were found in butanol/ acetic acid/water solvent. This is because the pH of this system is below that at which 5-methyl-5,6-DHF rearranges. No evidence for an organic peroxide was found in this study.

The identity of peak 1 was checked by t.l.c. with standard 4a-alcohol(8-dehydro-4a-hydroxy-5-methyl THF).

TABLE XXXIX

T.l.c. Solvent.	Sample	Rf	Inference
(i)	peak 1	0.92	4a-alcohol
	std	0.92	4a-alcohol
	"	0.87	5-methyl THF
	"	0.92	5-methyl-5,6-DHF
(ii)	peak 1	0.42	4a-alcohol
	std	0.42	4a-alcohol
	"	0.45	5-methyl THF
	"	0.37	5-methyl-5,6-DHF
(iii)	peak 1	0.30	4a-alcohol
	std	0.30	4a-alcohol
	"	0.42	5-methyl THF
	"	0.25	5-methyl-5,6-DHF

TABLE XXXX.

(iii) T.l.c. of oxidation products from Cu SO_4 solution.

T.l.c. solvent	Result of peroxide test.	Rf	Sample
(i)	+	0.95	Oxidation product
	+	0.45	
	+	0.95	5-methyl-5,6-DHF (std)
	+	0.45	$CuSO_4$ (std)
(ii)	+	0.35	Oxidation product
	+	0.11	
	+	0.35	5-methyl-5,6-DHF (std)
	+	0.11	$CuSO_4$
(iii)	+	0.30	Oxidation product
	-	0.32	5-methyl-5,6-DHF (std)
	+	0.30	$CuSO_4$ (std)

The kinetic plots manometrically and spectrophotometrically determined for each solvent are given in Diagrams XVII and XVIII. The rates for each method in a given solvent are identical. (Rates in 1M ammonium phosphate are 2.2 and 2.1 in $CuSO_4$ they are both 20.0 10^7 Mol O_2 min^{-1}). No time lag is observable between the plots, it must therefore be concluded that no intermediate is stable enough to be observed by this method. As no evidence for hydroperoxide formation can be obtained, it is doubtful if they are intermediates in the autoxidation of 5-methyl THF.

Diagram XVII.

Rate of O_2 uptake (determined manometrically) and rate of product formation (determined spectrophotometrically) in 1M ammonium phosphate pH 7.

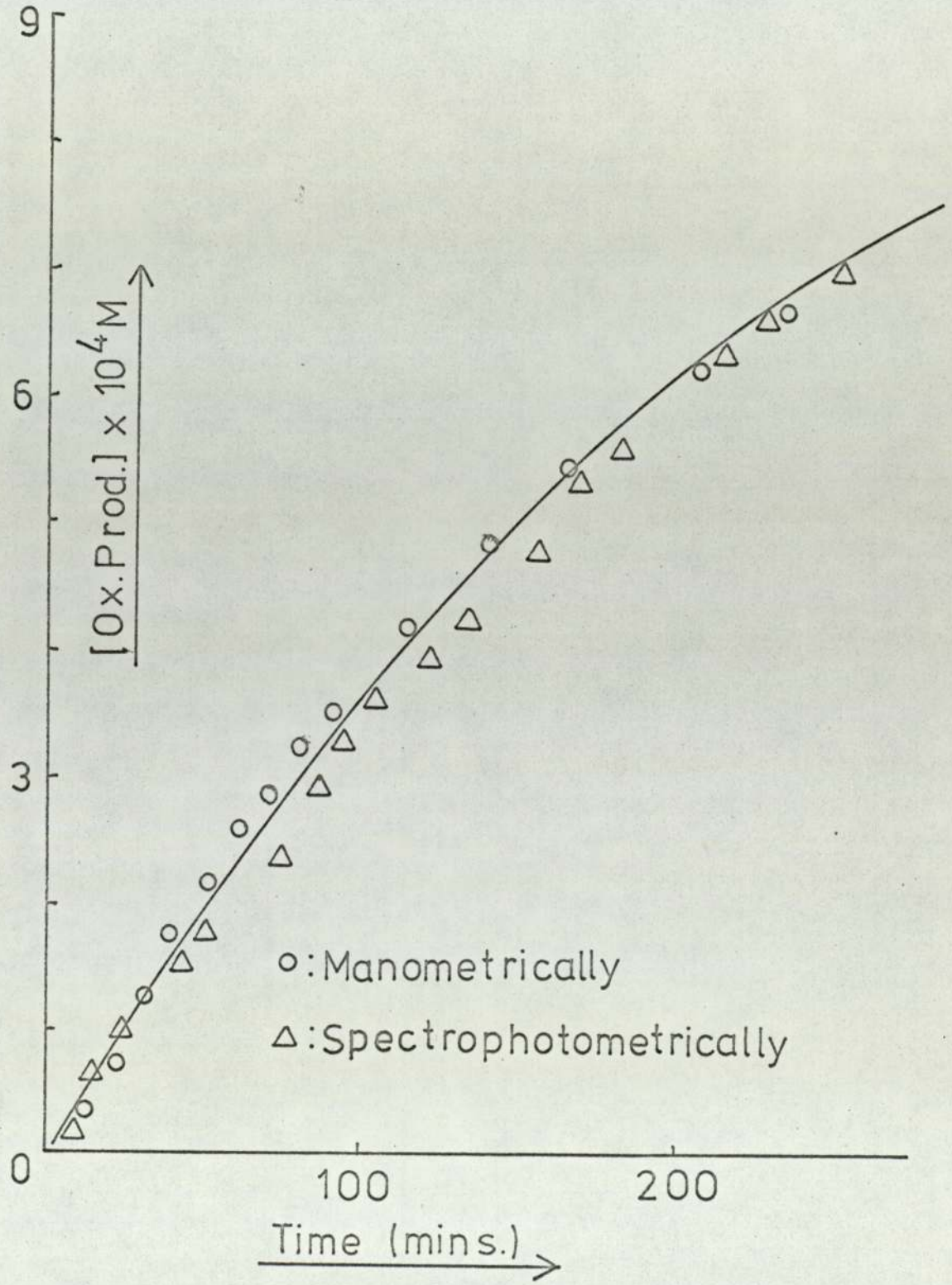
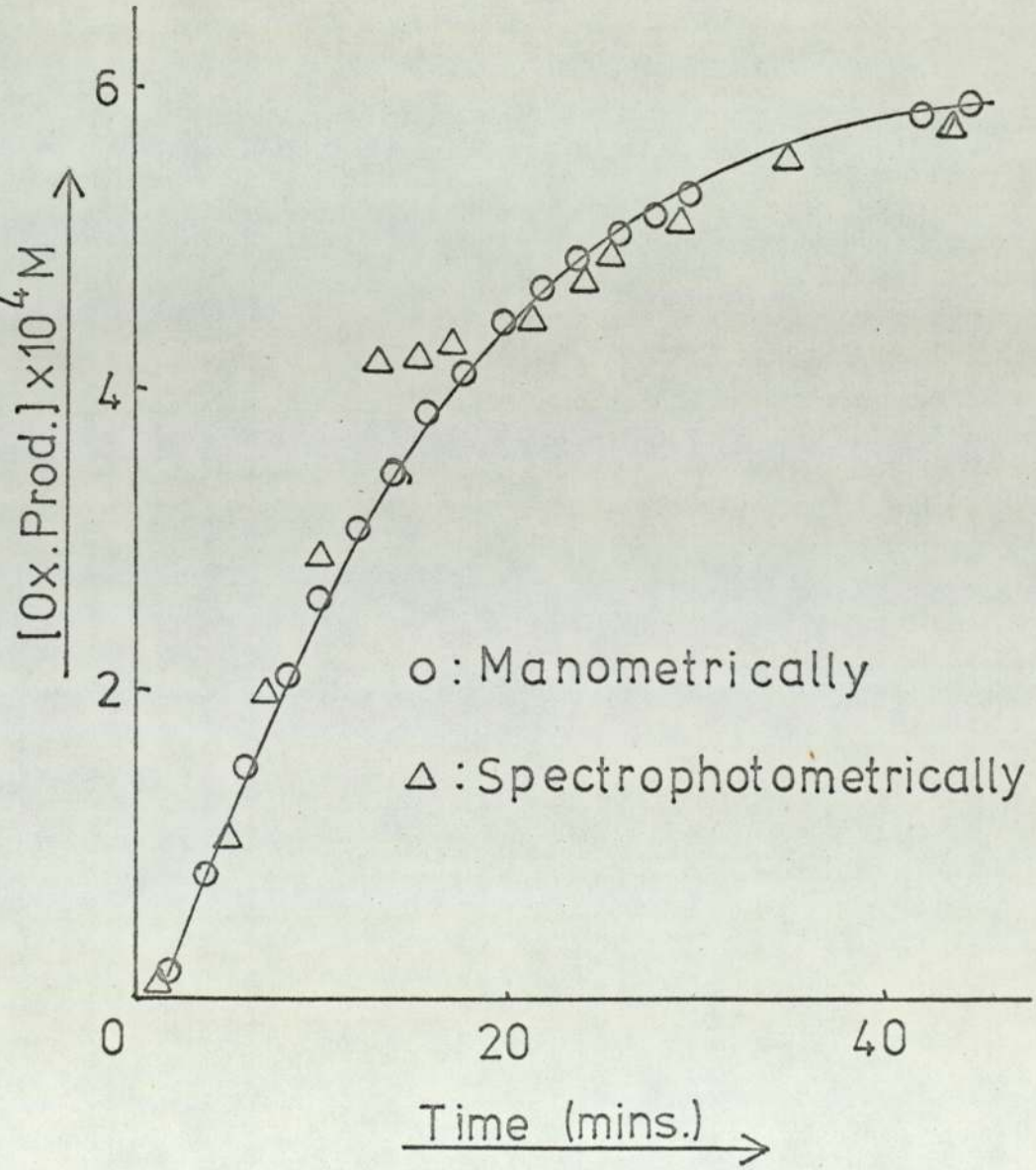


Diagram XVIII

Rate of O_2 uptake (determined manometrically) and rate of product formation (determined spectrophotometrically) in 10^{-4}M $CuSO_4$ pH 5.6.

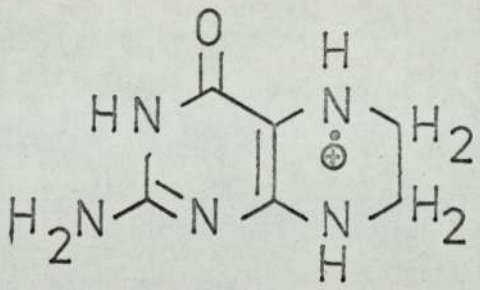


CHAPTER VI

Mechanism of Autoxidation of 5-methyl THF.

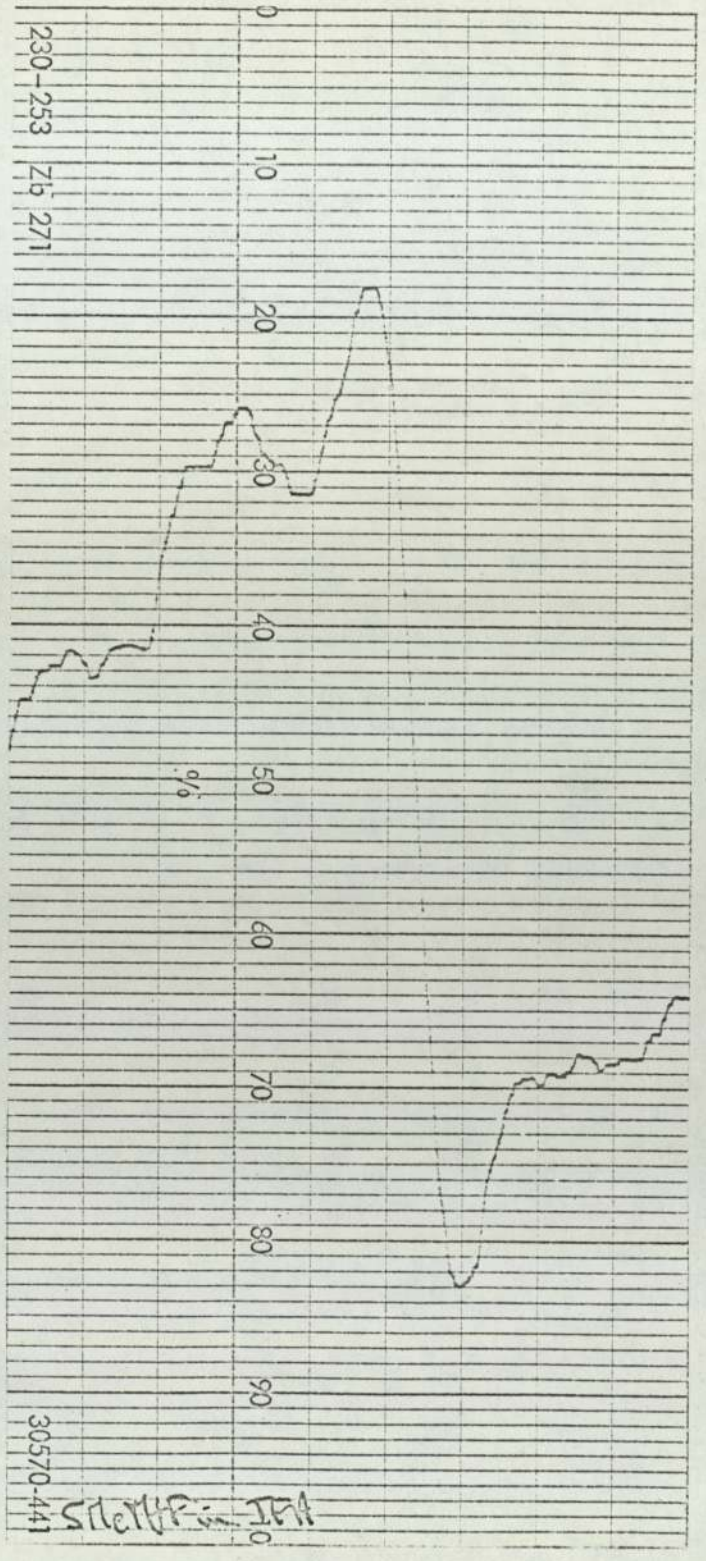
Postulated Mechanisms for the Oxidation of Reduced Pterins and Folates.

In agreement with the Perault and Pullman prediction[81] of easy formation of radical cations by folate coenzymes, Bobst[127,128] has demonstrated the existence of a tetrahydropterin radical cation (XXXII) formed after loss of an electron by electron spin resonance measurements.



(XXXII)

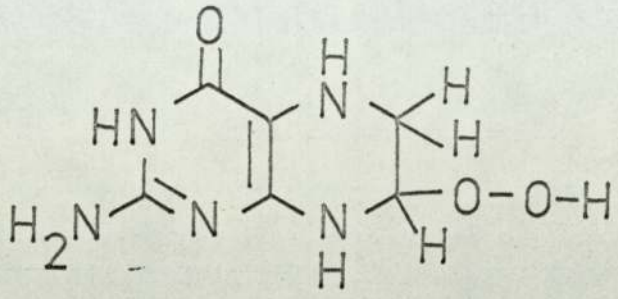
All of the proposed mechanisms of oxidation of tetrahydropteridines involve this radical as the initial product of oxidation. In these experiments it was possible to obtain an e.s.r. signal for 5-methyl THF, but not to resolve it into the predicted 7 line spectrum (Spectrum 22). Ehrenberg et al[115] showed by e.s.r. measurements that the unpaired electron is mainly confined to N (5) and the bridge carbons. This is in agreement with HMO calculations done by Pearson[114,119], who found the spin density on C (4a) to be slightly greater than C (8a) and slightly less than N (5). Despite the small amount of spin density at C (7), Hawkins[129] proposed this site for attack by oxygen to form a hydroperoxide intermediate (XXXIII) to explain Viscontini and



Spectrum 22.

E.s.r. spectrum of 5-methyl THF radical in TFA.

Weilenmann's results[130].



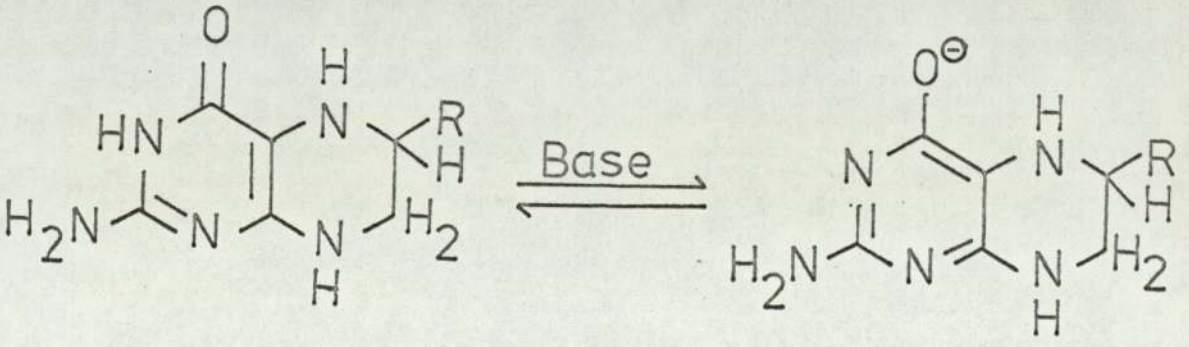
(XXXIII)

Later work[131] demonstrated that the initial stable product of oxidation of tetrahydropterin was 7,8-dihydropterin. This rules out loss of proton from C (7). Further evidence against this is supplied by Pearson[119], who did not detect any difference in rate of oxidation of THF and THF deuterated at C (7). Stocks-Wilson[118] has proposed hydroperoxide formation at C_6 and C_9, depending on the pH to explain the variation in product ratio with pH (see below).

TABLE XLI

pH of oxidation	Ratio of pterin:xanthopterin in the products.	
3	12.64	1.00
7	1.00	1.90
10	1.00	17.38

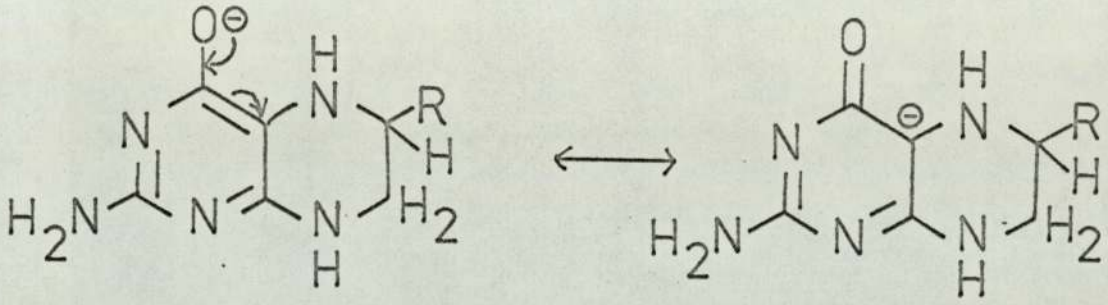
Evidence against these sites of attack and a mechanism consistent with the above observation have been put forward by Blair and Pearson[54]. These need not concern us as it has already been established that neither C (6) nor C (9) is involved as the site of attack in the oxidation of 5-methyl THF. The two possible sites for peroxide formation in 5-methyl THF are at C (8a) or C (4a). The first of these (or corresponding position) is proposed by Mager and Berends[106,107,109] for tetrahydropteridines, pyrazines, isoalloxazines, quinoxalines and flavins. It was proposed, however, on the false assumption that the unpaired electron in the tetrahydropteridine radical is localised at N (5). As mentioned earlier, this is not correct, and hence gives misleading results. In fact C (4a) has slightly more spin density than C (8a)[114,119] and the former would be therefore the position of oxygen attack. This is substantiated by the observation that the rate of oxidation of THF and THB exhibits first order dependence on enolate anion concentration formed by ionisation of the 3,4-amide group, viz:



VI

Kallen and Jencks[80] obtained the value pKa = 10.5 for this reaction determined spectrophotometrically; Pearson[119], using a similar method, obtained pKa = 10.8.

As expected, this species can lose an electron more easily than the neutral species.

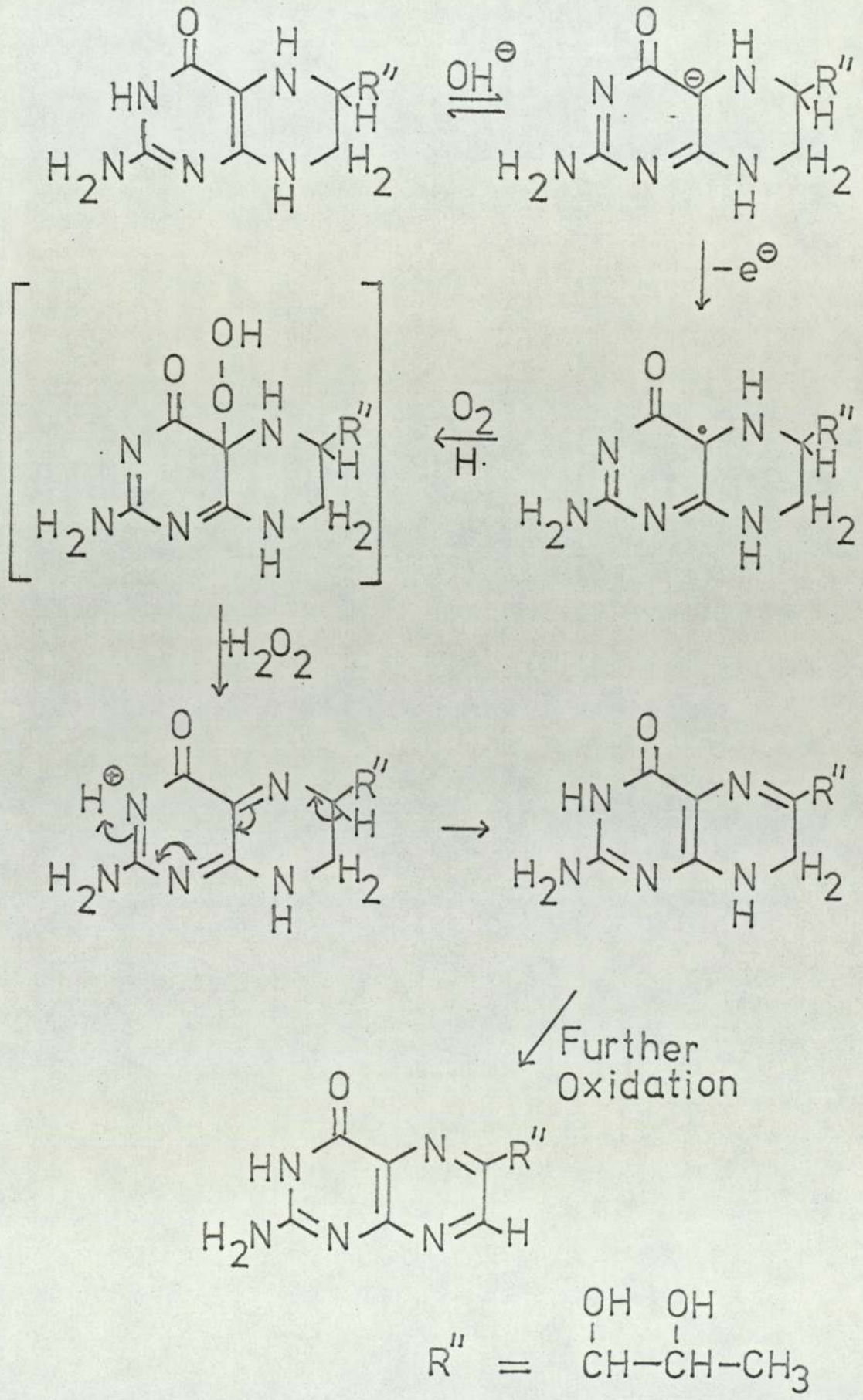


The only carbon atom on which the negative charge can reside is C (4a), hence this is the most likely site for attack by oxygen.

The mechanism for the oxidation of tetrahydrobiopterin proposed by Blair and Pearson[54,55] is shown overleaf.

Scheme V.

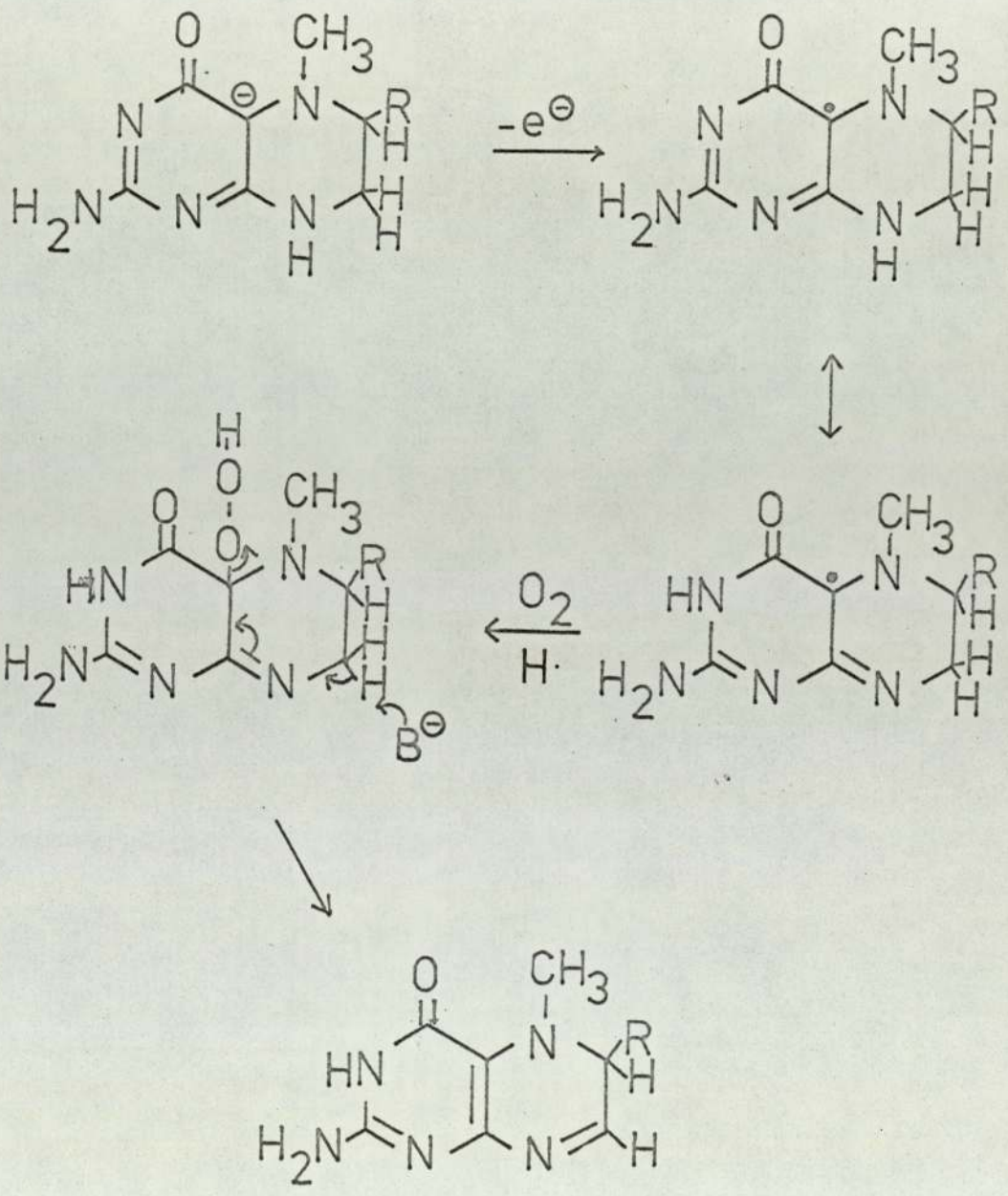
Mechanism proposed by Blair and Pearson[54] for the autoxidation of tetrahydrobiopterin.



Although the initial product of oxidation of 5-methyl THF is not a 7,8-dihydro structure, a similar mechanism can be applied.

viz:

Scheme VI.



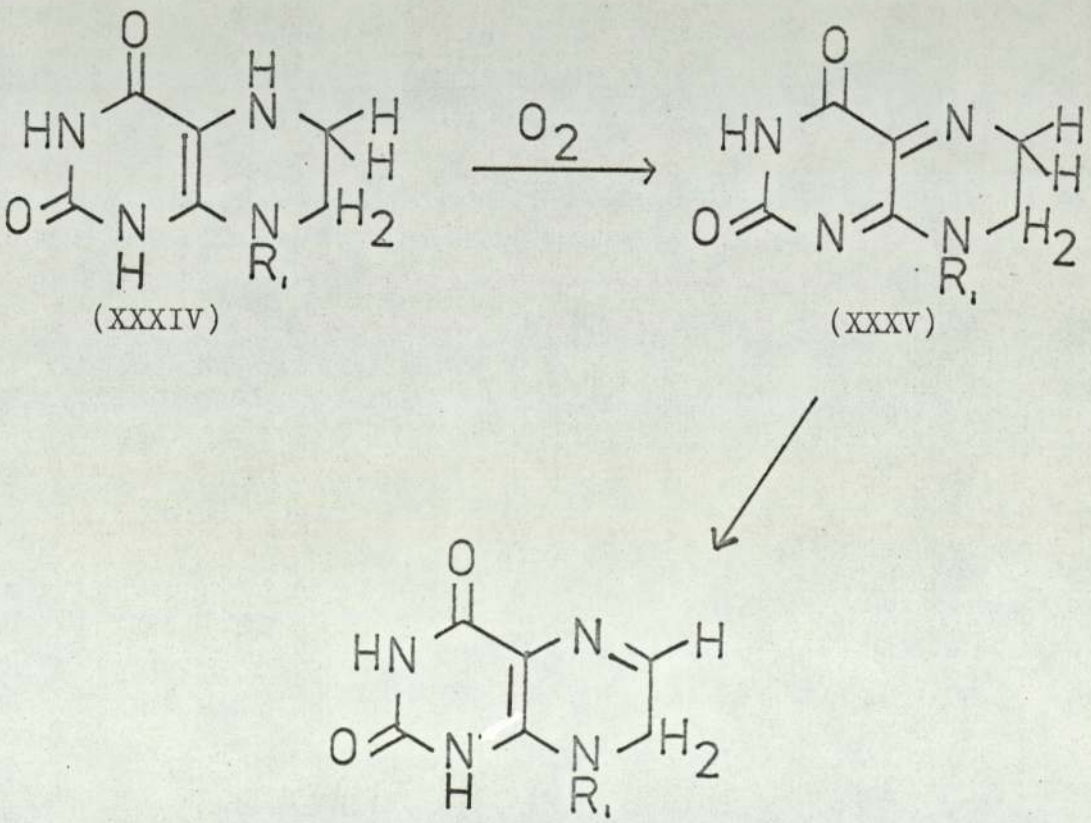
5-methyl-5,6-DHF.

Later studies by Pearson[119] on the kinetic characteristics of the free-radical intermediate showed that the reaction

$$AH^{\cdot} + O_2 \longrightarrow \text{chain carrier}$$

had a high activation energy (54 ± 8 kJ mol^{-1}). The addition of oxygen to the free radical to give an organic peroxy radical chain carrier would be expected to be a diffusion-controlled zero- (or low) activation energy process. The above observation does not support this possibility.

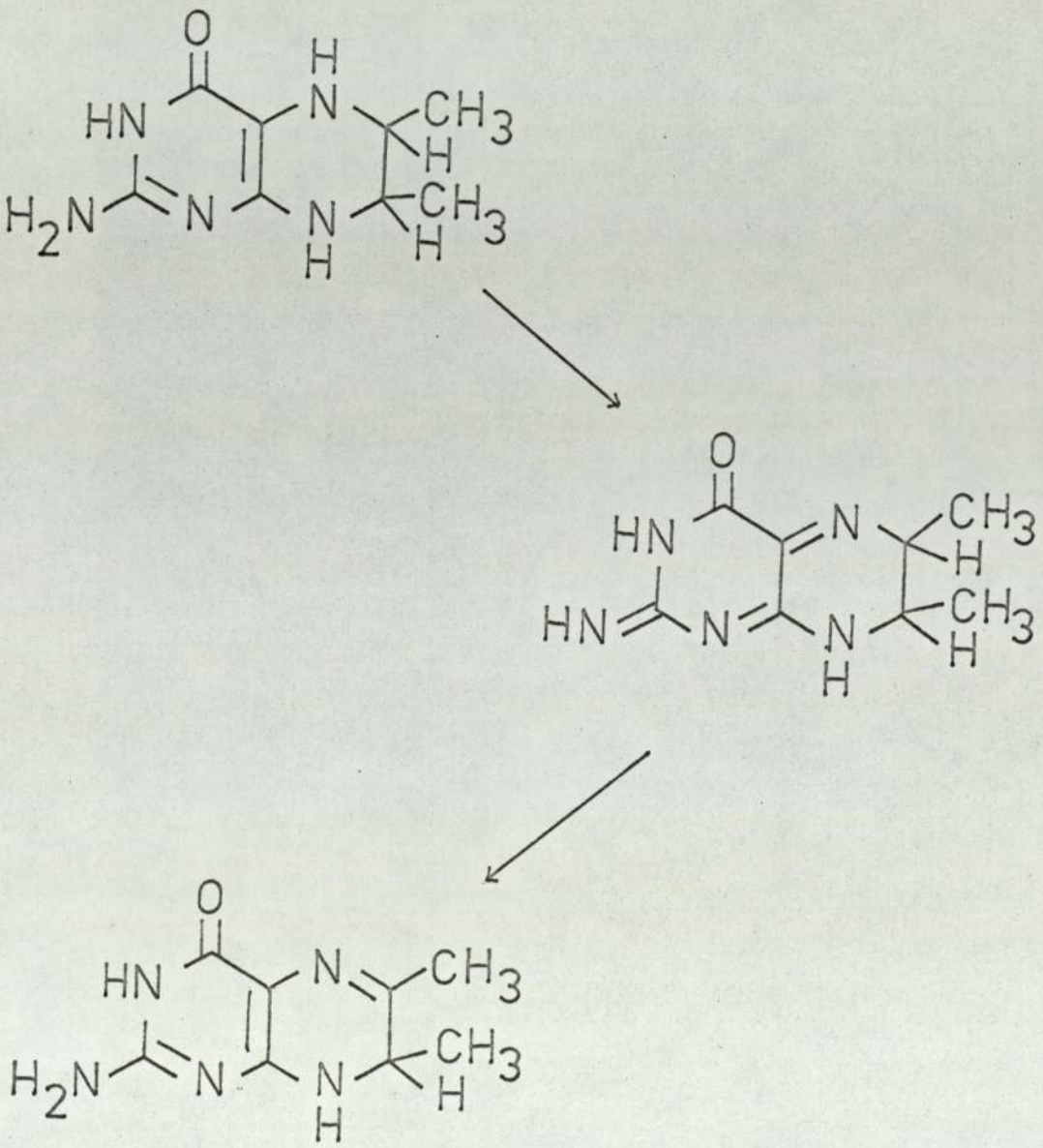
Hemmerich[105] studied the autoxidation of a tetrahydrolumazine (XXXIV) alkylated in position 8 and postulated the existence of a very labile quinonoid (XXXV) intermediate.



(XXXIV) (XXXV)

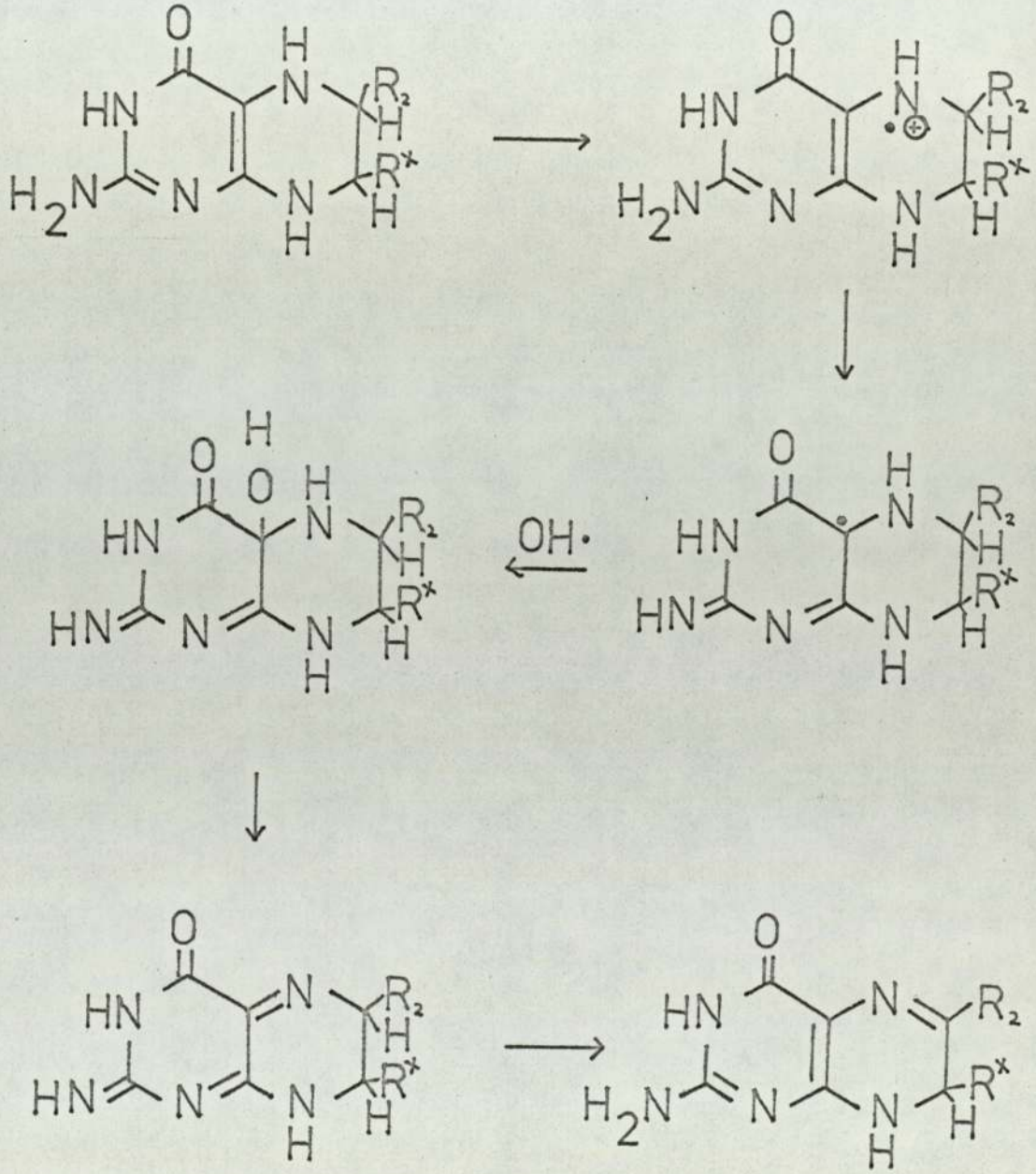
As his results were quite similar spectrophotometrically to those obtained by Kaufman (see Chapter III) in the oxidation with 2,6-dichlorophenolindophenol, he suggested that Kaufman's intermediate might also be a quinonoid structure. Kaufman[132] therefore proposed the following scheme for the oxidation of 6,7-dimethyltetrahydropterin, involving a quinonoid dihydropterin intermediate: -

Scheme VII.



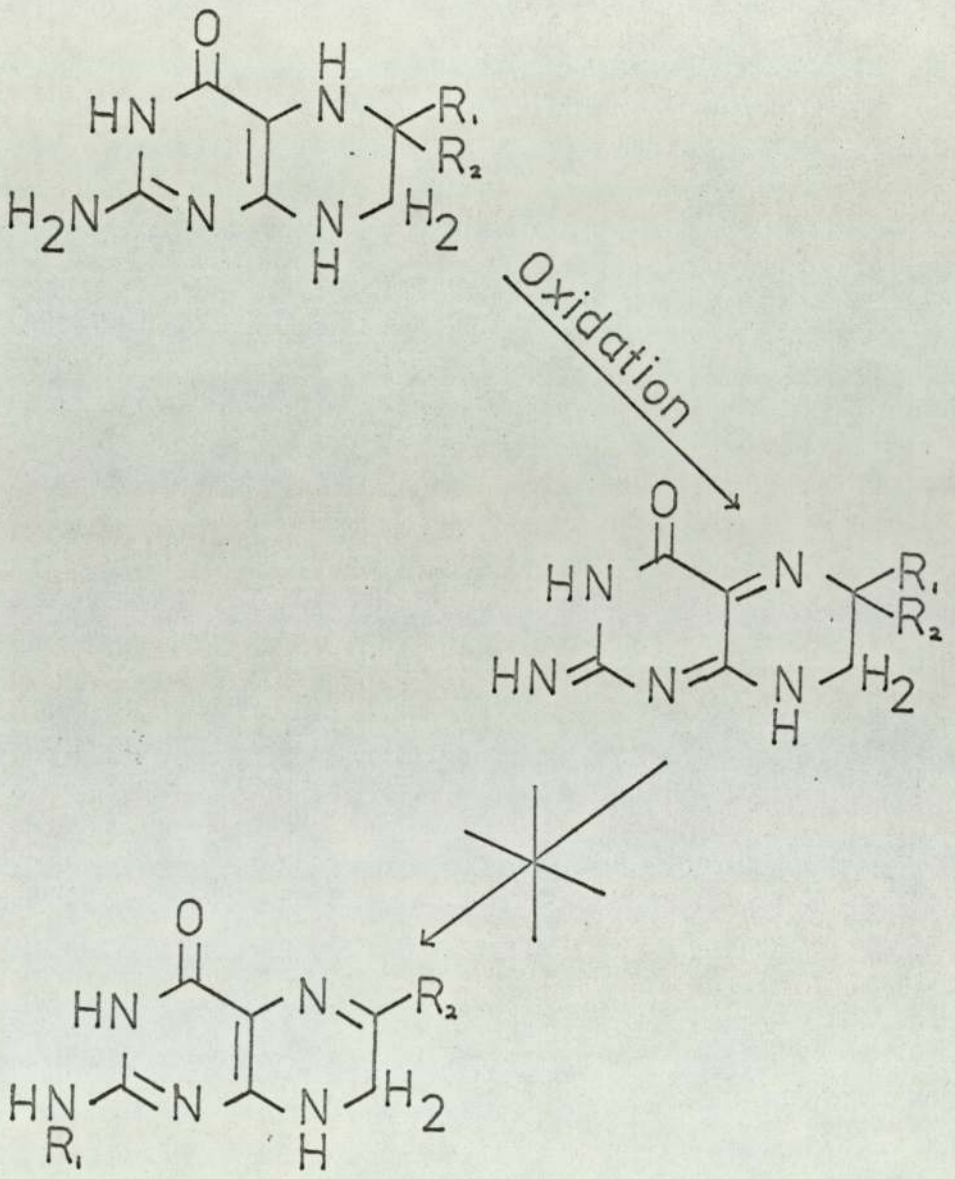
Viscontini has published several reports on the oxidation of tetrahydropterins in which a quinonoid intermediate is considered[133-138].

Scheme VIII.



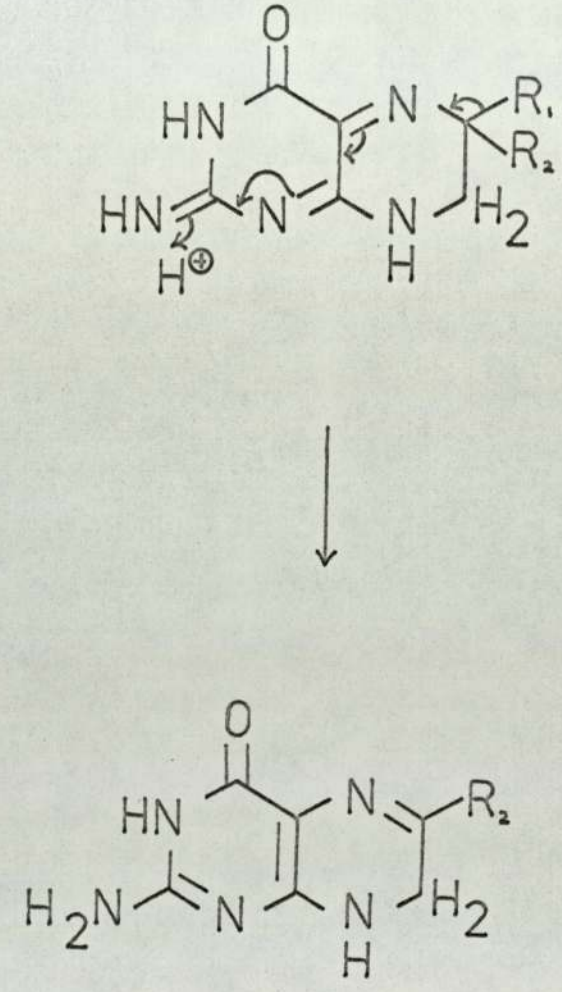
In a recent report, Viscontini et al[139] question the existence of a quinonoid dihydropterin intermediate. When 6,6-disubstituted-tetrahydropterins were oxidised no 2-substituted amino derivatives were observed. They expressed their results by the following scheme: -

Scheme IX.



This in fact is not a very likely product. The rearrangement of the quinonoid intermediate is likely to be a concerted reaction of the type (see over)

Scheme X.

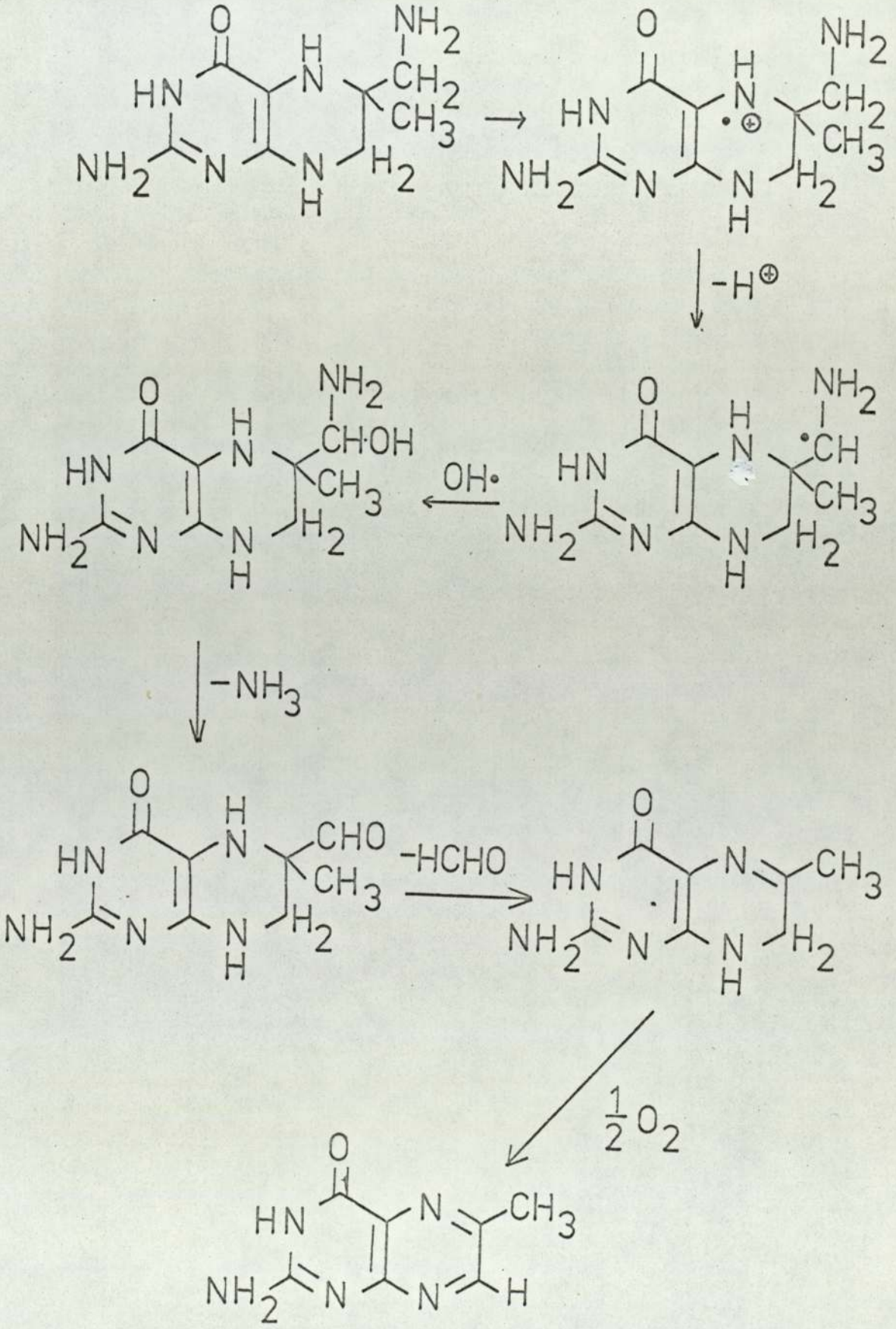


rather than a 1,7 alkyl shift. No 2-substituted amino derivative would be expected in this case. On aerial oxidation of 6-amino methyl-6-methyltetrahydropterin, the 6-aminomethyl chain was cleaved with the formation of 6-methyl-7,8 dihydropterin, formaldehyde and ammonia. When isolated, their yields accounted for 48, 30 and 35% respectively of the oxidised tetrahydropterin. When the tetrahydropterin was oxidised at pH 6.8 in the presence

of dimedone, a solid was obtained after four days, whose melting point differed from standard dimedone. Mixed melting points were not performed and the solid was regarded as the formaldehyde derivative of dimedone. No other evidence is available to support this idea of formaldehyde formation. A mechanism for this oxidation has been proposed by Viscontini and Argentini[140] (see over).

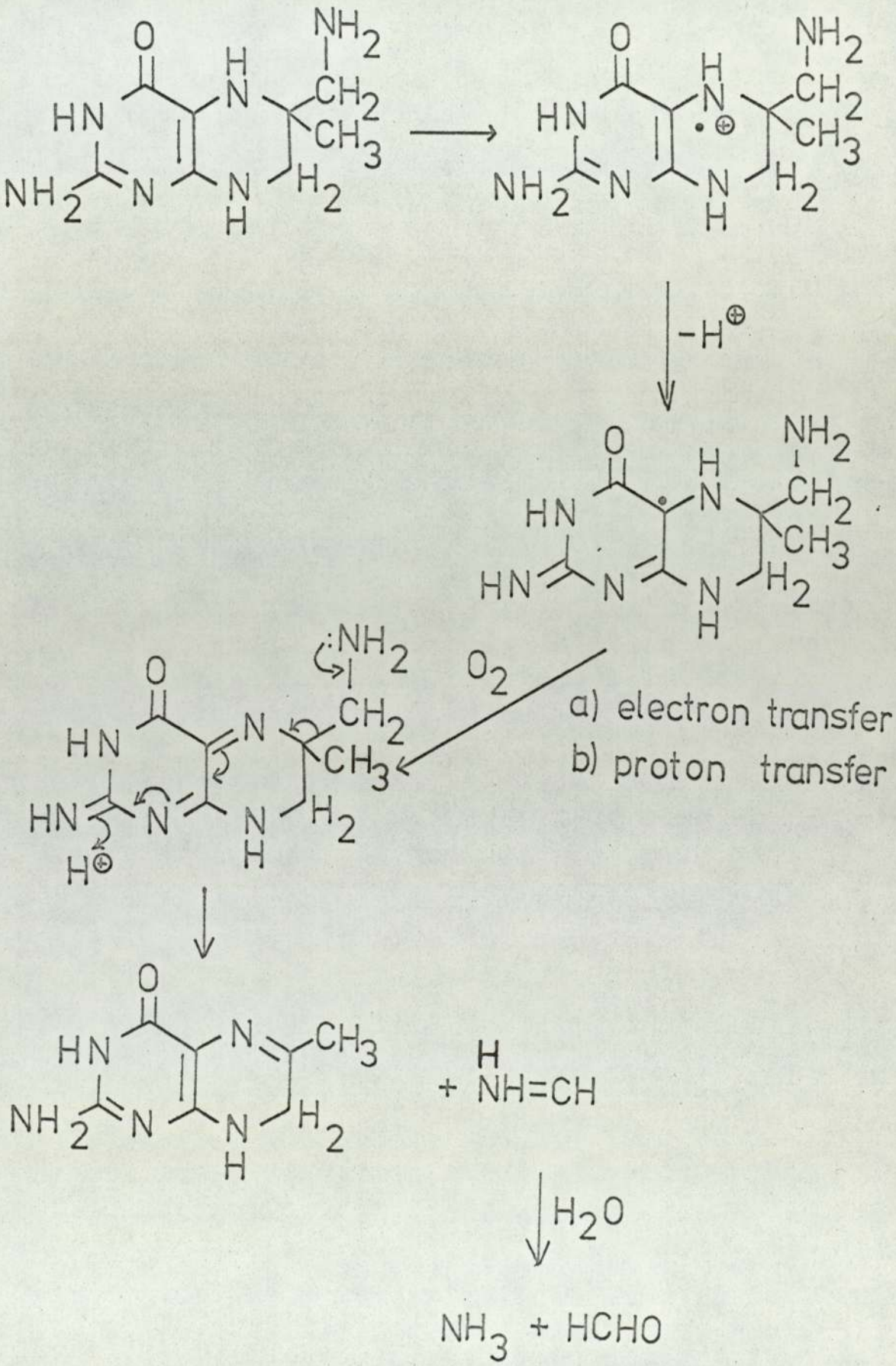
Scheme XI.

Oxidation of 6 amino methyl-6-methyl 5,6,7,8-tetra-hydropterin according to Viscontini and Argentini[140].



A more likely mechanism is: -

Scheme XII.

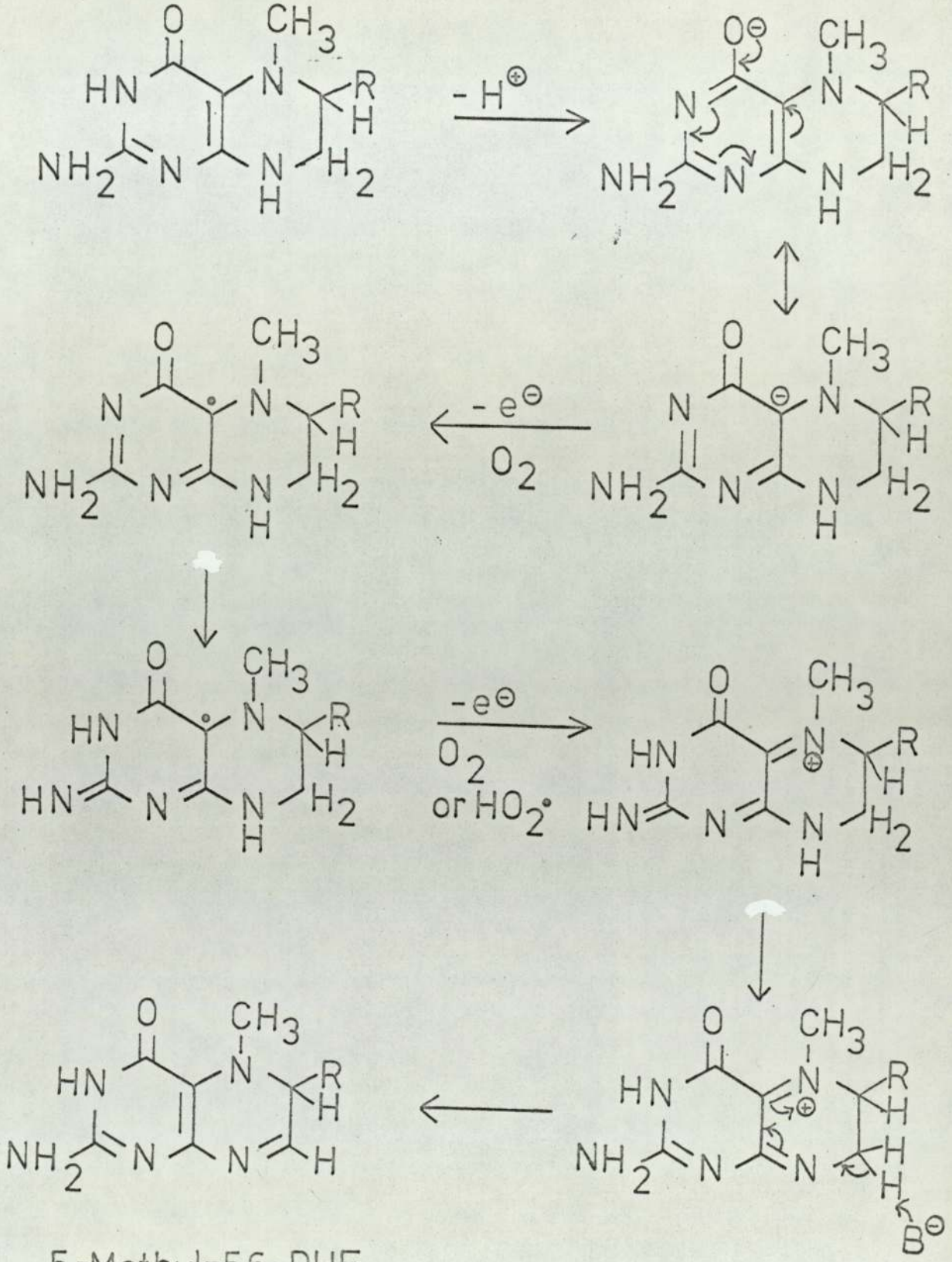


Vonderschmitt and Scrimgeour[126] have shown that Fe^{3+} oxidised tetrahydropterin to an intermediate which rearranges to 7,8-dihydropterin. This intermediate is regarded as a quinonoid dihydropterin, on the basis of the absorption spectrum and polarographic wave of the intermediate being similar to those of a quinonoid dihydropterin, the latter prepared by oxidation of tetrahydropterin with indophenol according to the method of Kaufman[141]. Using polarography, Archer and Scrimgeour[142] have studied the reduction potentials of tetrahydropterins, and interpreted the results on the basis of a quinonoid dihydropterin-tetrahydropterin couple. They have also anaerobically oxidised in situ with potassium ferricyanide 6,7-dimethyl-tetrahydropterin, 6-methyltetrahydropterin, and tetrahydropterin[140]. The changes in absorption spectra observed with such solutions are credited to the involvement of quinonoid dihydropterin intermediates. Kinetic measurements of the rearrangement of the quinonoid dihydropterin to the 7,8-dihydropterin show that the reaction is buffer catalysed, the rate of reaction being dependent on the pKa value for each buffer[117], and the rate of oxidation of reduced pterins being solvent dependent has been known for many years[143]. Isotopic experiments show that cleavage of the C-H bond at position 6 is rate-limiting in the rearrangement of the intermediate dihydro quinonoid to 7,8-dihydropteridine. Stocks-Wilson[118] misinterpreted this isotope effect by

thinking it applied to the formation of the intermediate, i.e. cleavage of C (6)-H to form C (6)-O-O-H rather than the disappearance of the intermediate. Chippel and Scrimgeour[144] have examined the oxidation of DHF and THF using potassium ferricyanide under anaerobic conditions. They propose that oxidation of THF by ferricyanide occurs via two one-electron steps, to produce a quinonoid dihydrofolate. Rembold et al[145] could not observe quinonoid intermediates in their studies on the oxidative degradation of hydrogenated pteridine cofactors. However, they believe that the assumption of a quinonoid dihydropterin does explain their results and a scheme incorporating such intermediates is now presented for the oxidation of 5-methyl THF.

Scheme XIII

Proposed mechanism for the autoxidation of 5-methyl THF.



5-Methyl-5,6-DHF

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APPENDIX

Index

Tests on the Reversal of Methotrexate (XI) Toxicity in Mice using 5-isobutyl THF.

Experimental (carried out by Dr. Searle, Department of Cancer Studies, Birmingham University).

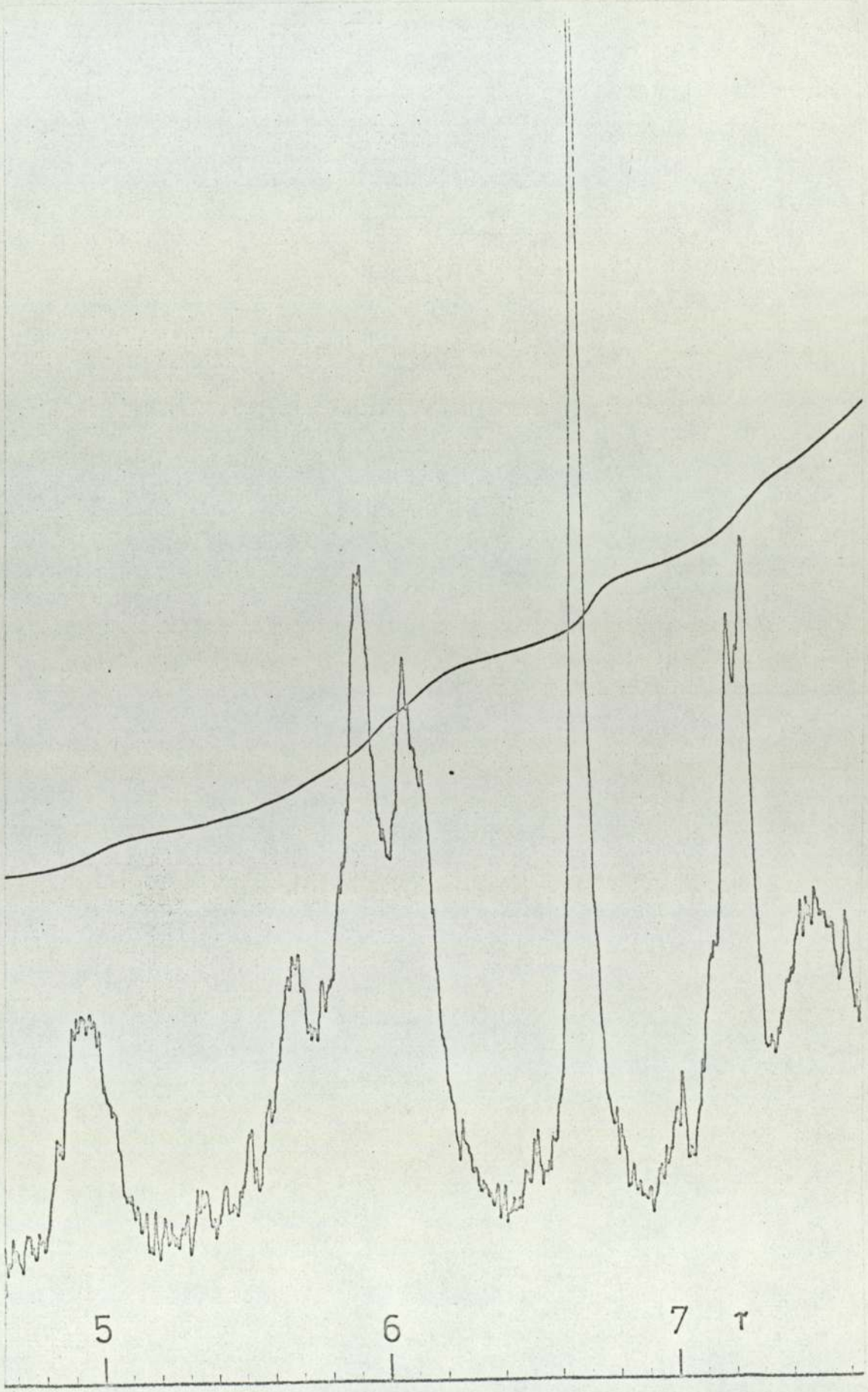
Male C57BL/BcrXIF/BcrF$_1$ hybrid mice were housed in plastic (Perspex) boxes, each containing 4 mice, and were fed cube diet 41B and tap water ad libitum. Groups comprised 8 mice, each weighing 27 - 29 g. at the time of the first injections. They were weighed several times before starting the experiments, and daily during the experiments. Deaths were also recorded daily. In all experiments methotrexate was administered on 5 consecutive afternoons (days 1 - 5) and the test compound was injected 5 hours earlier into the opposite flanks of the animals. 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. 5-isobutyl THF was administered within an hour of dissolution in 0.9 per cent saline. Control mice received the same volume of saline. The dosages and results are given in Table VI.

The programme used to calculate the theoretical percentage concentrations of 5-methyl THF zwitterion is given below.

```
10A C,P1,DP,P2,K1P,K2P,K3P
20SET K1=FEXP(-K1P*FLOG(10))
30SET K2=FEXP(-K3P*FLOG(10))
40SET K3=FEXP(-K3P*FLOG(10))
50TYPE !!!,"FOR CONCENTRATION ",%7.06,C
60T !,"    PH      H3A          H2A        HA     A",!
70FOR PH=P1,DP,P2;DO 80/150
76QUIT
80SET H=FEXP(-PH*FLOG(10))
90SET ONE=K1/H; SET TWO=K1*K2/H 2
100SET THREE=K1*K2*K3/H 3
110SET A3=1/(1+ONE+TWO+THREE)
120SET A2=A3*ONE
130SET A1=A3*TWO
140SET A0=A3*THREE
150T !,%4.02,PH.%11.08,C*A3,C*A2,C*A1,C*A0
```

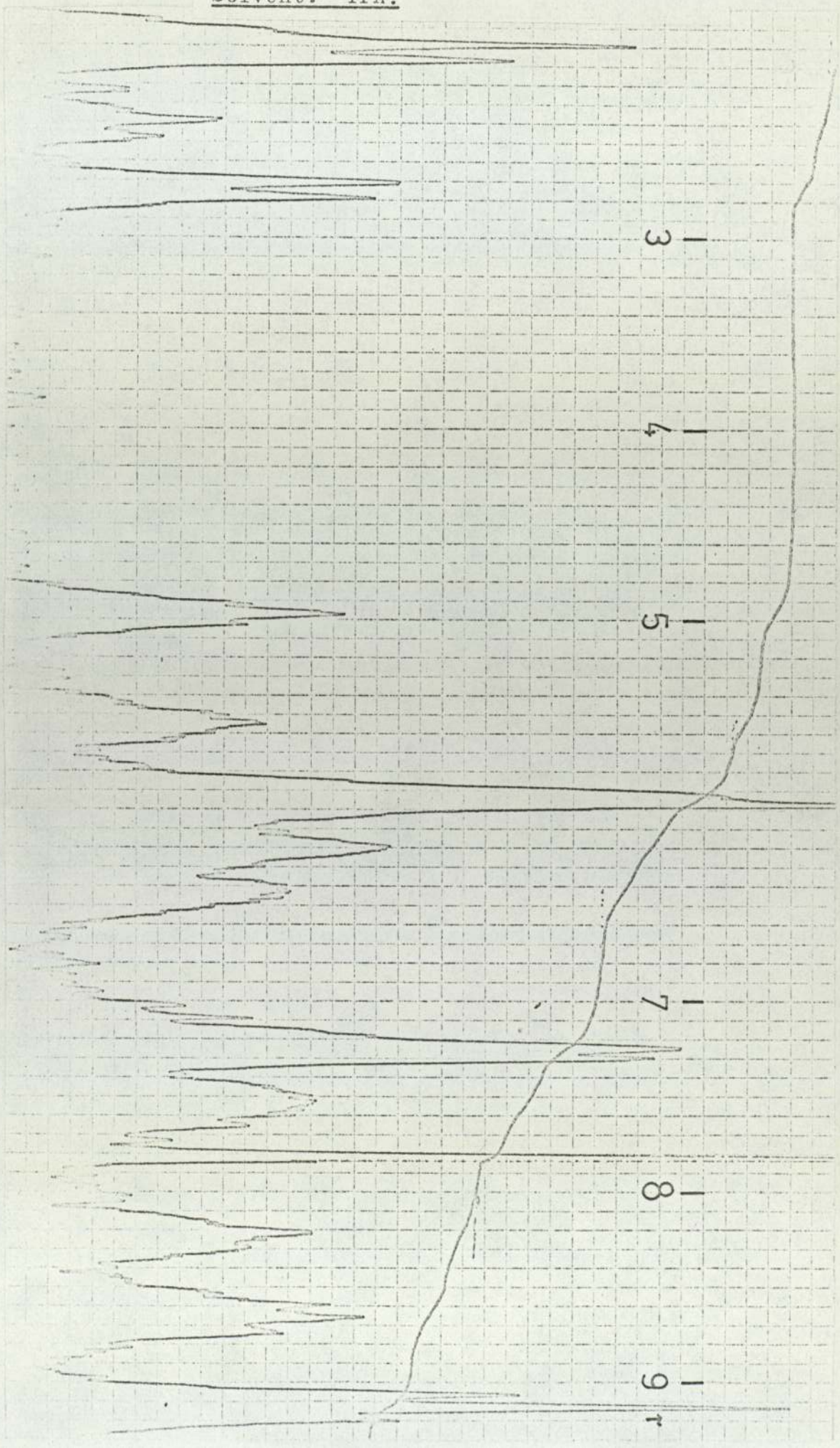
^{1}H n.m.r. spectrum of 5-methyl THF.

Solvent: TFA.



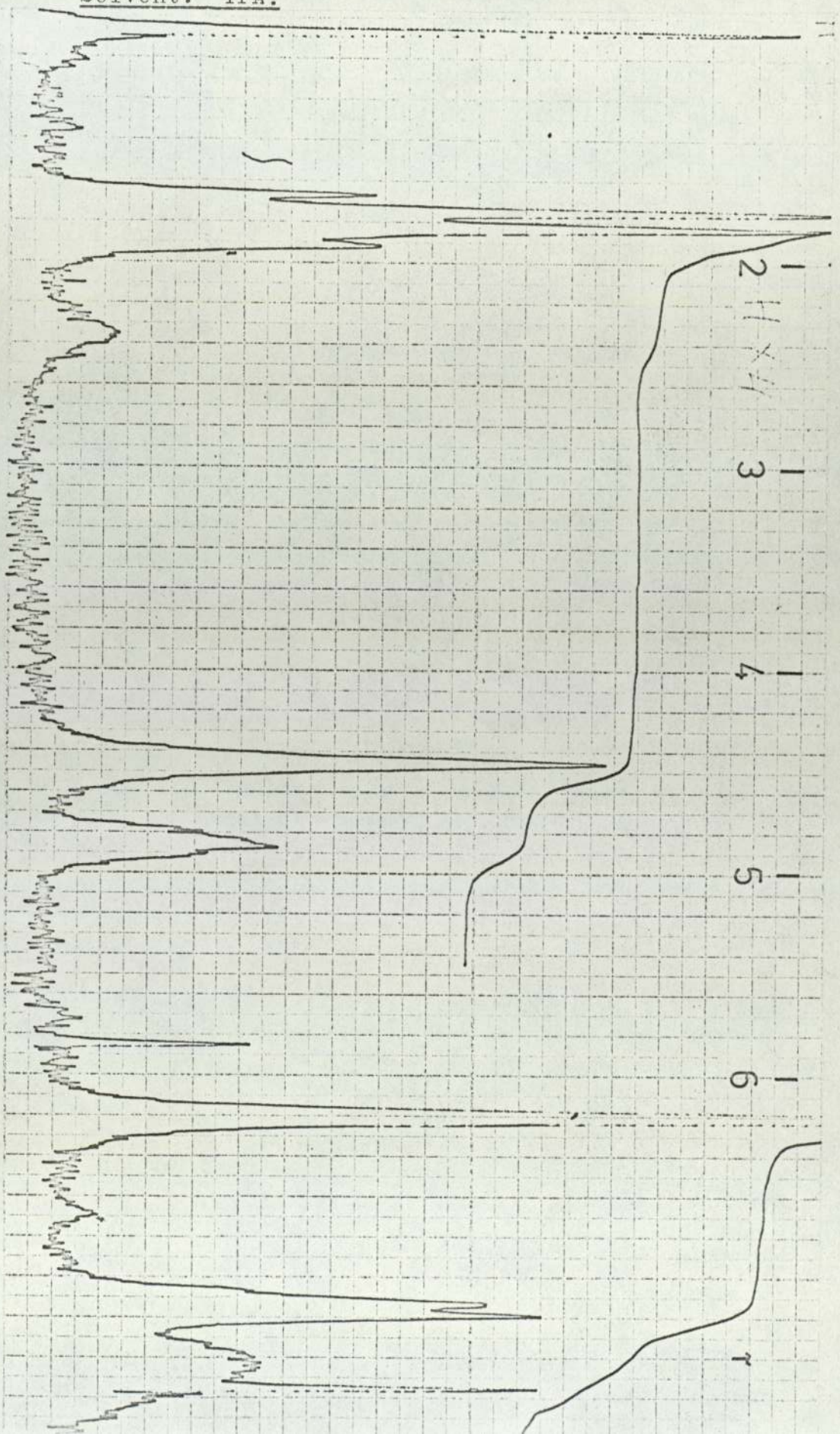
^{1}H n.m.r. spectrum of 5-n-butyl THF

Solvent: TFA.



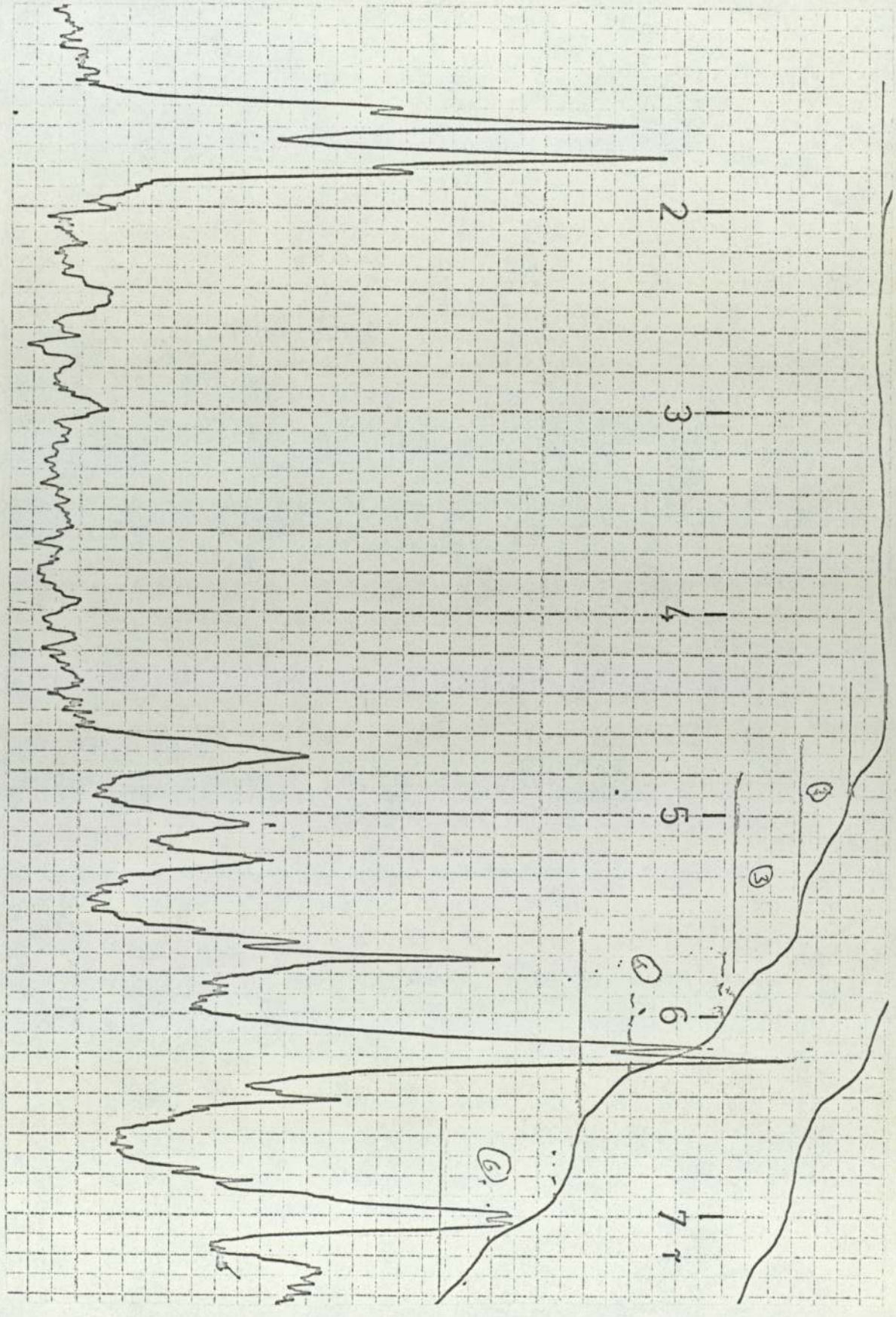
^{1}H n.m.r. spectrum of methotrexate.

Solvent: TFA.



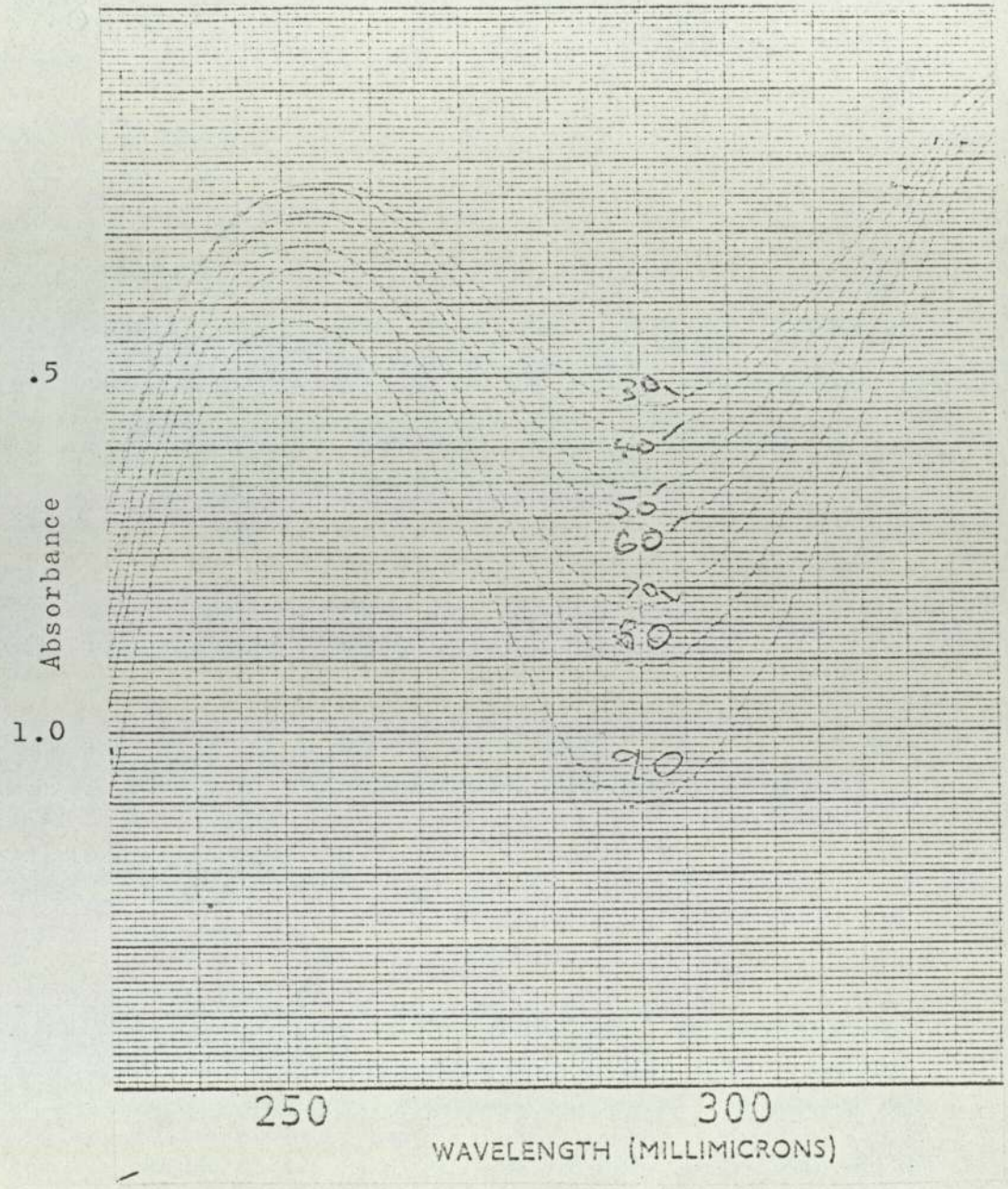
^{1}H n.m.r. spectrum of tetrahydromethotrexate.

Solvent: TFA.



U.v. spectra of fractions coming off the column in a preparation of 5-methyl THF.

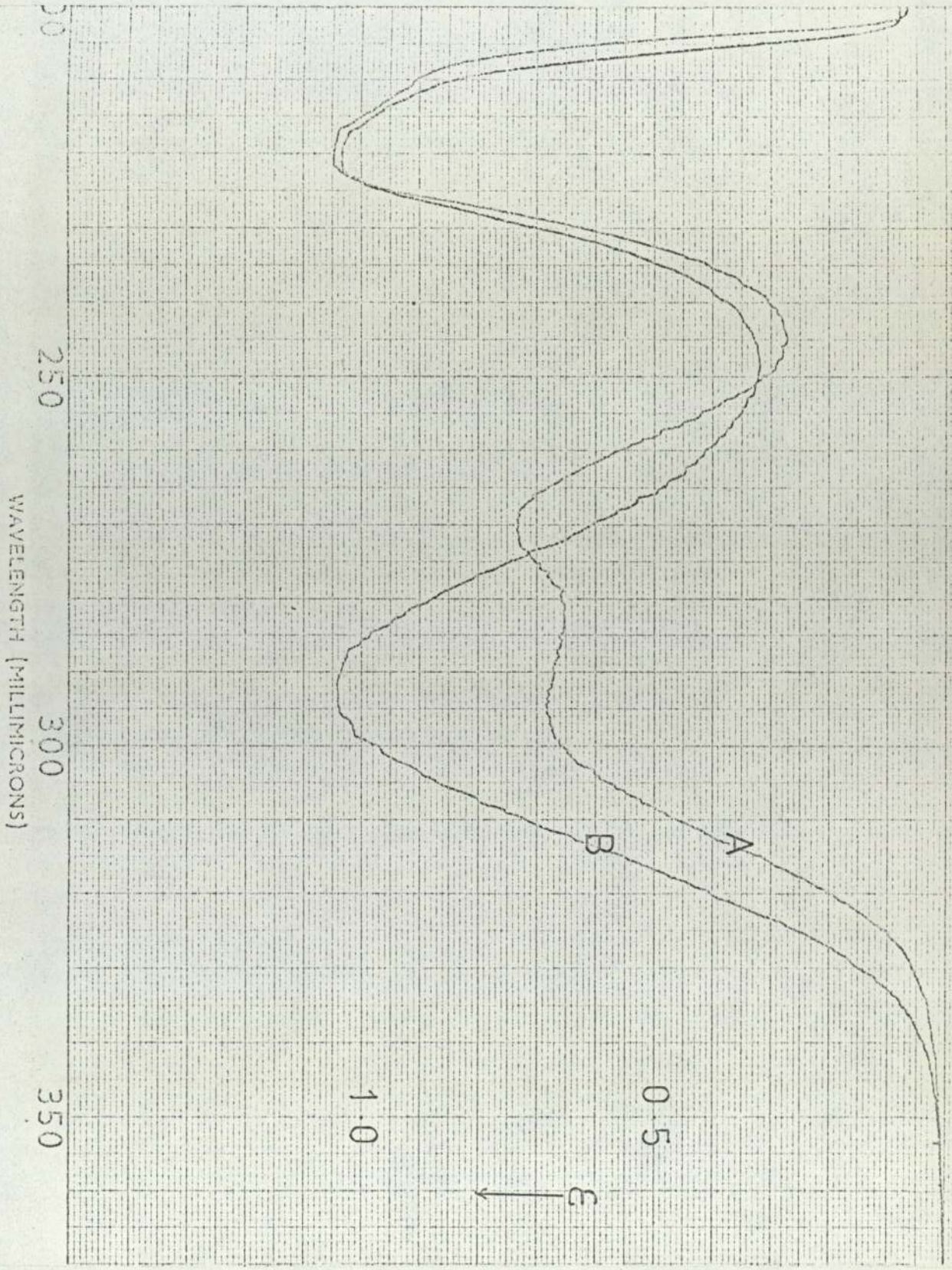
Solvent: 0.1M phosphate buffer pH 7.



U.v. spectra of 5-methyl THF (Ba salt).

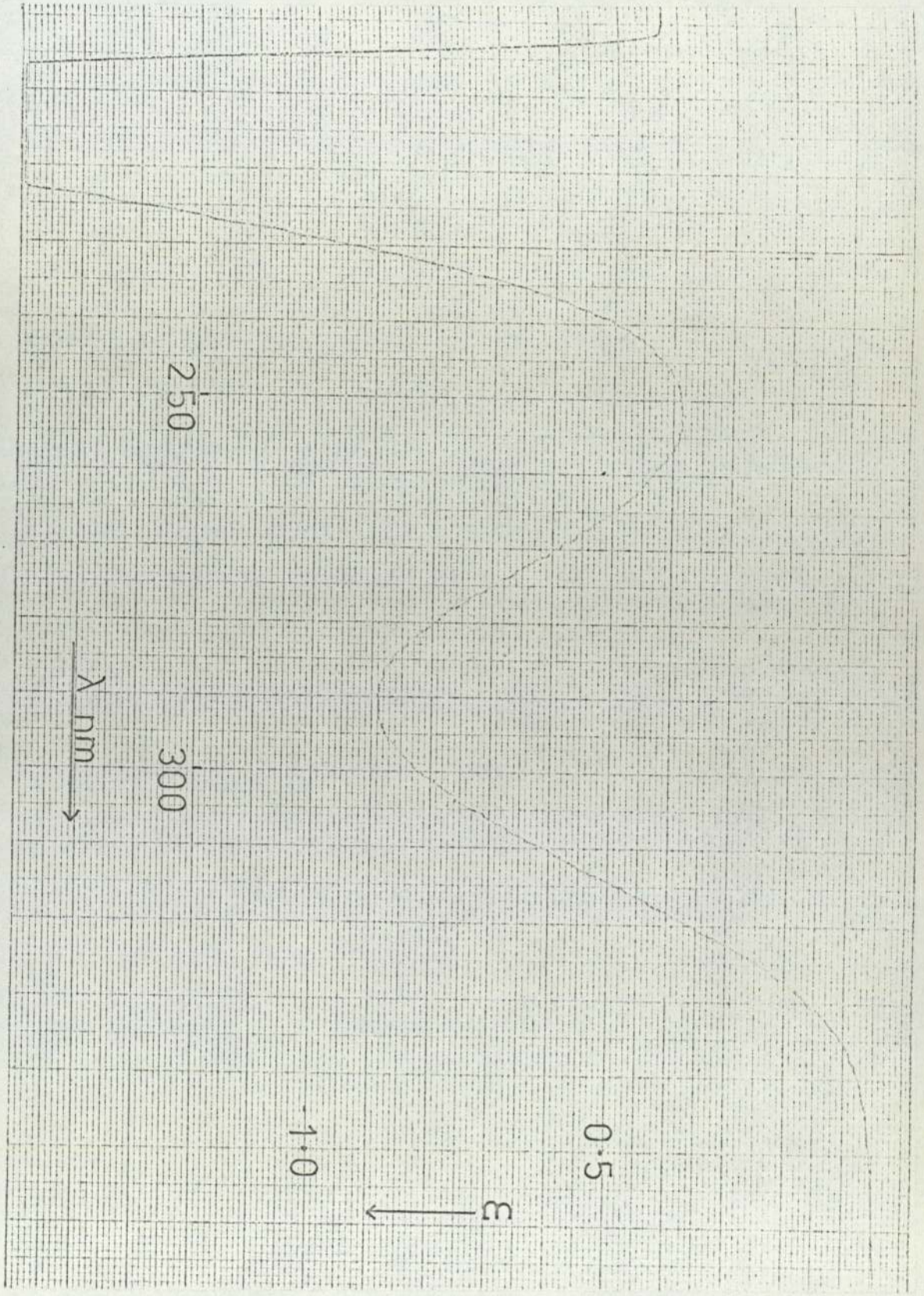
Solvents: 0.1M HCl pH 1; curve A.

0.1M phosphate buffer pH 7 curve B.



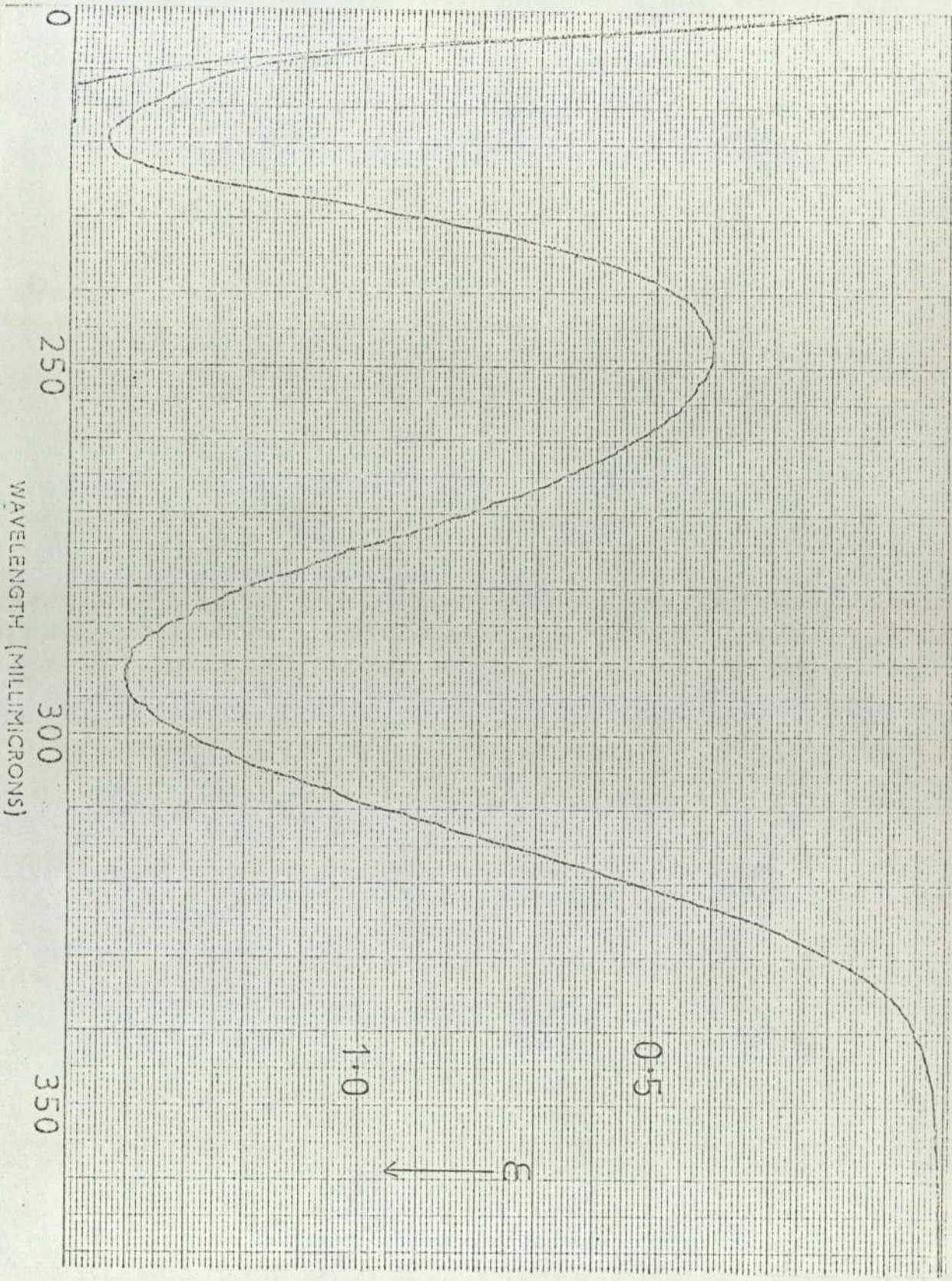
U.v. spectrum of 5-methyl THF (Ba salt)

Solvent: 0.1M NaOH pH 13.



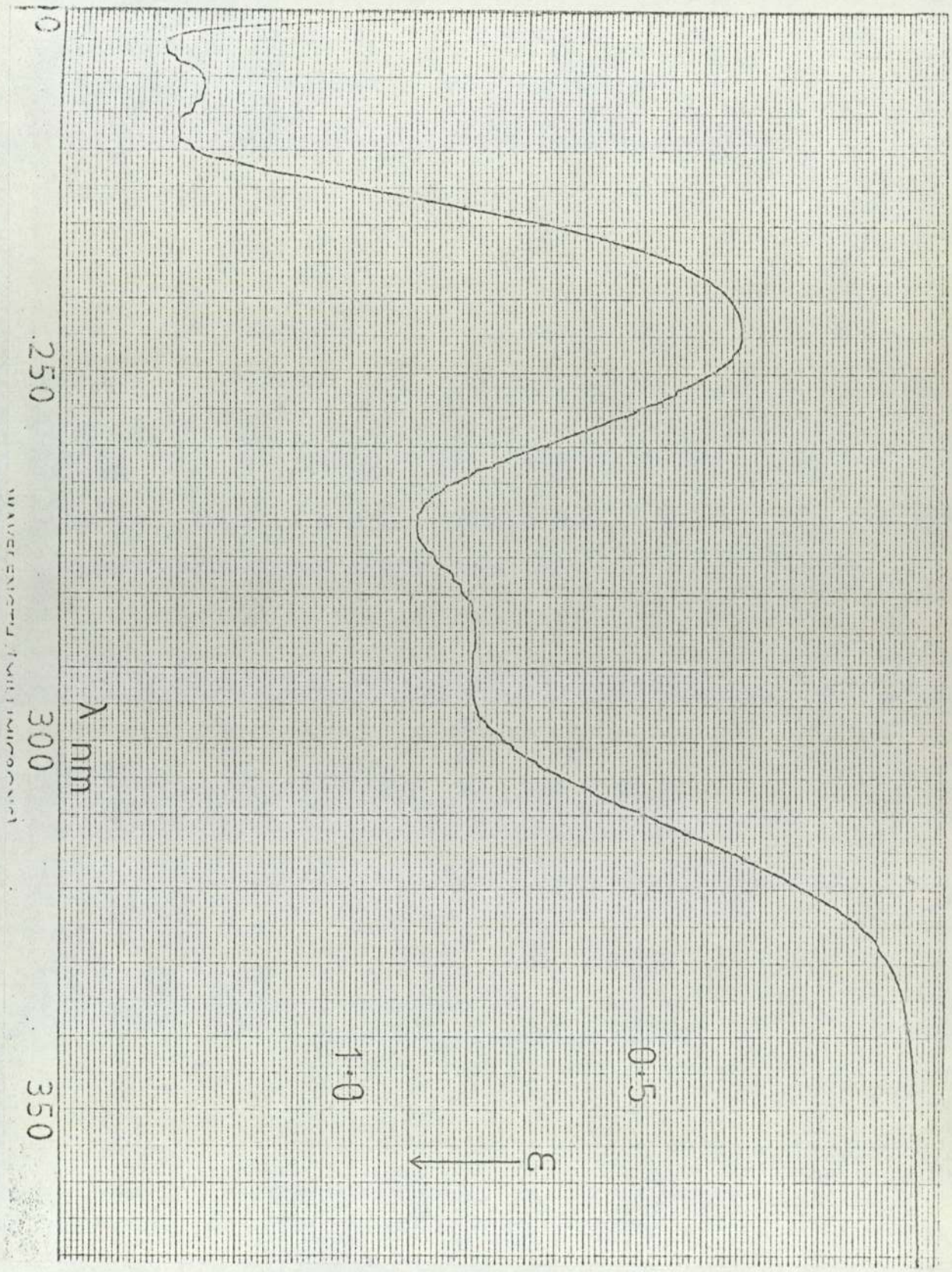
U.v. spectrum of 5-methyl THF (Ca salt)

Solvent: 0.1M phosphate buffer pH 7.



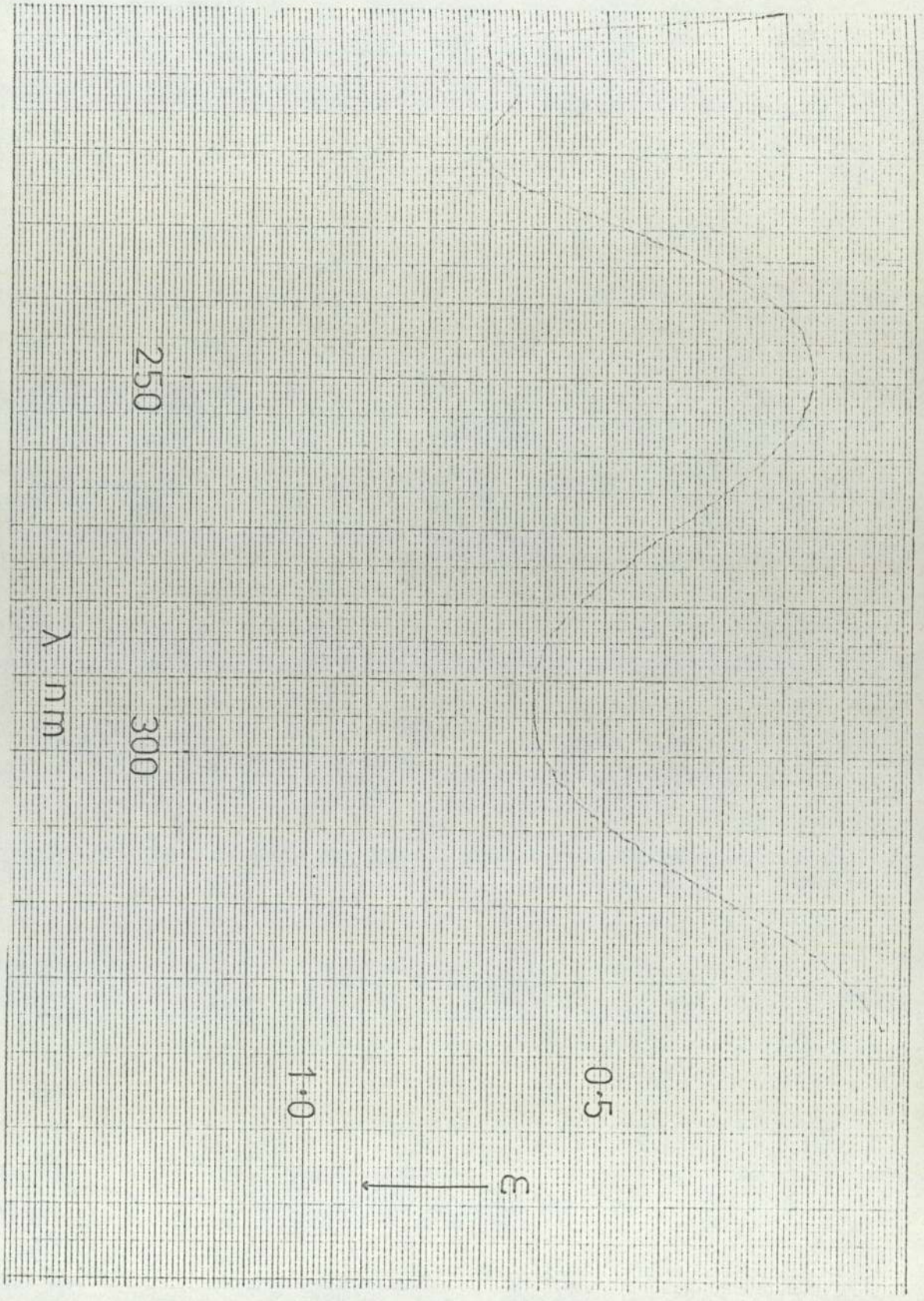
U.v. spectrum of 5-n-butyl THF (Ba salt)

Solvent: 0.1M HCl pH 1.



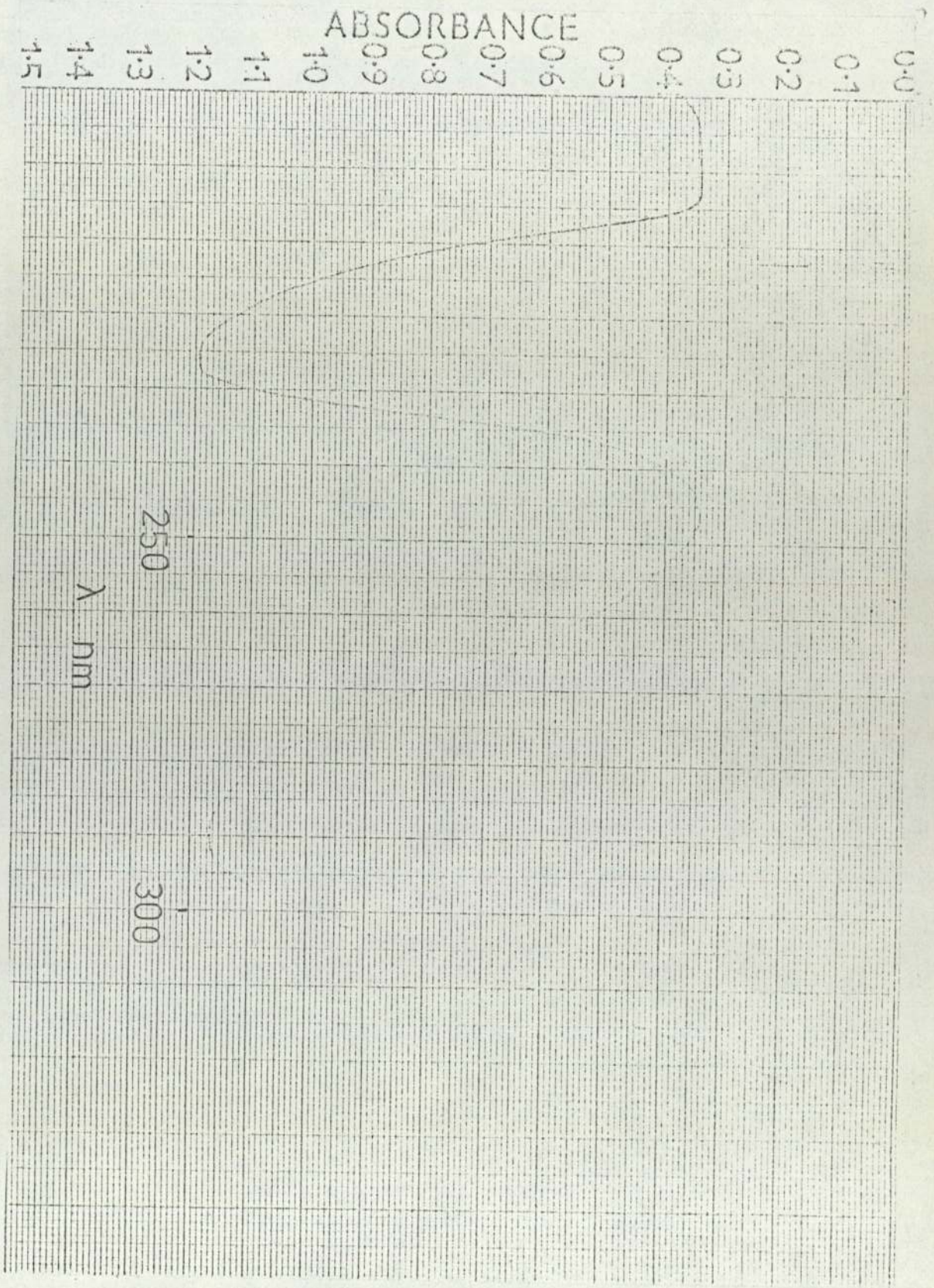
U.v. spectrum of 5-n-butyl THF (Ba salt).

Solvent: 0.1M phosphate buffer pH 7.



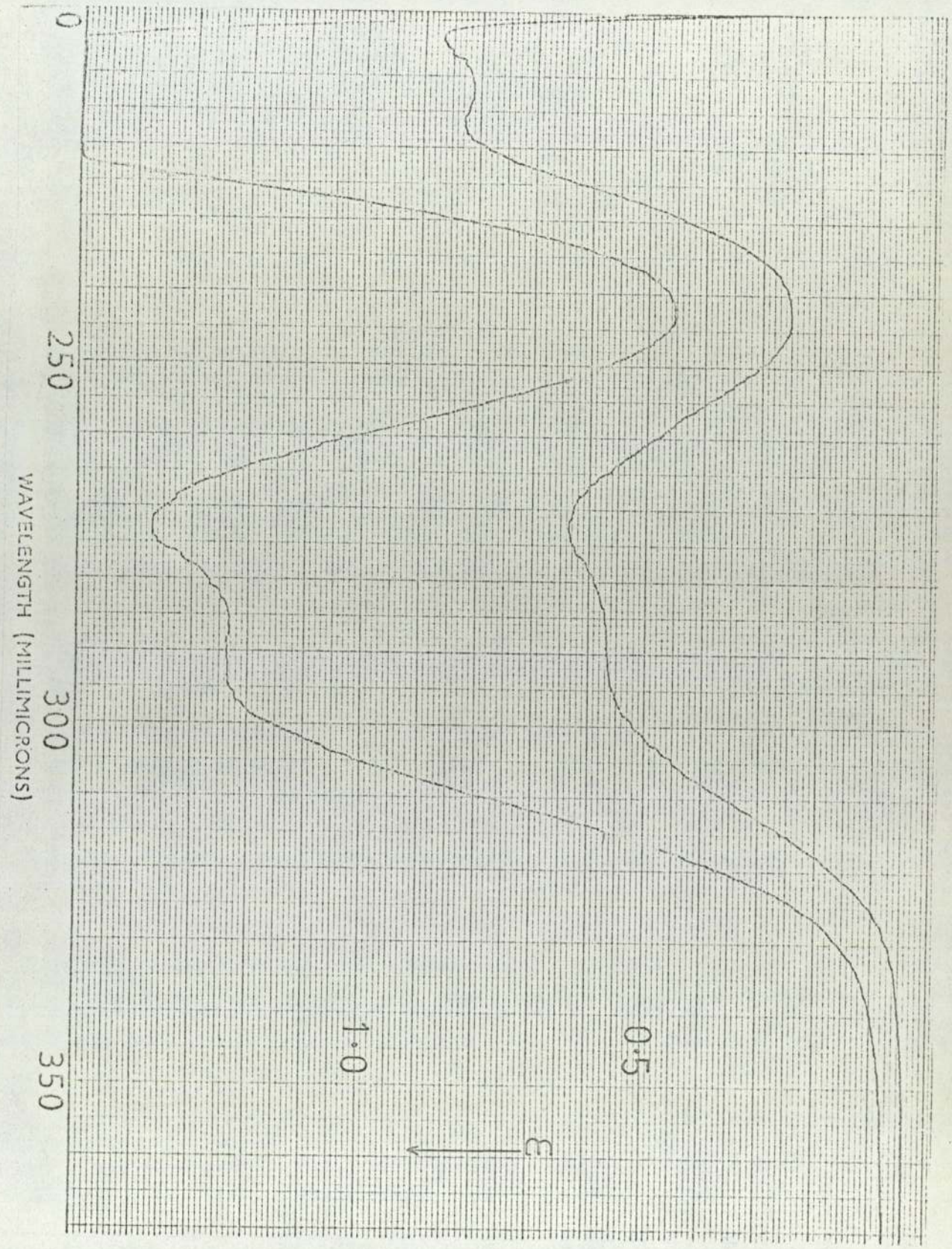
U.v. spectrum of 5-n-butyl THF (Ba salt).

Solvent: 0.1M NaOH pH 13.



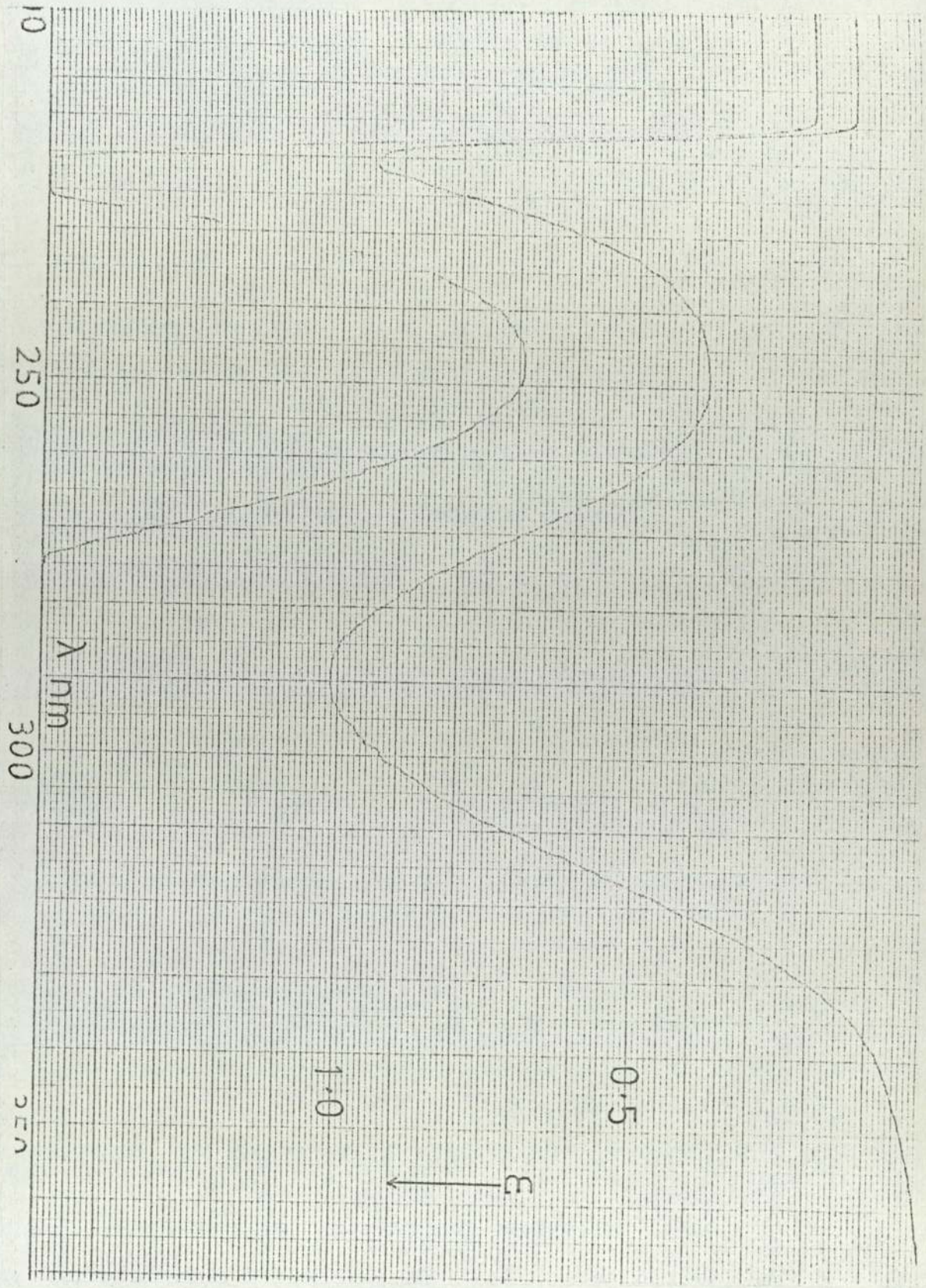
U.v. spectrum of 5-isobutyl THF (Ba salt)

Solvent: 0.1M HCl pH 1.



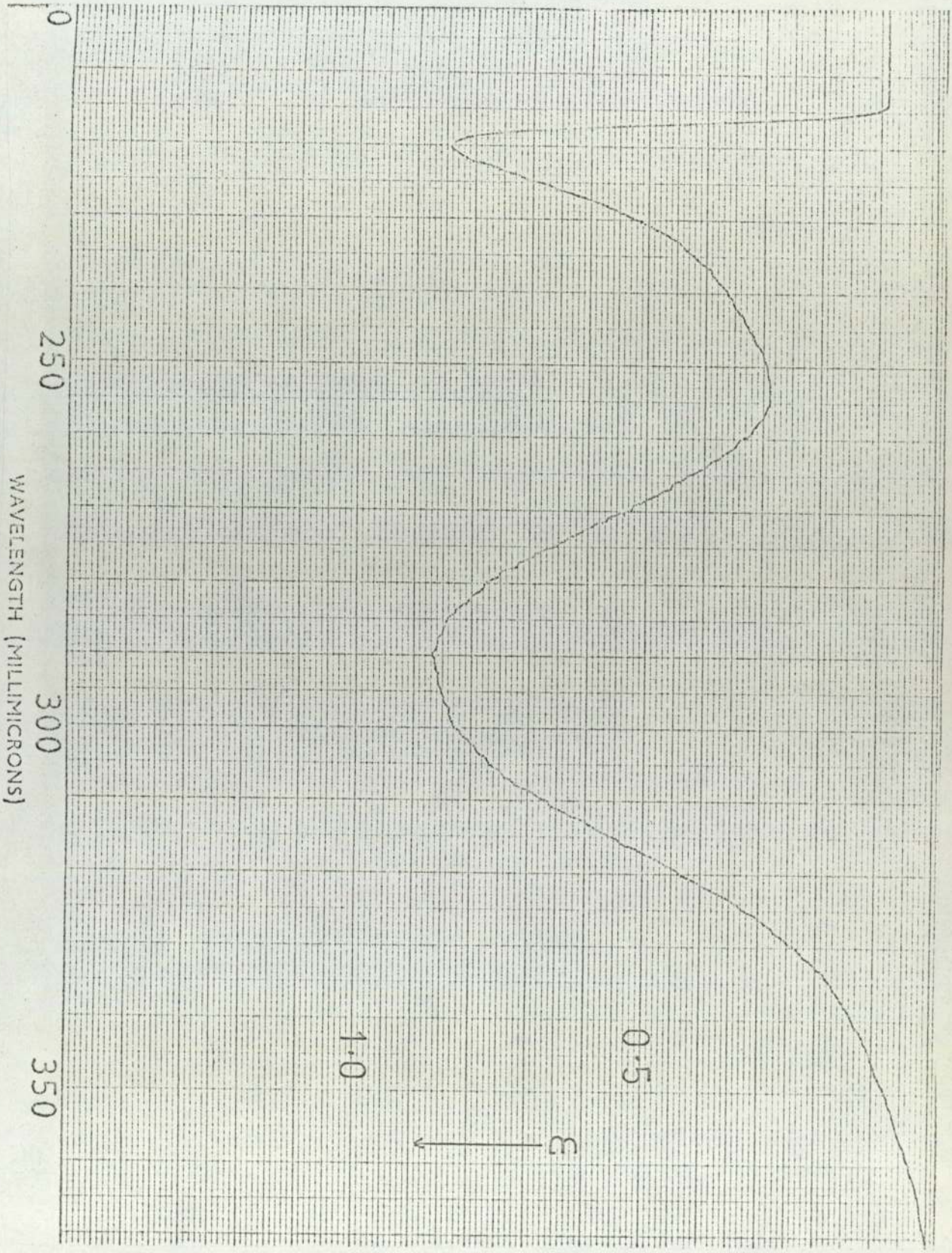
U.v. spectrum of 5-isobutyl THF (Ba salt)

Solvent: 0.1M phosphate buffer pH 7.



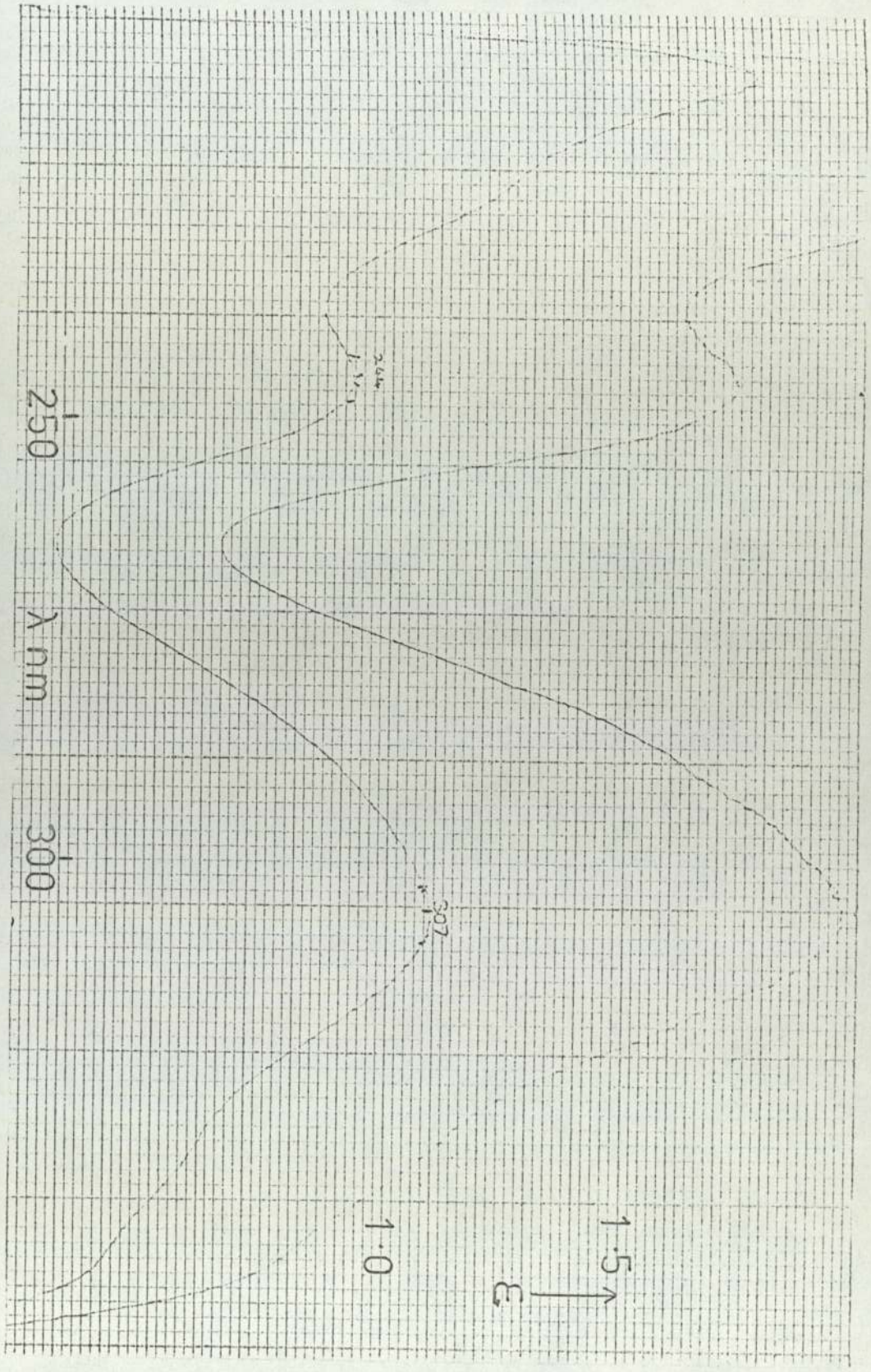
U.v. spectrum of 5-isobutyl THF (Ba salt).

Solvent: 0.1M NaOH pH 13.



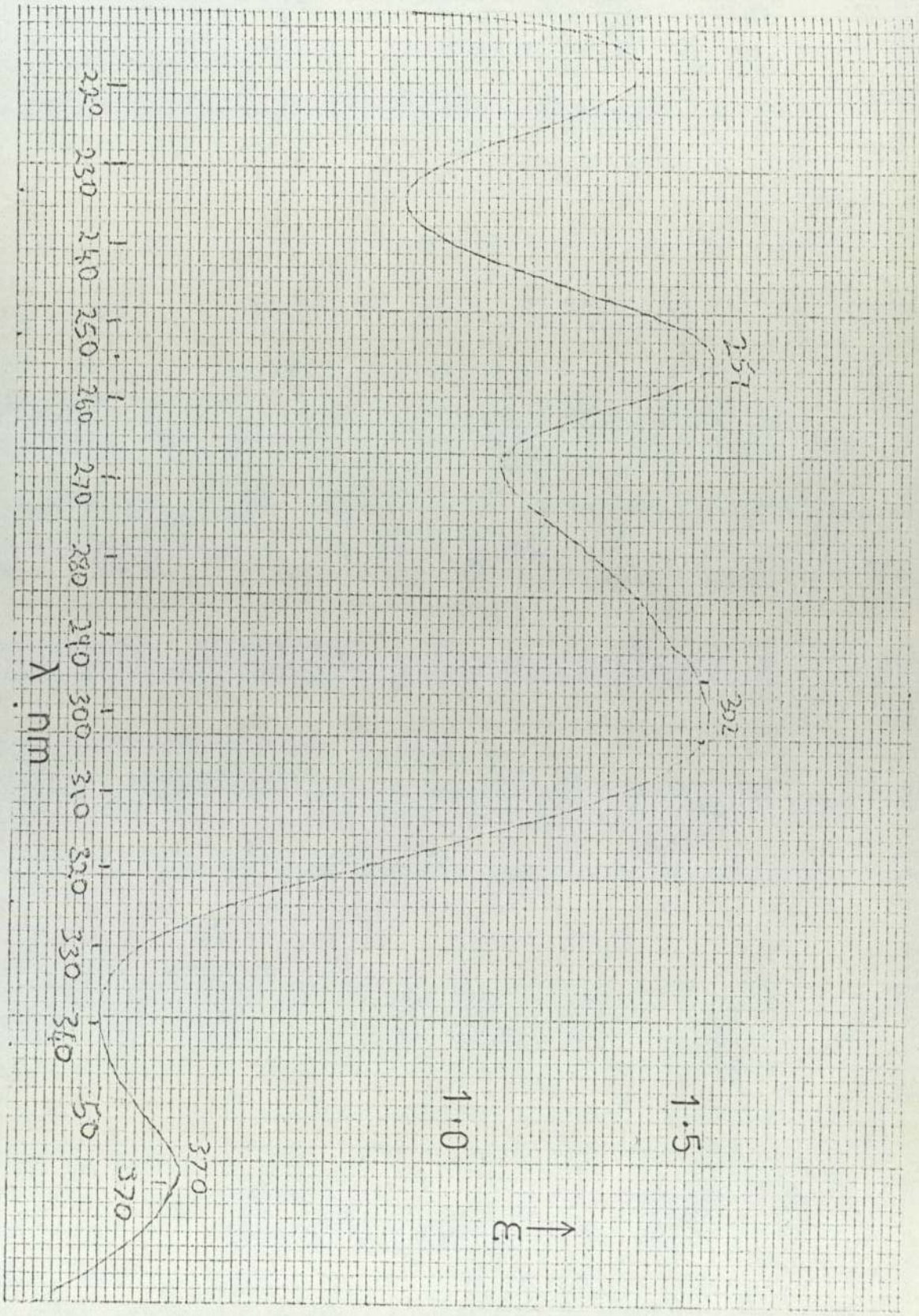
U.v. spectrum of Methotrexate.

Solvent: 0.1M HCl pH 1.



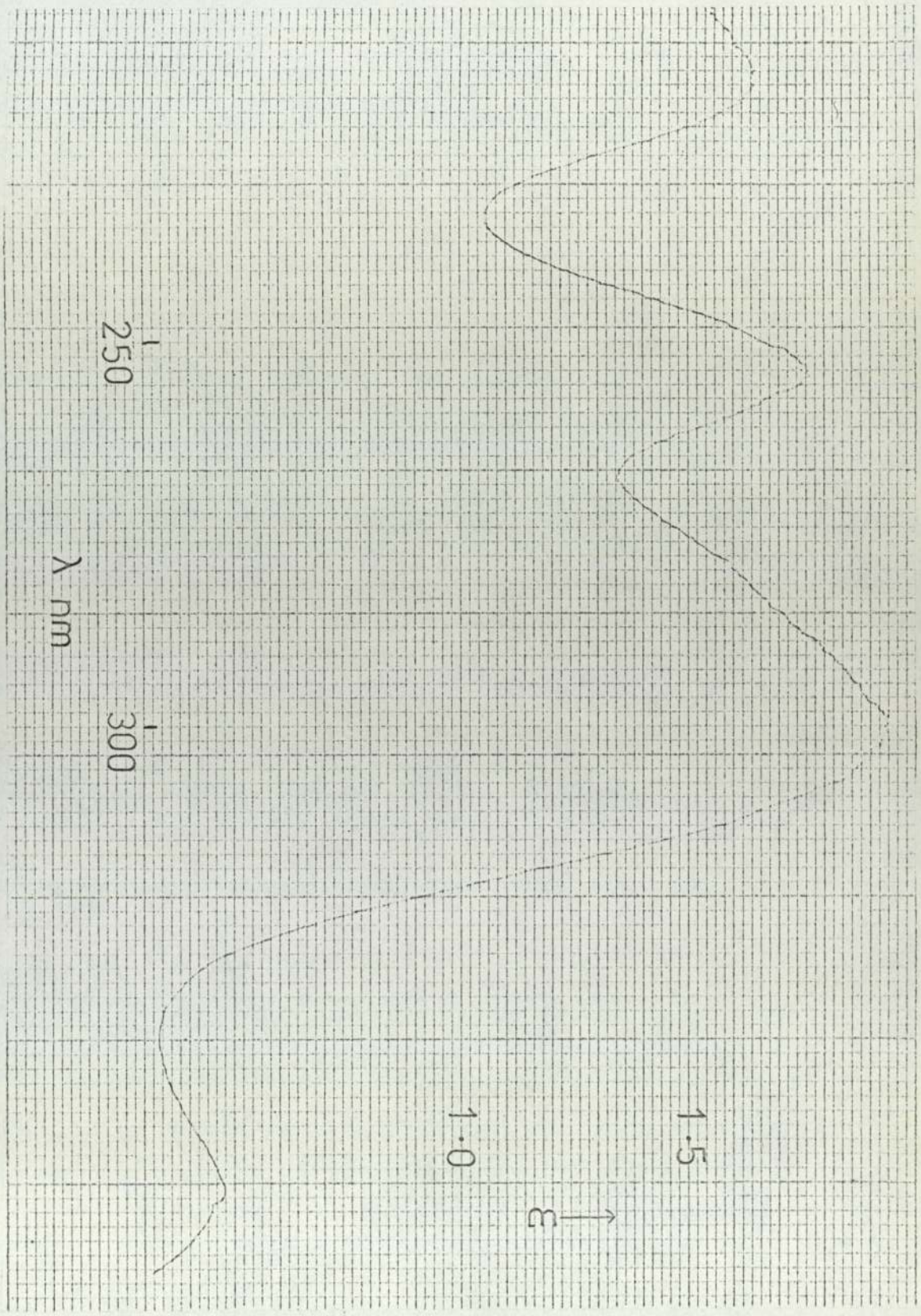
U.v. spectrum of Methotrexate.

Solvent: 0.1M phosphate buffer pH 7.



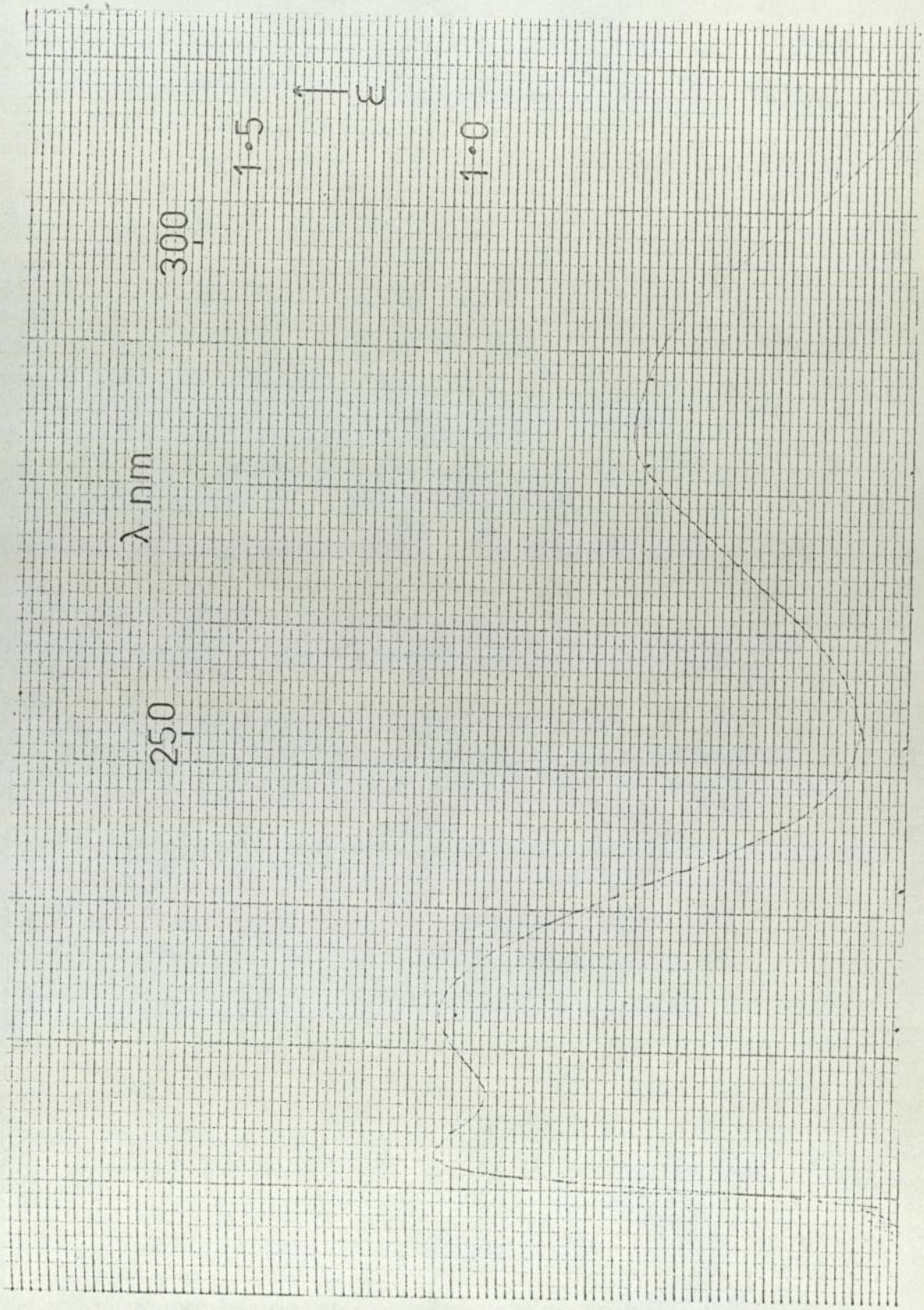
U.v. spectrum of Methotrexate.

Solvent: 0.1M NaOH pH 13.



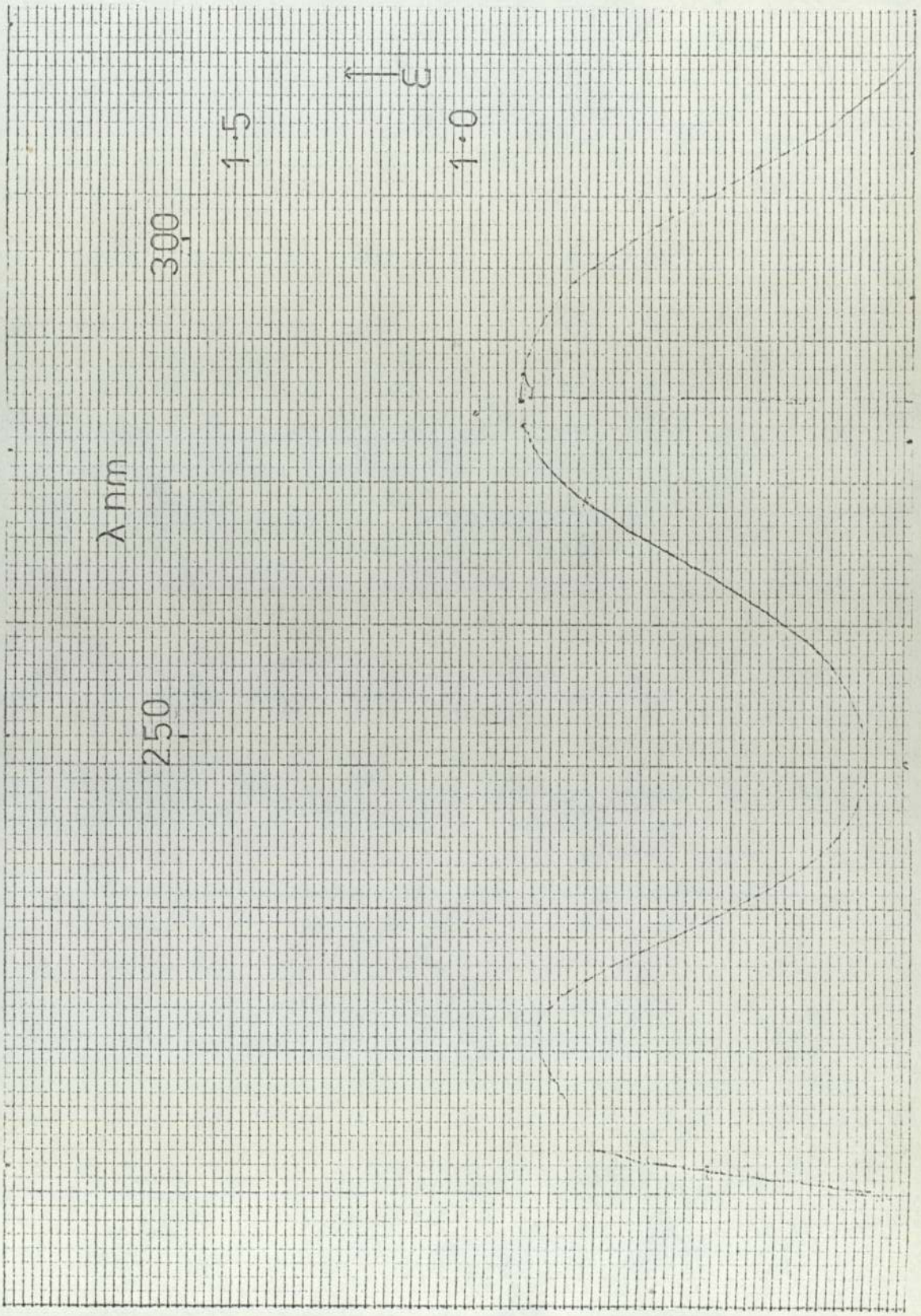
U.v. spectrum of THM

Solvent: 0.1M HCl pH 1.



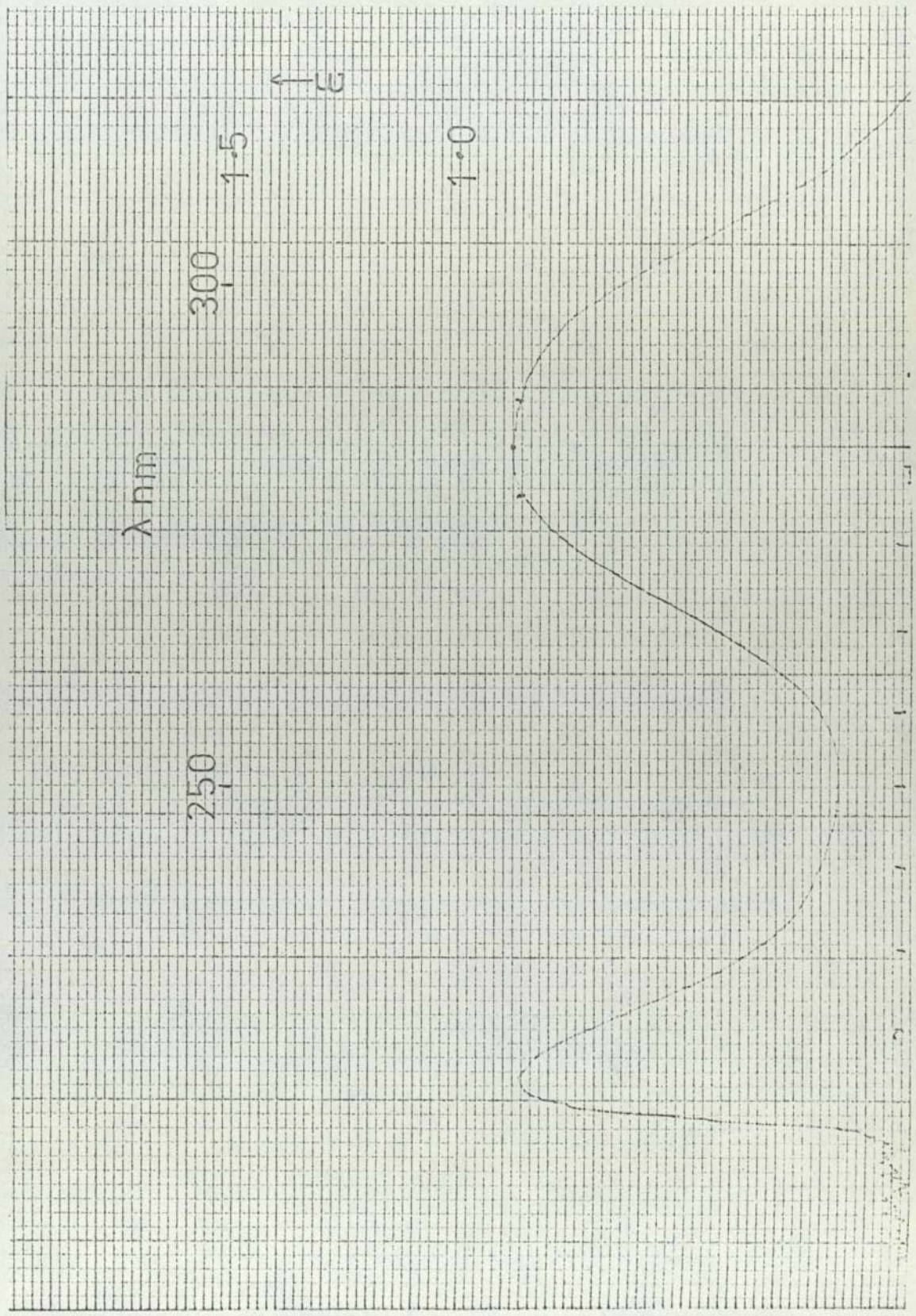
U.v. spectrum of THM.

Solvent: 0.1M phosphate buffer pH 7.



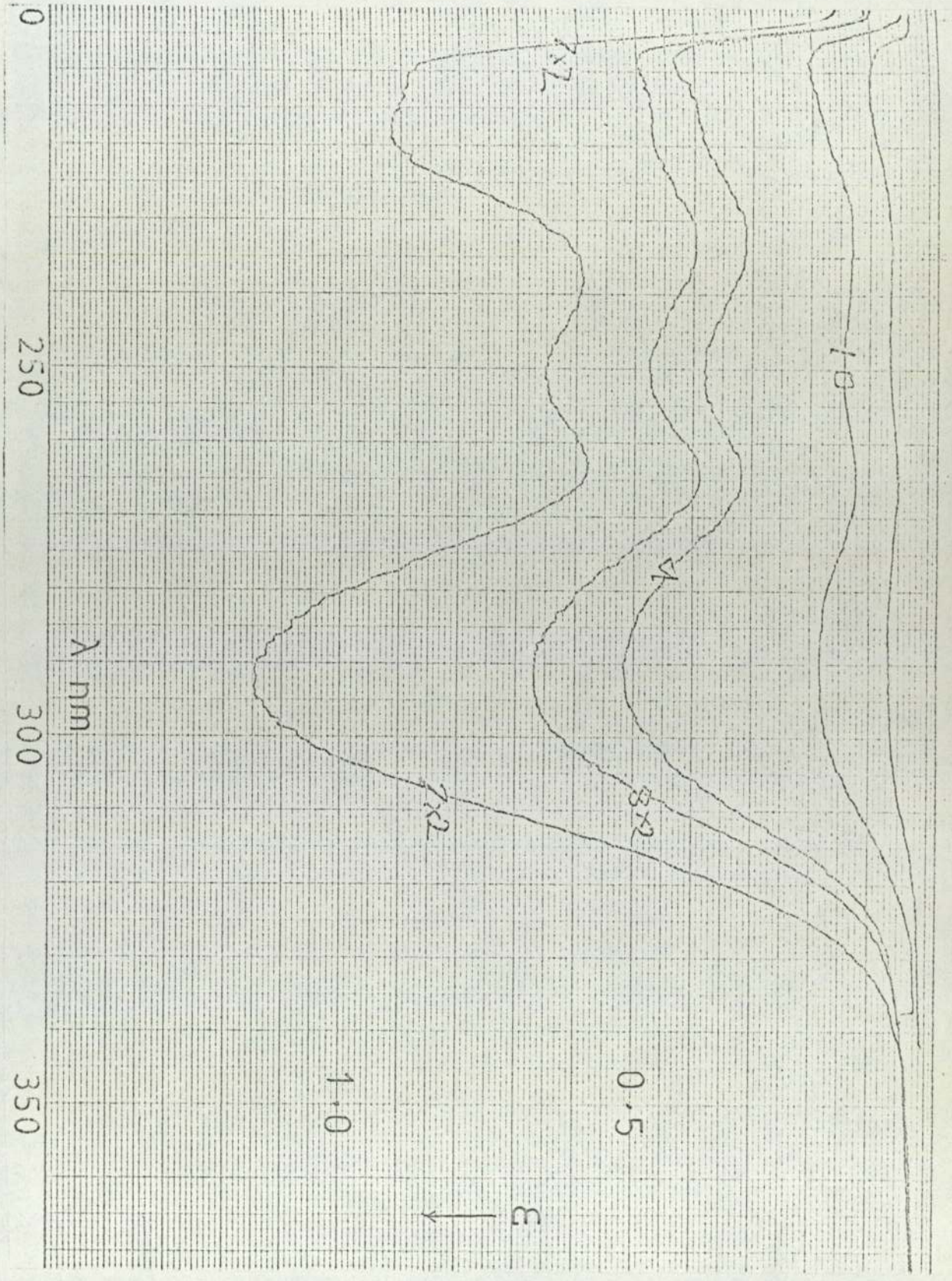
U.v. spectrum of THM.

Solvent: 0.1M NaOH pH 13.



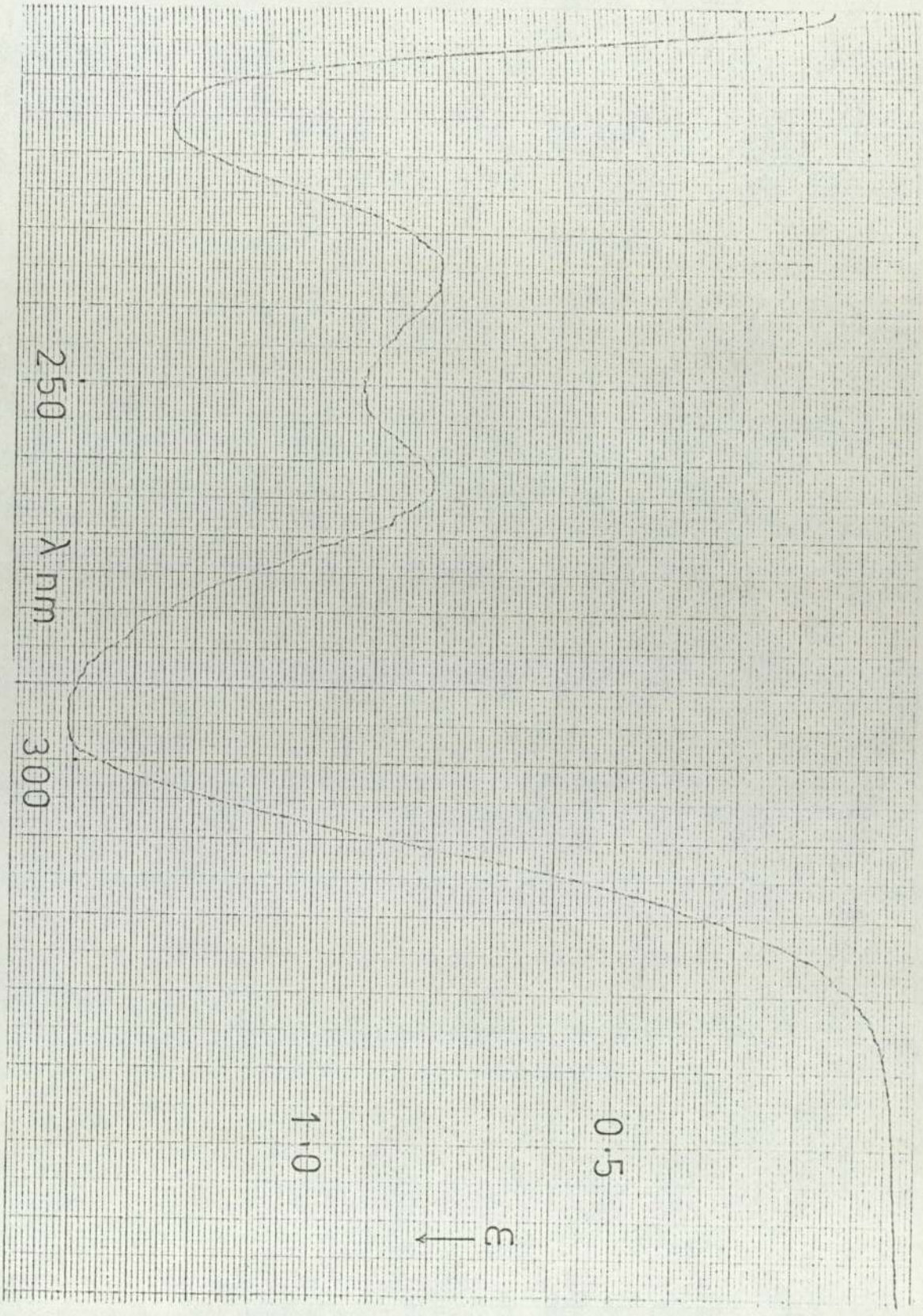
U.v. spectra of fractions off the column in a preparation of 5-methyl-5,6-DHF.

Solvent: 0.1M phosphate buffer pH 7.



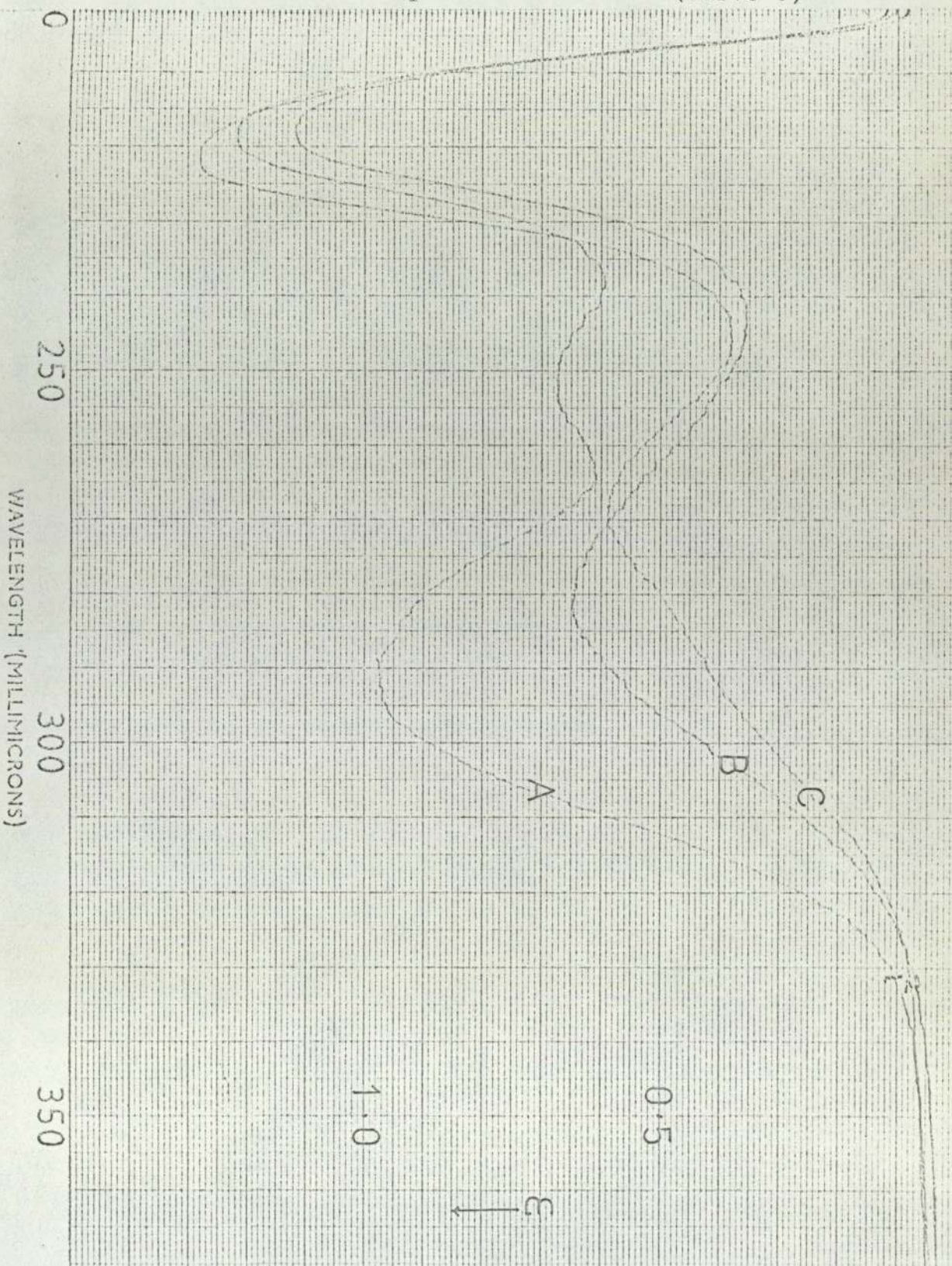
U.v. spectrum of 5-methyl-5,6-DHF prepared by autoxidation of 5-methyl THF in 10^{-4}M $CuSO_4$.

Solvent: 0.1M phosphate buffer pH 7.



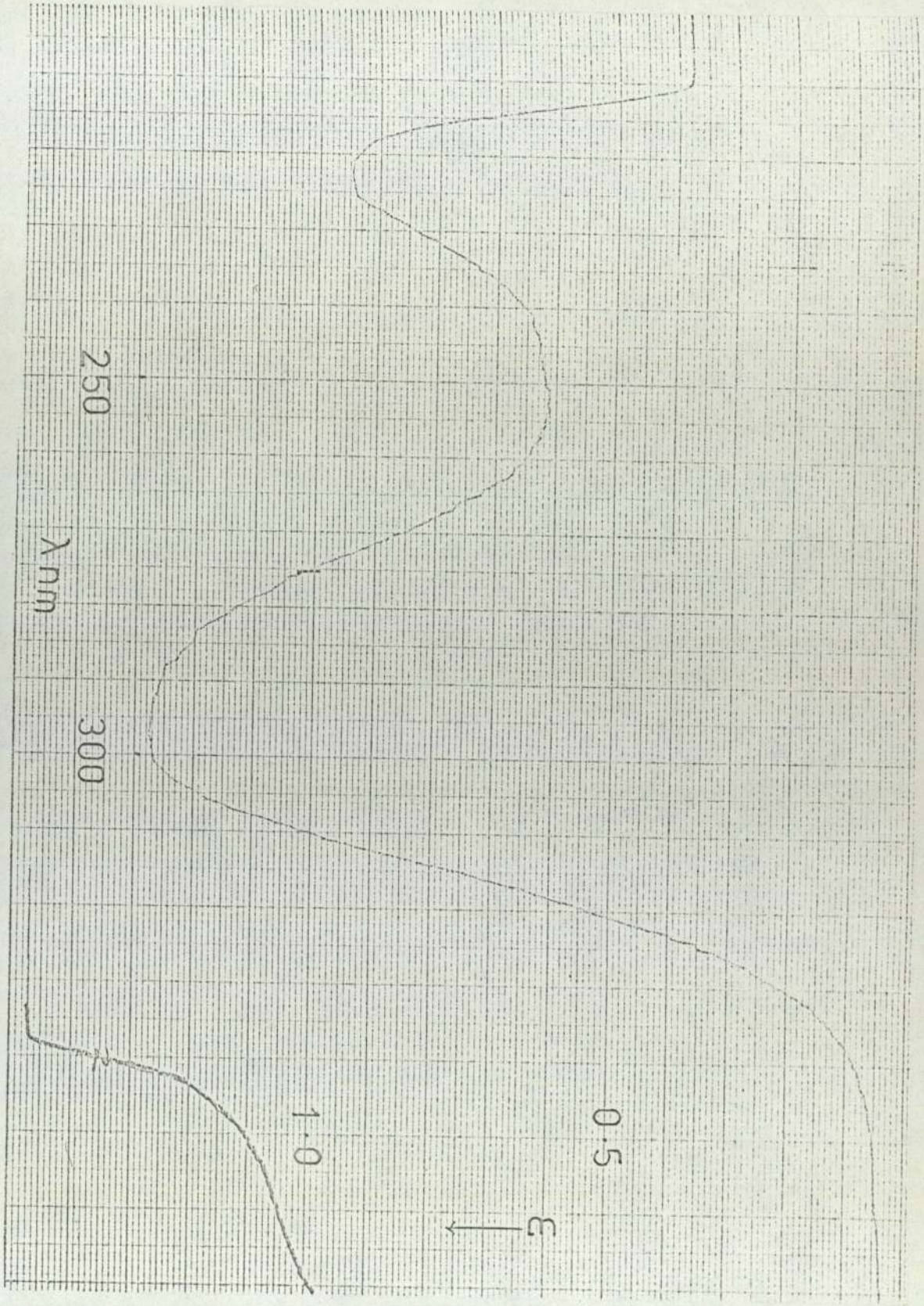
U.v. spectra of 5-methyl-5,6-DHF (curve A) and its acid catalysed rearrangement product (5-methyl-5,8-DHF) (Curves B and C).

Solvents: 0.1M phosphate buffer pH 7 (curve A).
" " " " (curve B)
0.1M HCl pH 1 (curve C)



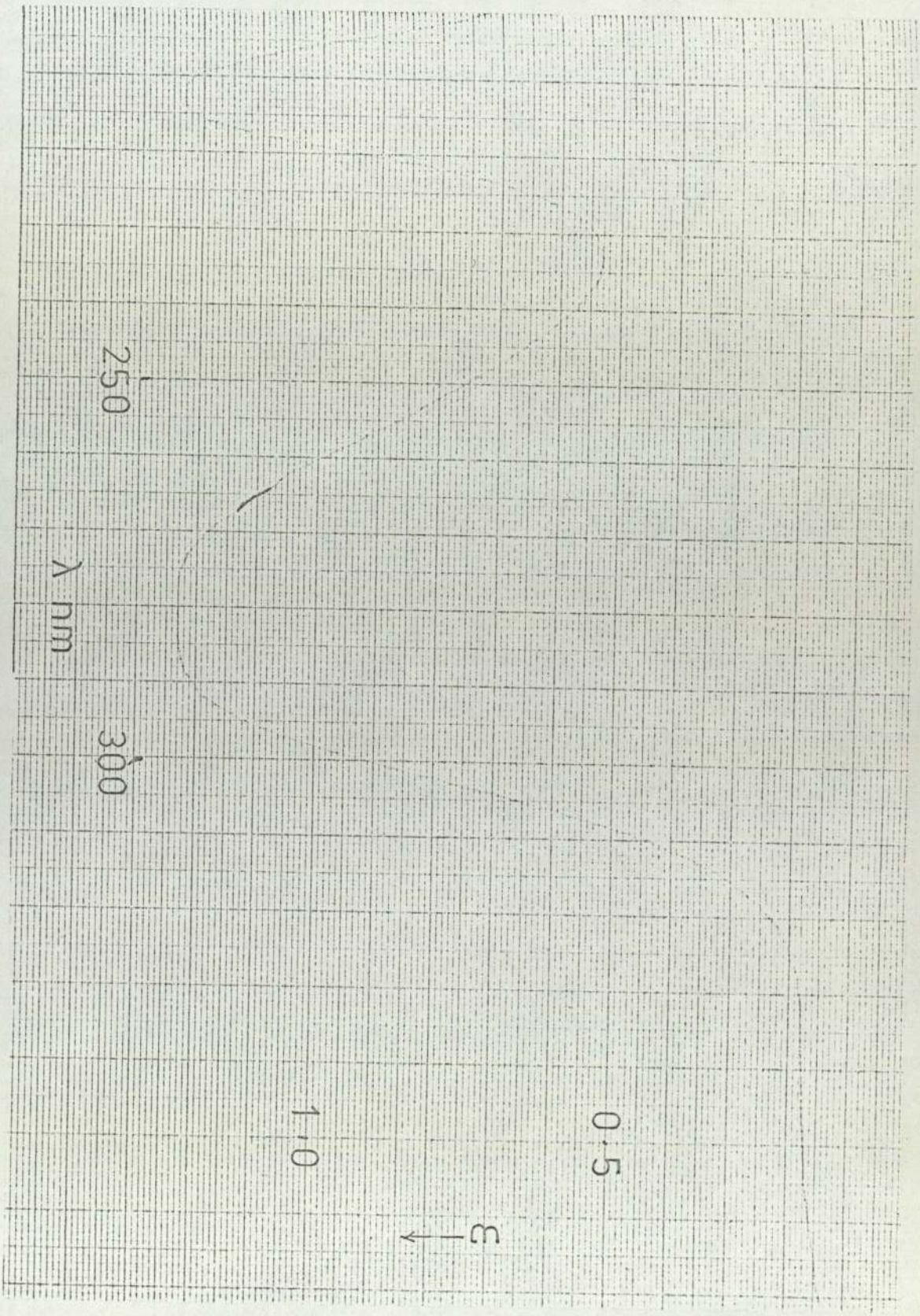
U.v. spectrum of 5-methyl-5,6-DHF.

Solvent: 0.1M NaOH pH 13.



U.v. spectrum of 8-dehydro-4a-hydroxy-5-methyl THF.

Solvent: 0.1M phosphate buffer pH 7.



U.v. spectra for a time study of the reaction

5-formyl THF $\overset{H^+}{\rightleftharpoons}$ 5-10-methenyl $THF^+ + H_2O$.

Curves 1 —— 9 were recorded every 3 mins.

Curve 10 was recorded after 48 hrs.

Solvent: 0.1M HCl pH 1.