Oxidation of Tetrahydropteridines and Tetrahydrofolates

by

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Summary

The autoxidation of tetrahydropteridines in aqueous media has been studied. Particular attention has been paid to tetrahydrofolic acid, which is readily oxidised in solution by air. At pH's 3, 7, 10 and 13 the products were p-aminobenzoylglutamic acid, 2-amino-4-hydroxypteridine, xanthopterin, folic acid and dihydrofolic acid. These were identified by t.l.c., mass spectra, u.v. spectra and fluorescence spectra and estimated using fluorescence spectra. Although 2-amino-4-hydroxypteridine and xanthopterin accounted for 50 - 60% of the tetrahydrofolic acid used, their ratio was pH dependent. In the presence of aqueous ammonia 2,6-diamino-4-hydroxypteridine was formed. A mechanism involving hydroperoxide intermediates has been proposed for these reactions.

Dihydrofolic acid was oxidised to give folic acid and 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine, the latter compound being further oxidised to xanthopterin. A buffer effect on the rate of oxidation was observed. The formation of hydrogen peroxide was shown to occur during the oxidation of dihydrofolic acid.

Oxidations of tetrahydropteridines were followed using time-based u.v. spectral studies. The effects of pH, buffer and deaeration of the solution were observed using this technique. Each compound showed the most rapid and marked spectral changes when in phosphate buffer, pH 7. The results are explained and discussed on the basis of hydroperoxide involvement.

Previous work has postulated the formation of a quinonoid dihydropteridine intermediate from the tetrahydropteridine by a

reversible step, which irreversibly changes to the dihydropteridine. The oxidation of tetrahydropteridines by 2, 6-dichlorophenolindophenol and the appearance of a transient peak at λ_{max} 303nm. when oxidised by ferricyanide have been attributed to such an intermediate. This evidence has been examined more fully and the results obtained are not in accord with the quinonoid dihydropteridine scheme. All the evidence supporting such a scheme is considered and a better interpretation, on the basis of a scheme involving hydroperoxide intermediates, is discussed in detail.

Acknowledgements

I sincerely thank Dr. J. A. Blair for his supervision and encouragement of this work. I am grateful to the following people for the services they provided:- Mr. E. Hartland and Mr. M. Houghton for the N. M. R. and mass spectra they prepared; Dr. A. Z. Britten for use of a Unicam SP-800 spectrometer and an Aminco-Bowman fluorescence spectrophotometer; and Dr. R. F. Long (Roche Products Ltd., Welwyn Garden City) for the generous gift of samples of biopterin, tetrahydrobiopterin, 2-amino-4-hydroxy-6-methyl-7,8-dihydropteridine and 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine.

I also sincerely thank my wife for typing this manuscript.

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This work was carried out between 1968 and 1971 at the University of Aston in Birmingham. It has been done independently and has not been submitted for any other degree.

R. Stocks-Wilson.

R. Stocks-Wilson

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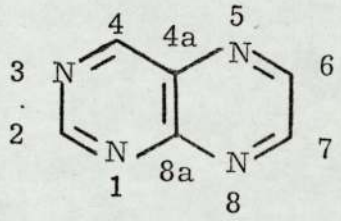
Introduction

1. Nomenclature and Abbreviations

The pteridine ring system is systematically termed pyrimido[4,5-b]-pyrazine. The numbering system for pteridine (I) was put forward by Kuhn and Cook[1] and is based on the rules applied to all other aza derivatives of naphthalene. It is the one in common use today and has been approved by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry[2]. Some publications, especially in the German literature, have used a different system (II) designed to relate pteridine to purine (III).



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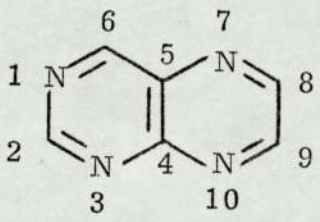

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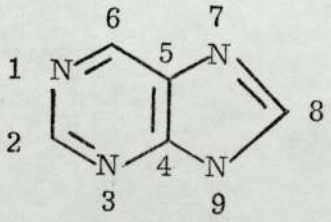
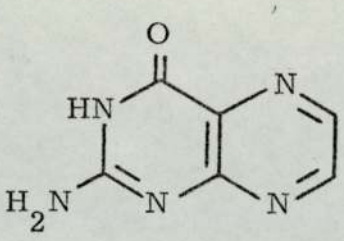


(III)

The term 'pterin' was originally used as a collective term for the butterfly wing pigments[3] or even for insect pigments in general[4]. Pterin has also been used occasionally as an abbreviation for pteridine. Pfleiderer[5] has suggested that since the natural pteridines designated by trivial names '.....pterin' are all derivatives of 2-amino-4 -hydroxypteridine (IV) use of the term 'pterins' should be restricted to

include only derivatives of 2-amino-4-hydroxypteridine.

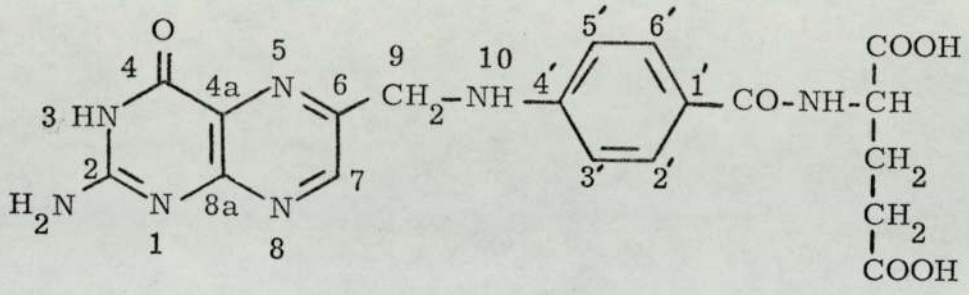


(IV)

As most of the pteridines that occur naturally, and particularly those known to be involved in metabolic processes, are derivatives of 2-amino-4-hydroxypteridine such a use of the term 'pterin' for the basic structure (IV) simplifies the names of pteridines. The term 'pterin' is more frequently used in recent publications and is accordingly used with this meaning in the remainder of the dissertation.

Although structure (IV) is referred to as being a 4-hydroxy pteridine, the structure is written as the tautomeric 4-oxo derivative. Evidence indicates that the equilibrium between these tautomeric forms lies in favour of the 4-oxo form, except in a few special cases such as O-methylated derivatives [6-16]. Structures of pterins are therefore written in the 4-oxo form. Similarly, 6- and 7-hydroxypterins are written in the form of 4, 6- and 4, 7-dioxopteridines.

Folic acid is a compound containing a p-aminobenzoylglutamic acid residue attached to the pterin ring. It has been shown to possess structure (V), i. e. N-[4-{[(2-amino-4-hydroxy-6-pteridinyl)methyl] amino}benzoyl]glutamic acid.



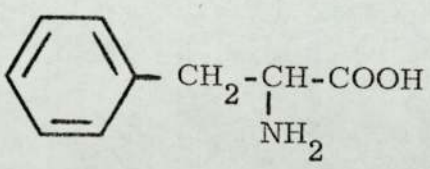
(V)

The chemists who elucidated structure (V) proposed for it the name 'pteroylglutamic acid'[17]. The alternative name, 'folic acid', was first proposed by Mitchell et al[18]. It is standard practice to use 'folic acid' to indicate the compound with structure (V) and to refer to the related compounds as the appropriate folic acid derivatives[19-22]. Since the folic acid derivatives participating in most biochemical reactions are derivatives of 7,8-dihydrofolic acid or 5,6,7,8-tetrahydrofolic acid, the practice[20] of abbreviating the names of these compounds to DHF and THF respectively, will be adopted and used in the remainder of the dissertation.

2. Biological Occurrence and Importance of Pterins and Folates

Pterins are widespread in nature, occurring as red, orange and yellow pigments in the wings and eyes of insects, as pigments in the skin of reptiles, and in amphibia[23] and fish[24] they play a role in melanin synthesis. In comparison, little is known of the occurrence of pterins in mammalian species. Isoxanthopterin is a constituent of human urine[141], probably arising from folate degradation, and biopterin is a normal constituent of human urine[142-145].

Pterins have an important role as metabolic cofactors. The first such role for a pterin was established in 1963 when it was shown that the phenylalanine-hydroxylation cofactor isolated from rat-liver extracts is dihydrobiopterin[25]. Subsequent studies showed that in vivo the cofactor is tetrahydrobiopterin[32]. Earlier studies showed that the enzymatic conversion of phenylalanine (VI) to tyrosine (VII) required the participation of two liver enzymes and a reduced pteridine cofactor[26-31].



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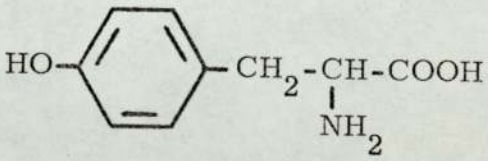
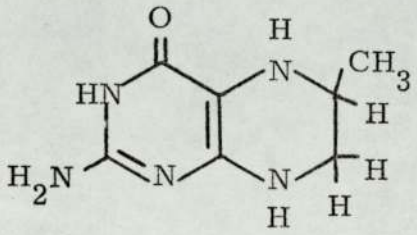


(VII)

In this system reduced nicotinamide adenine dinucleotide phosphate (NADPH) acts as the terminal electron donor, molecular oxygen is required and the stoichiometry of the overall reaction satisfies equation (1)[26].

$$\text{Phenylalanine} + \text{NADPH} + H^+ + O_2 \longrightarrow \text{NADP}^+ + H_2O + \text{tyrosine} \quad (1)$$

THF[29] and reduced model pterins such as tetrahydro-6,7 -dimethylpterin or tetrahydro-6-methylpterin can replace the natural cofactor[30,31]. Tetrahydro-6-methylpterin (VIII) is much more effective than THF whereas the isomeric 4-amino-2-hydroxy -tetrahydro-6-methylpteridine (IX) is inactive[31].



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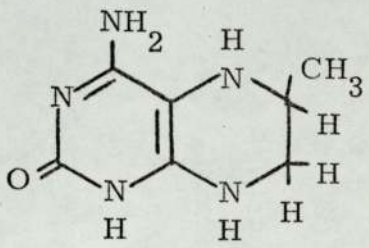


(IX)

Folic acid, DHF, 5-formyl THF and non-reduced pteridines are inactive.

When substrate amounts of a tetrahydropterin are used in the system, substantial hydroxylation of phenylalanine occurs when only the rat liver enzyme and oxygen are present[30]. This suggests that the hydroxylation is catalysed by the enzyme purified from rat liver and proceeds according to equation (2).

$$\text{Tetrahydropterin} + \text{phenylalanine} + O_2 \longrightarrow \text{'oxidised' pterin} + \text{tyrosine} + H_2O \qquad (2).$$

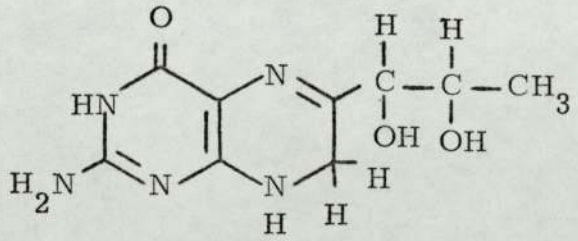
The function of the sheep liver enzyme and NADPH is to reduce the 'oxidised' pterin back to tetrahydropterin according to equation (3).

$$\text{'Oxidised' pterin} + \text{NADPH} + H^+ \longrightarrow \text{tetrahydropterin} + NADP^+ \qquad (3).$$

In accordance with this scheme, the initial rate of phenylalanine

hydroxylation is unaltered by omission of NADPH from the reaction mixture, but under these conditions, the reaction soon stops[30]. In the presence of NADPH the reaction proceeds at a uniform rate over a long period. 'Oxidised' pterin represents an intermediate whose existence was inferred from kinetic studies. In the absence of an efficient reducing system, the 'oxidised' pterin is rapidly converted nonenzymatically to the inactive 7,8-dihydropterin[30].

As far as is known, the cofactor functions in the hydroxylase system in the same way as do the synthetic pterins. One difference is that the cofactor must be reduced to the tetrahydro form before it can take part in the reaction. This initial reduction of dihydrobiopterin (X) is catalysed by dihydrofolate reductase[25,32], an enzyme with an established role in one-carbon metabolism. There is some evidence that in liver tissue[32] (and in insect eyes[33]) most of the biopterin is present as the tetrahydro compound and that it is oxidised to the dihydro compound (X), or the aromatic form, during its isolation.



(X)

Subsequently it has been shown that tetrahydropterins may function in many other oxygenase reactions in a similar manner, an example is the oxidation of long-chain alkyl ethers of glycerol to fatty acids and free glycerol[34]. Tietz et al.[34] have shown that the oxidation of batyl alcohol to stearic acid takes place with the intermediate formation of

stearaldehyde according to equations (4) and (5).

$$RCH_2OCH_2R^1 + O_2 \xrightarrow{\text{Tetrahydropterin}} RCH_2OH + R^1CHO \qquad (4)$$

(Batyl alcohol) (Glycerol) (Stearaldehyde)

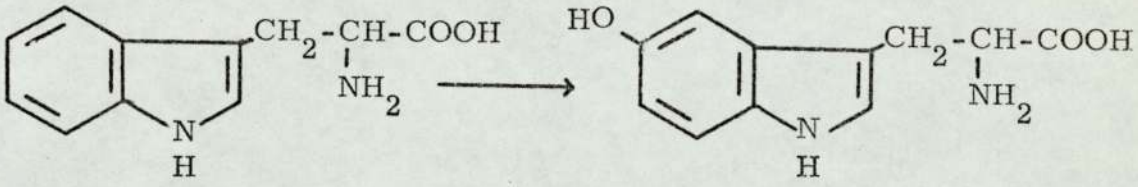
$$R^1CHO + NAD^+ + H_2O \longrightarrow R^1COOH + NADH + H^+ \qquad (5)$$

Further examples are:-

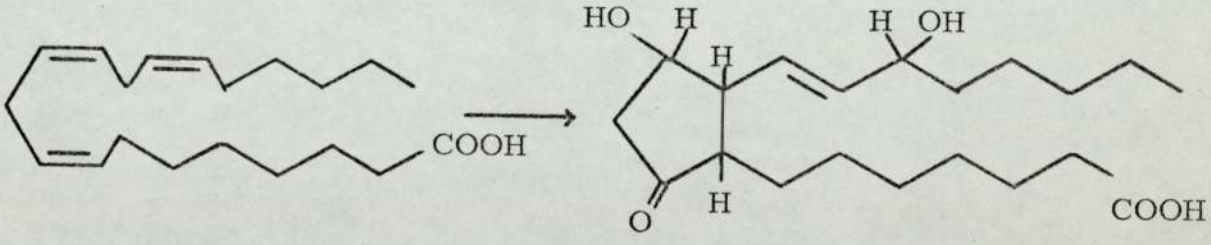
The conversion of tyrosine to 3,4-dihydroxyphenylalanine[36-38].

$$\text{Tyrosine} + \text{tetrahydropterin} + O_2 \xrightarrow{Fe^{2+}} \text{3,4-dihydroxyphenylalanine} + \text{'oxidised' pterin} + H_2O.$$

The hydroxylation of tryptophan to 5-hydroxytryptophan[40].



The conversion of 8,11,14-eicosatrienoic acid to prostaglandin[41].



for which Samuelsson[164] has shown, using ^{18}O and mass spectra, that all the oxygen atoms are derived from molecular oxygen, and the possible involvement of a hydroperoxide intermediate is in agreement with ^{18}O experiments and ^{3}H tracer experiments.

Other oxidations are the 17- α -hydroxylation of progesterone[35] and the hydroxylation of cinnamic acid to p-coumaric acid[39].

Folic acid exists in plant and animal tissues, largely in the form of conjugates with more than one glutamic acid residue. It is, however, the reduced forms of folic acid and their derivatives that play such an important role as co-enzymes in biochemical systems. The active co-enzyme forms are derivatives of THF, many enzymic reactions require as substrates not THF itself but formyl, methyl or other derivatives of THF, and they are involved in the biological transfer of one carbon atom units at the oxidation levels of formate, formaldehyde, and methanol (see Table I), and in the interconversion of these different oxidation states[42,43]. The general aspects of folic acid metabolism are covered in the review by Stokstad and Koch[44].

TABLE I

THF Derivatives and the Attached One Carbon Unit

Site of Attachment			One Carbon Unit	Oxidation State
N-5	N-10	N-5, N-10		
H-C=O	H-C=O		formyl	formate
H-C=NH			formimino	
		=CH	methenyl	
		-CH$_2$-	methylene	formaldehyde
-CH$_3$			methyl	methanol

3. Reduction of Pterins and Folates

A number of reduced pterins have been prepared either directly by appropriate syntheses from pyrimidine precursors or by the reduction of pterins with various agents. Methods of reduction employed are:- powdered zinc in alkali[30, 45-48], or in acid[25, 48]; sodium or potassium borohydride[49-55, 63]; sodium dithionite[45, 46, 49]; catalytic hydrogenation over platinum, palladium or Raney nickel[30, 49, 56-62]; and sodium or potassium amalgam[49]. Although some of these reducing agents are believed to act through hydride ions, some through protons and some through free radical formation, they generally give the same product from any given pterin. Exceptions to this generalisation are the fact that only sodium amalgam can attack a double bond linking a carbon which bears a hydroxyl group, and only this reagent attacks the 7,8-double bond in certain 6,7-dihydroxypteridines[49]. The inability of other reducing agents to attack such bonds is due to the predominance of the cyclic amide tautomeric structure. With few exceptions[64] reduction takes place in the pyrazine ring, as would be expected from the fact that pyrazines are reduced relatively easily, whereas pyrimidines are difficult to reduce, particularly in alkaline medium. Catalytic hydrogenation usually results in the formation of a 5,6,7,8-tetrahydropterin although a dihydro compound is formed from a 6- or 7-hydroxypterin[49] and sometimes from other pterins, especially at high pH[65]. Zinc appears to give a 7,8-dihydro derivative with most pterins[25, 30, 46-48]. and borohydride is unable to reduce some pteridines[49, 52], with others gives a mixture of dihydro and tetrahydro compounds[49], but usually gives a tetrahydro product.

Folic acid and many of its derivatives can easily be reduced by a variety of agents to the corresponding dihydro or tetrahydro compounds. Catalytic hydrogenation of folic acid over platinum or palladium yields either DHF or THF as the main product, depending on the conditions[65,66]. In glacial acetic acid or in neutral aqueous solution two molecules of hydrogen are rapidly consumed with the formation of THF[65-70], but there is a slow further uptake of hydrogen with the formation of unknown products[65-67,71,72]. After removal of the catalyst the solution is lyophilised or, if glacial acetic acid is used as solvent, the product can be precipitated with ether[71]. If acetic acid is the solvent the product is THF diacetate[70,73]. Catalytic reduction of folic acid in O.1N. NaOH yields mainly DHF[65] but the uptake of hydrogen beyond one mole is rapid[66,74] so that it is impossible to prepare pure DHF by this method. When catalytically hydrogenated in 98% formic acid, folic acid takes up two moles of hydrogen[67], but significant formylation of folic acid also occurs under these conditions so that the product is a mixture of THF, 5-formyl THF and 5,10-methenyl THF. Catalytic hydrogenation of folate or pterins in trifluoroacetic acid is rapid and allows subsequent isolation of salts of the reduced products[60].

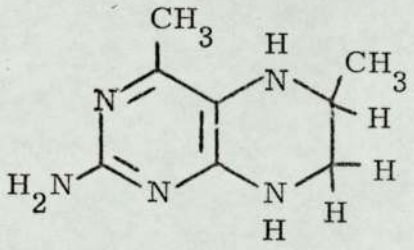
Folic acid can also be reduced by dithionite and at room temperature the product is DHF which can be isolated by precipitation at low pH in the presence of ascorbate[75-77] or mercaptoethanol[78]. Under carefully controlled conditions, a microcrystalline product is obtained[79]. At elevated temperatures the main product of dithionite reduction is THF[80,81]. Reduction of folic acid by borohydride yields THF together with smaller amounts of DHF, the proportion of DHF depending on the reaction conditions, particularly the borohydride concentration and the

temperature[82-84]. Reduction of 7-methyl folate by zinc dust in molar NaOH has been used to prepare 7-methyl DHF[85], dithionite being unable to reduce the 7,8-double bond in the presence of the methyl group at position 7.

4. Oxidation of Pterins and Folates

The stability of reduced pteridines, like that of the parent compounds, is influenced by the number of electron-liberating substituents which are present (for example NH_2 and OH), but whereas these groups increase the stability of the parent compounds they diminish the stability of the reduced compounds.

Tetrahydropteridines bearing a single amino or hydroxyl group, such as (XI), are also stable in acid solution, though less stable in alkali, but some of the corresponding 7,8-dihydro compounds oxidise to the parent pteridine under air in acid or, more rapidly, in alkaline solution[49,59,86].

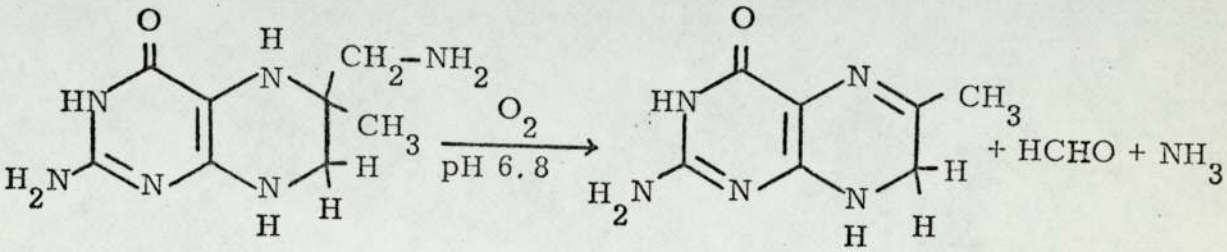


(XI)

Tetrahydropterin is rapidly oxidised in solution under air[56,87,132], and attempts to isolate the solid yield a complex mixture of oxidation products[56]. The tetrahydro derivatives of 6-methyl pterin[51], 6,7-dimethyl pterin[51], 6,7-diphenyl pterin[54,88,90], 6,8-dimethyl pterin[91], 8-(2-hydroxyethyl)-6,7-dimethyl pterin[51], 2,4-dihydroxy-pteridine[49], 2,4-diaminopteridine[53], 6-aminopterin[58], lumazine[94] and 6,7-diphenyl lumazine[88] are also readily oxidised to the dihydro and parent compounds. In acid, 5-methyl-6,7-diphenyl-5,6-dihydro-pterin rapidly loses its methyl group and a proton and is oxidised to 6,7-diphenyl pterin[54,88,89].

5-Methyl-6,7-diphenyl-tetrahydropterin behaves similarly, but the demethylation is much slower, all conditions being equal[54, 88, 89].

6-Aminomethyl-6-methyl-tetrahydropterin (XII) is oxidised to 6-methyl-7,8-dihydropterin (XIII), formaldehyde and ammonia[62]:-

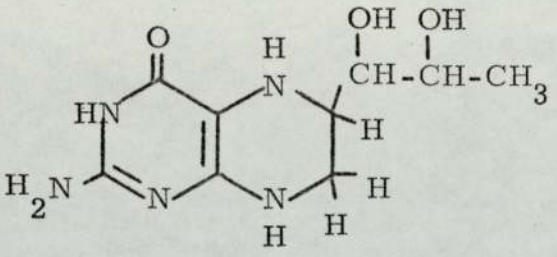


(XII) (XIII)

Similarly, 6-aminomethyl-6-methyl-7 substituted-tetrahydropterins areoxidised to 6-methyl-7 substituted-7,8-dihydropterins[92].

5,6-Dimethyl- and 5,7-dimethyl-tetrahydropterins are stable in air[63].

Xanthopterin yields a dihydro derivative which is oxidised by air in alkaline solution back to xanthopterin, but which on catalytic oxidation in glacial acetic acid yields leucopterin (6,7-dihydroxypterin)[65]. Dihydro and tetrahydropterins are also readily oxidised to the parent compounds by manganese dioxide, alkaline permanganate, hydrogen peroxide or iodine[47, 48, 52, 53, 93]. Tetrahydrobiopterin (XIV)[61] and tetrahydro-neopterin (XV)[94] are oxidised by boiling in air at pH 7.5, to give pterin and dihydroxanthopterin or xanthopterin as the major products.



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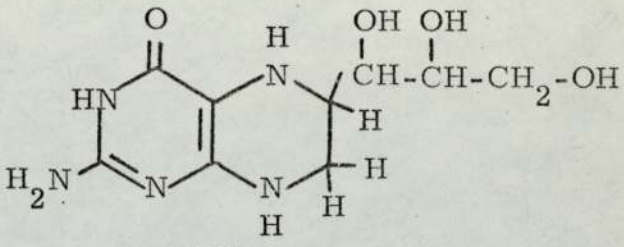
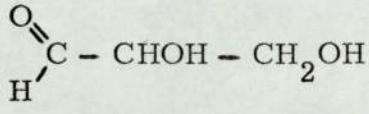


(XV)

When tetrahydrobiopterin is oxidised with iodine the major product is biopterin, with little pterin and no dihydroxanthopterin[61]. Experiments with tetrahydroneopterin having a ^{14}C - labelled side chain show that cleavage of the side chain produces glyceraldehyde (XVI) and is coupled to an oxidative process[61].



(XVI)

Investigators using the coenzymes DHF and THF have been aware of the instability of both of these compounds, and there have been many attempts to characterise the products of degradation. Both DHF and THF are readily oxidised by air. Although O'Dell et al.[65] and Zakrzewski[77] reported that DHF is reoxidised by air in alkaline solution to folic acid, and less rapidly oxidised in acid solution, crystallised DHF obtained by dithionite reduction of folic acid is reported as being relatively stable in solution at pH 9.5 as judged by ultraviolet absorption and nuclear magnetic resonance spectra[82]. Decomposition of DHF at pH 4 to 7 is retarded by thiols, transition metal cations or

ascorbate and accelerated by EDTA. The effect of metal ions is probably due to complex formation which EDTA reverses, for pteridines are known to chelate with metals[95]. DHF is oxidised to p-aminobenzoylglutamic acid[66,75,96] and, in phosphate buffer, pH7, a pterin with absorbance maxima at about 270 and 420 nm. and yellow-green fluorescence[97,98]. This pterin reacts with thiols, bisulphite or dimedone to give colourless products (the thiol adduct has λ_{max} 277 nm). This pterin was suggested[97], and shown[98], to be 6-formyl-7,8-dihydropterin. However, neither of the latter groups has reported either the amount of 6-formyl-dihydropterin formed from DHF or the presence of any other pteridine degradation products. An increased absorbance above 400 nm. is also observed for acid solutions of DHF[99-102].

The instability of DHF means that care must be exercised in the storage of the compound. The solid may be stored in a vacuum desiccator over P_2O_5 and solid KOH at O° in the dark or as a suspension in 5mM. hydrochloric acid at 1°. Storage as a frozen suspension in 1mM. hydrochloric acid containing 10 mM. mercaptoethanol[103], or as a suspension in 10% potassium ascorbate, pH 6.0, at 4° have also been recommended[104].

Numerous investigators[65,66,77,94,105-110] have attempted to identify the products of oxidation of THF, but they report only limited success. THF is readily oxidised in solution by air. p-Amino-benzoylglutamic acid[66,77,105,109], pterin[77,94,108], xanthopterin[66,94,108], and 6-methyl pterin[66] have been observed among the products. Some reoxidation to DHF[77,106,107,110] and folic acid[66,110] also occurs. In addition, formaldehyde can be detected in the solutions of oxidised

THF[108]. In acid solution, cleavage to p-aminobenzoylglutamic acid is quantitative in the absence of protective agents[110]. Blakley[66] examined the products of oxidation in air of THF and found small amounts of folic acid and large amount of p-aminobenzoylglutamic acid, (52% p-aminobenzoylglutamic acid from THF after one hour), but could only account for a small proportion of the pteridines as xanthopterin (17% of THF used). He reports that the oxidation was not appreciably affected by changes in pH. Futterman and Silverman[109] found that THF formed by enzymic reaction was converted at pH 6 to stoichiometric quantities of p-aminobenzoylglutamic acid, and to several pteridine compounds. Zakrzewski[77] isolated pterin, p-aminobenzoylglutamic acid and DHF (10-20% of THF used) from THF solutions aerated at pH 7.7 (0.1 molar ammonium acetate) for one hour. Rembold et al.[94] oxidised THF at pH 7.5 (0.1 molar Tris-HCl buffer) and after two hours the reaction was stopped by boiling. Xanthopterin plus dihydroxantho -pterin (20% of THF used) and pterin (11% of THF used) were isolated, identified and estimated. DHF and THF have been degraded oxidatively using ferricyanide under alleged anaerobic conditions[111]. Depending on the pH, temperature and buffer system, DHF can give folic acid, p-aminobenzoylglutamic acid, dihydroxanthopterin, 6-formyl-dihydropterin and formaldehyde. Each of these products was identified and isolated in high yield. Although it is more susceptible to cleavage than is DHF, THF gives similar products. In addition, moderate quantities of pterin were isolated from the oxidation of THF at pH 5.6

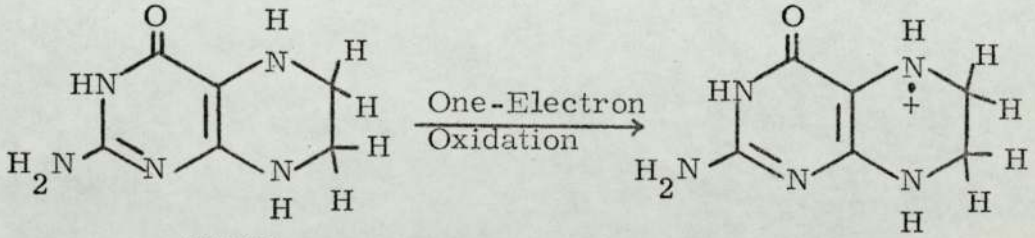
Dry samples of THF deteriorate 20 - 25% in air in two to three days[110]. Oxidative degradation of THF results in rapid change in the absorption spectrum and is greatly retarded by 2,3-dimercaptopropanol,

and to a lesser extent by mercaptoethanol[112]. Thiols are less effective anti-oxidants in acid solution[113]. Cysteine is also effective[20]. This effect of thiols does not seem due to binding of heavy metals since other sequestering agents are relatively ineffective[112], and it has been postulated that a stable thiol-THF complex of unknown structure is formed[110]. Ascorbate is even more effective than thiols in protecting THF from oxidative degradation, and two-thirds of the THF in such protected solutions remain after four days at 0-5° and pH 7.4, the pH of maximum stability. Ascorbate has been used to retard THF degradation during chemical synthesis of 5-formyl THF[67] and in the course of the extraction and separation of THF derivatives from biological materials[115]. 2-Mercaptoethanol is often used because of its volatility and subsequent removal during the isolation of samples by lyophilisation. The protective action of ascorbate may be due in part to removal of dissolved oxygen, but both thiols and ascorbate may protect by reducing any intermediate which is the primary product of oxidation.

5. Current Mechanisms for the Oxidation of Reduced Pterins and Folates

The mechanism of oxidation of tetrahydropterins is of considerable importance for the mechanism of nonenzymatic oxidation of tetrahydro -pterins is intimately related to the role of reduced unconjugated pterins as hydroxylation coenzymes, since it has been shown that the first detectable product is the same in the nonenzymatic and in the phenyl -alanine hydroxylase - catalysed oxidation of tetrahydropterins[116]. Considerable effort has been expended in attempts to elucidate the mechanism of oxidation, but has met with only limited success.

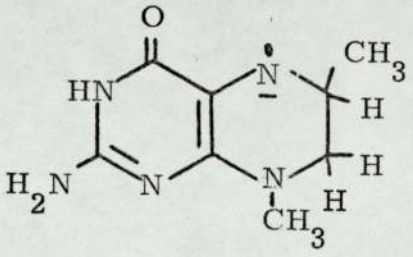
Perault and Pullman[117] have predicted the easy formation of radical cations by folate coenzymes. Bobst has discussed the formation of radicals in the oxidation step of hydrogenated pteridines[118] and he has also found[119, 120], by taking electron spin resonance measurements, that tetrahydropterin (XVII)can be oxidised by a one-electron step to form the tetrahydropterin radical cation (XVIII):



(XVII) (XVIII)

Ehrenberg et al.[121] have found that 5-methyl-6, 7-diphenyl-5, 6-dihydro -pterin is oxidised by hydrogen peroxide in trifluoroacetic acid with formation of the 8-monohydropterin radical cation, which is sufficiently stable for detection and structural elucidation by electron spin resonance hyperfine analysis. The corresponding tetrahydropterin also gives a

radical cation under these conditions, and the structure in this case is believed to be the 6, 7, 8-trihydropterin derivative. Later studies[122] have shown that in the monohydropterin radical the unpaired electron is delocalised over the whole pyrazine part, whereas in the trihydropterin radical the electron is mainly confined to position N-5 and possibly the bridge carbons. Viscontini and Okada[89] have also suggested oxidation involving radical cations. Different mechanisms involving radical anions (XIX) have also been postulated[91].

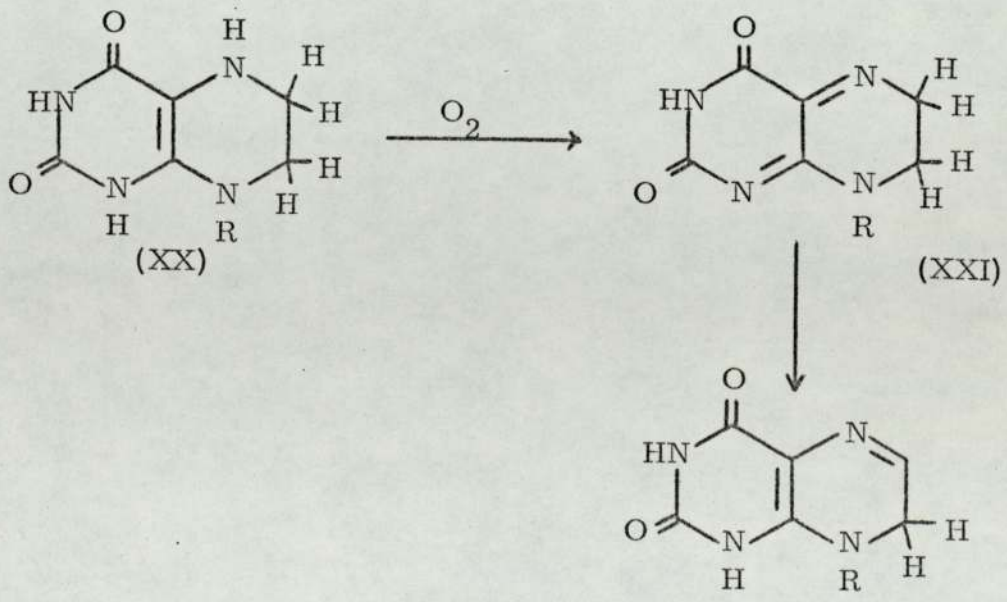


(XIX)

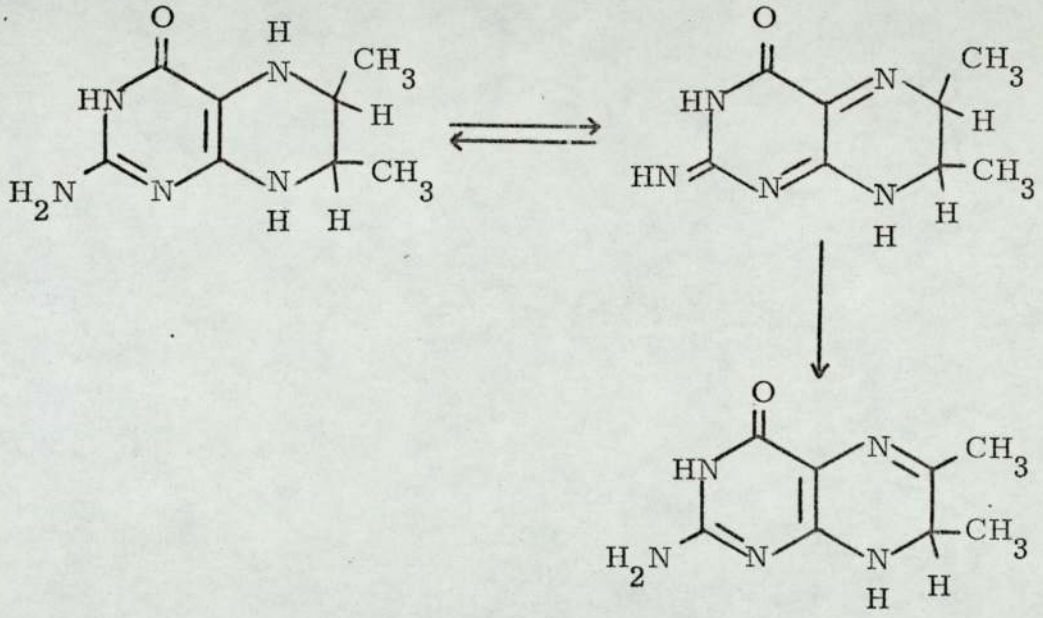
Electron spin resonance spectra[123, 124] of solid THF, 5-methyl THF, 5-ethyl THF and 5-n-propyl THF show an unpaired electron on a nitrogen atom. As 5-formyl THF does not give a spectrum, this electron may be assigned to N-5. Evidence has been obtained to suggest that Fe^{3+} rapidly oxidises tetrahydropterin to form an intermediate, believed to be quinonoid-dihydropterin, which then tautomerises to 7, 8-dihydropterin relatively slowly[125]. It is considered that free radicals are obligatory intermediates both in oxidation of reduced pterins by air, H_2O_2 or Fe^{3+} and in the enzymic oxidation of reduced pterin by phenylalanine hydroxylase.

Quinonoid dihydropteridines have been discussed by many authors as possible primary oxidation products of tetrahydro derivatives.

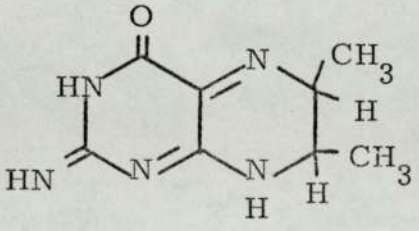
Oxidation of the tetrahydropterin cofactor during the phenylalanine hydroxylase reaction[30,116] produces an intermediate which is different from, but rapidly converted to, the 7,8-dihydropterin. Two important properties of the intermediate are a) it can be irreversibly converted to the dihydropterin and b) it must be an oxidising agent capable of being reduced to the tetrahydro level[30]. The intermediate is too unstable to be isolated from the enzyme reaction mixture, but a compound that has similar chemical and spectral properties, and that reacts in the hydroxylation system in similar manner to the intermediate, is formed by the oxidation of 6,7-dimethyltetrahydropterin by 2,6-dichlorophenol -indophenol[116]. After removal of the dye from the solution by ether extraction an absorbance maximum at 302-305nm. was observed, and the spectrum changed to give a spectrum of 6,7-dimethyl-7,8-dihydro -pterin. The unstable intermediate is also formed by oxidation of the tetrahydropterin by oxygen or hydrogen peroxide. Hemmerich[126] studied the autoxidation of a tetrahydropteridine (XX), alkylated in position 8, and postulated the existence of a very labile quinonoid intermediate (XXI):-



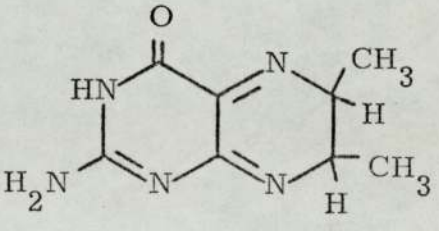
As his results were quite similar spectrophotometrically to that obtained by Kaufman in the oxidation with 2, 6-dichlorophenolindophenol, Hemmerich suggested that Kaufman's intermediate might also be of quinonoid structure. Evidence involving racemisation[116] and ^{3}H-labelled compounds[51] ruled out the possibility of 5, 8-dihydro - or 5, 6-dihydropterins. Kaufman[51] therefore proposed the following scheme for the oxidation of 6, 7-dimethyl tetrahydropterin, involving a quinonoid dihydropterin intermediate:-



At least two quinonoid forms (see below) are possible, but for both spectral[51, 126] and molecular orbital reasons[118] the structure is assumed to be para.



p-Quinonoid



O-Quinonoid

From a consideration of the relative rates of oxidation of the tetrahydro -pterins in air, and the relative rates of reduction of the corresponding dihydropterins by NADPH, Kaufman[51] concluded that the primary site of oxidation of the tetrahydropterins is N-5 and that the primary oxidation product has a quinonoid dihydropterin structure.

Viscontini has published several reports on the oxidation of tetrahydropterins in which a quinonoid intermediate is considered[54, 87, 88, 90, 92, 127]. He also considers radicals, radical cations and pterin intermediates with a hydroxyl group at position 4a. One of his reports[92] contains a scheme, reproduced in Figure I, which includes all of these intermediates. Viscontini and Leidner[88] report the oxidation of 6, 7-diphenyl-5, 6-dihydropterin as proceeding via a monohydrogenated radical:-

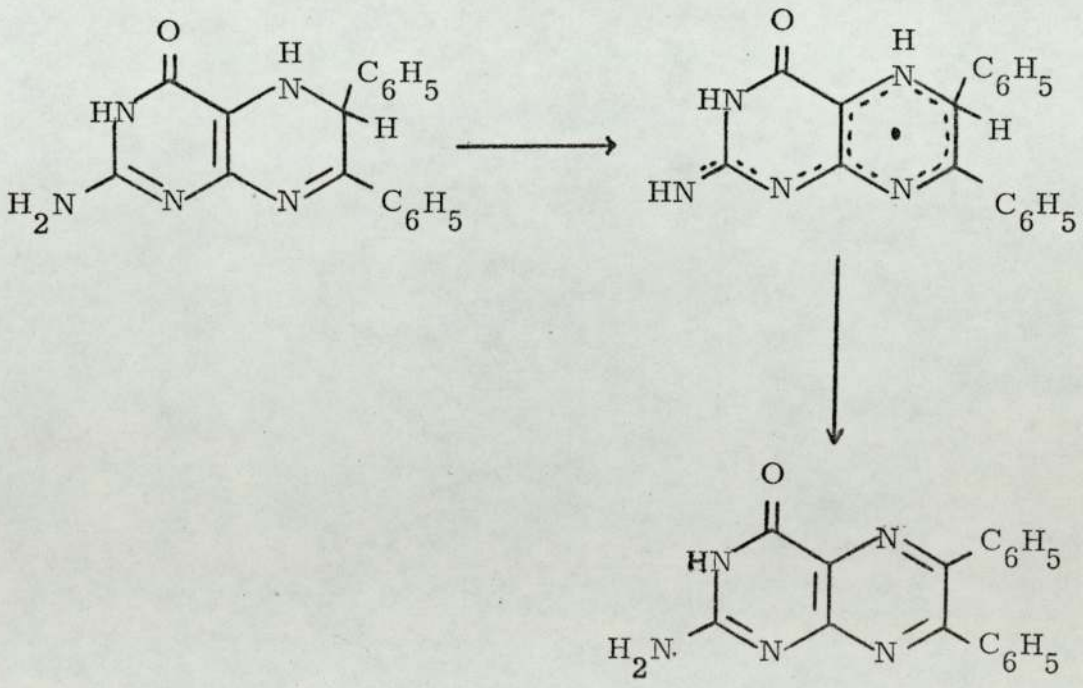
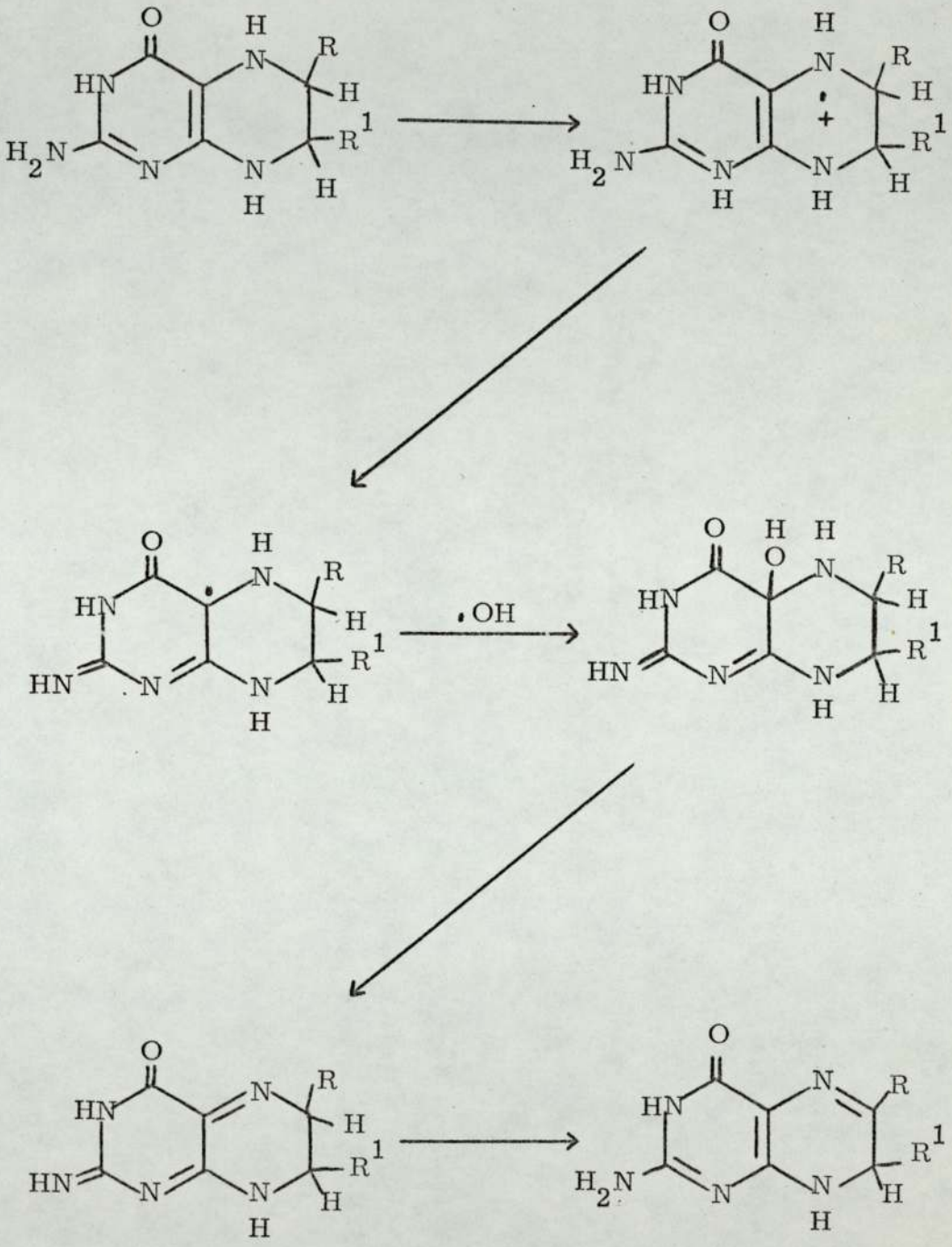
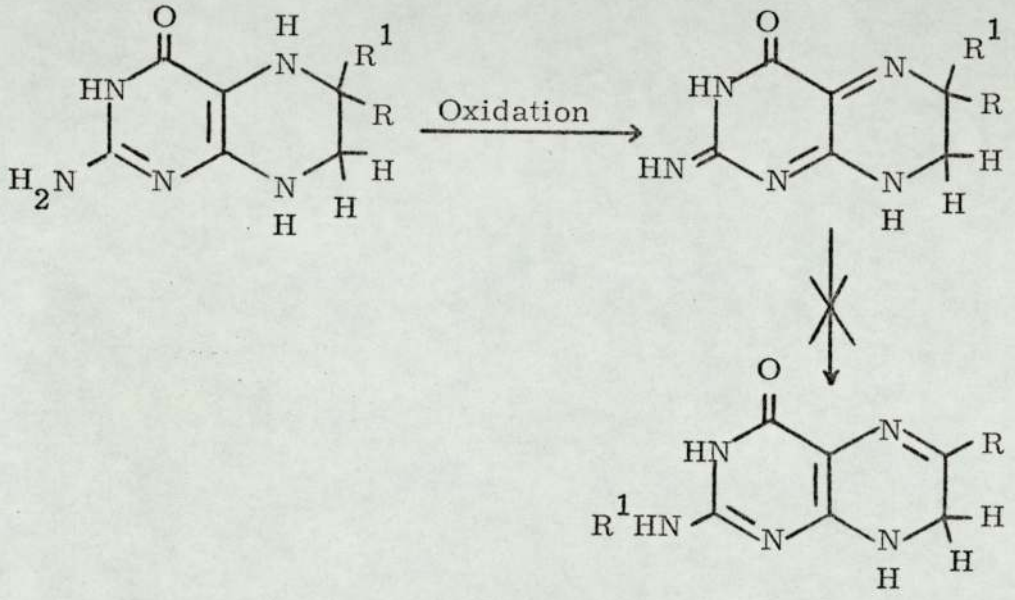


Figure I Oxidation of Tetrahydropterins
Scheme According to Viscontini et al. [92]

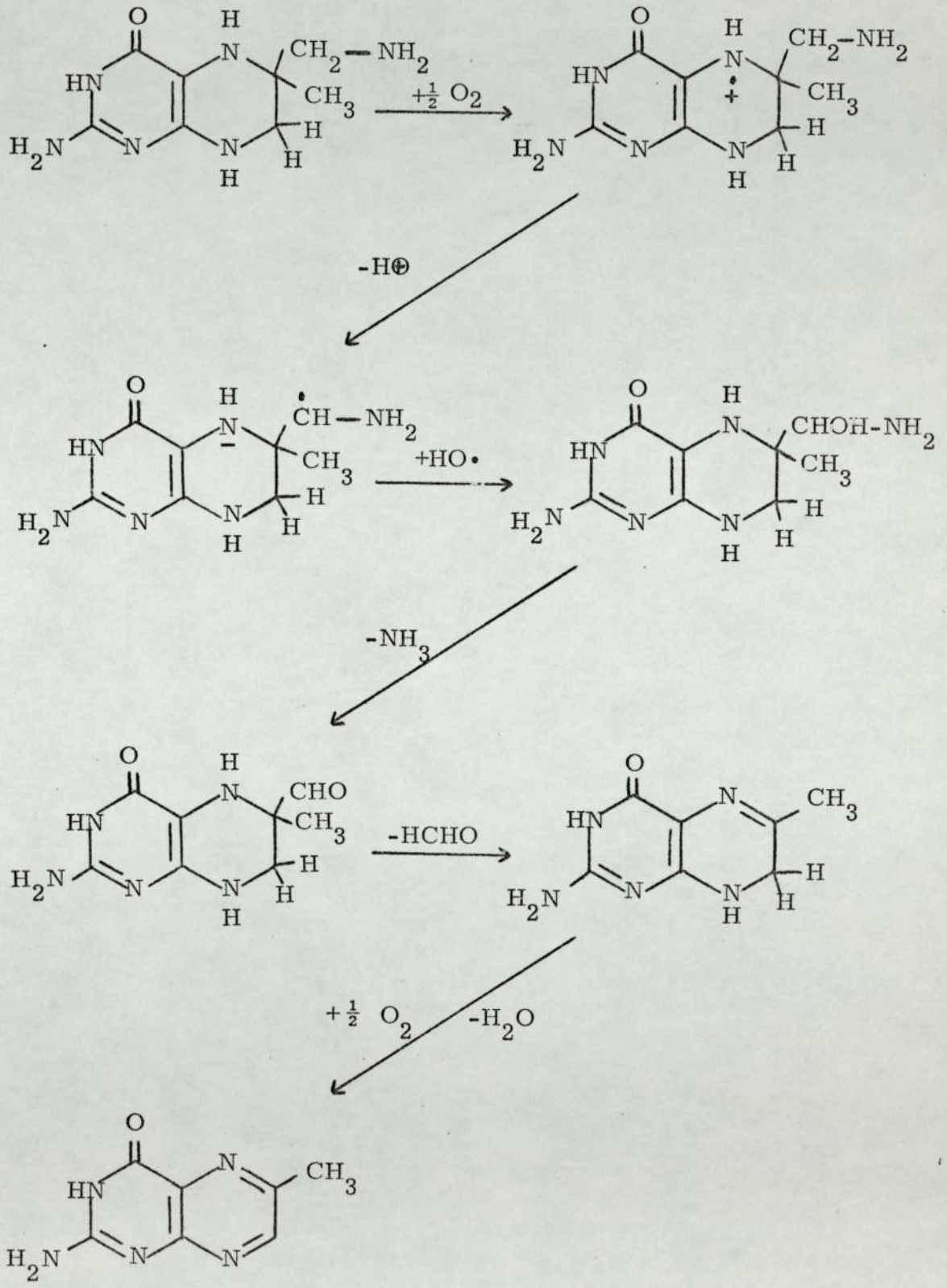


In a recent report Viscontini et al.[62] question the existence of a quinonoid dihydropterin intermediate. When 6,6-disubstituted-tetra-hydropterins were oxidised no 2-substituted amino derivatives were observed, a product expected if a quinonoid intermediate had been present. Viscontini et al. expressed their results by the following scheme:-



On aerial oxidation of 6-aminomethyl-6-methyl tetrahydropterin 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 the idea of formaldehyde formation. A mechanism for this oxidation has been proposed by Viscontini and

Figure II Oxidation of 6-aminomethyl-6-methyl -5, 6, 7, 8-Tetrahydropterin[157].



Argentini[157] (see Figure II).

Vonderschmitt and Scrimgeour[125] have shown that Fe^{3+} oxidises tetrahydropterin to an intermediate which rearranges to 7,8-dihydropterin. The intermediate is regarded as a quinonoid dihydropterin solely 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[30]. It is important to realise that this evidence, as well as certain other evidence for quinonoid intermediates, is dependent on the assumption that Kaufman is correct in regarding his intermediate as being a quinonoid dihydropterin.

Using polarography, Archer and Scrimgeour[128] have studied the reduction potentials of tetrahydropterins, interpreting 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-methyl-tetrahydropterin and tetrahydropterin[129]. 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 of each buffer[129]. The rate of oxidation of reduced pterins being solvent dependent has been known for many years[86]. Isotopic experiments show that cleavage of the C-H bond at position 6 is rate-limiting in the oxidation of tetrahydropterins[129]. Chippel and Scrimgeour[111] have examined the oxidation of DHF and THF using potassium ferricyanide under alleged anaerobic conditions. They presume that oxidation of THF by

ferricyanide occurs via two one-electron steps, to produce a quinonoid dihydrofolate.

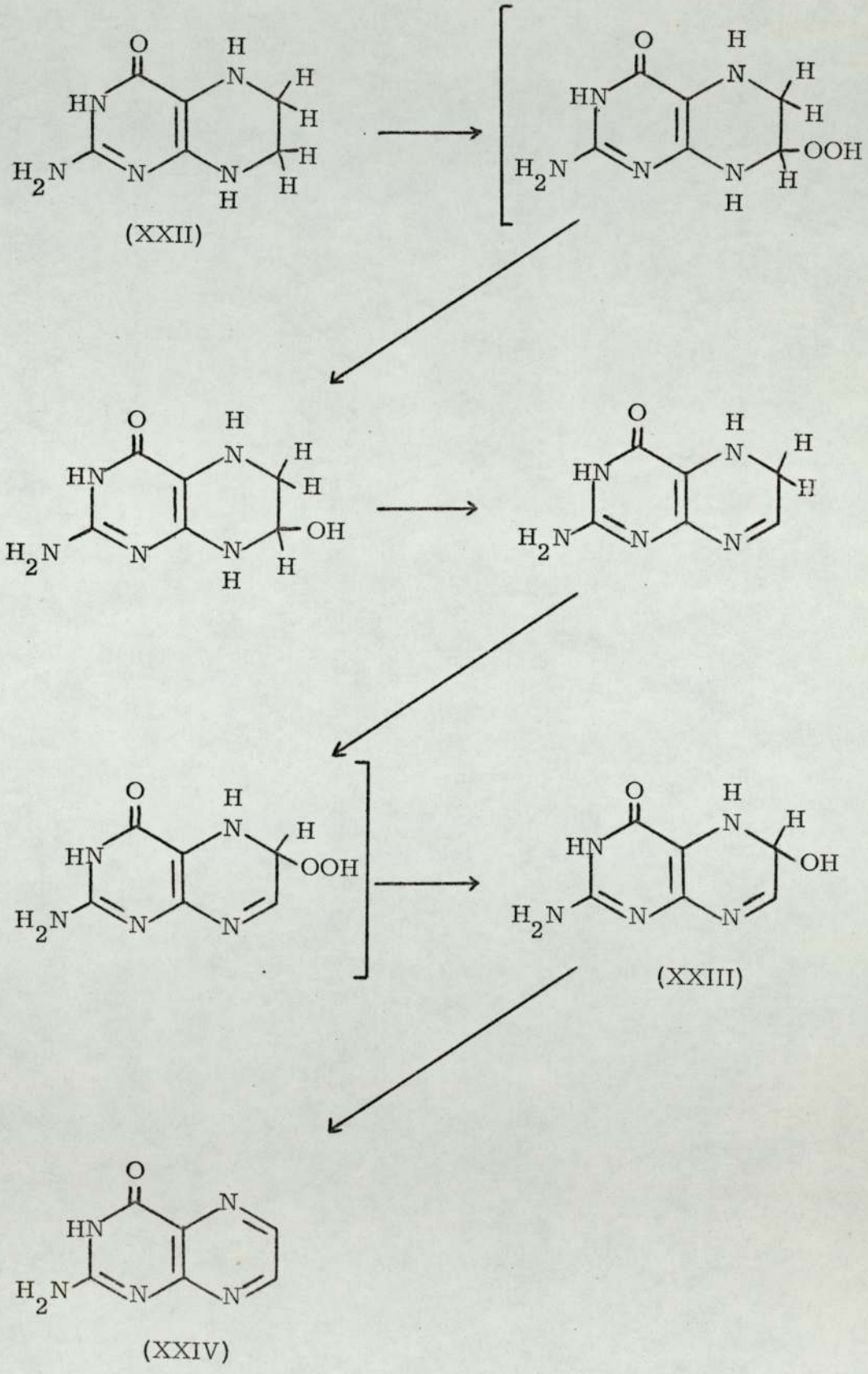
Some reluctance in accepting the idea of a quinonoid dihydropterin intermediate has been shown, Huennekens[130] believes that oxidation of THF occurs initially at the 5,6-position, particularly position 6, and Kaufman[131] has mentioned that the proposal by others (see below) of a hydroperoxide intermediate in the aerobic oxidation of tetrahydropterins remains to be established.

Rembold et al.[94] could not observe quinonoid intermediates in their studies on the oxidative degration 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 presented. The validity of their scheme is dependent on one vital assumption, which seems unlikely on the evidence of previous reports (see Chapter IV).

Hydroperoxides are the second type of intermediate that have been proposed to account for the oxidation of reduced pterins. Viscontini and Weilenmann[132] have studied the oxidation of tetrahydropterin. Their results are interpreted by Hawkins[133] as evidence for a scheme involving the formation of hydroperoxides (see Figure III). Although peroxidic compounds have not been isolated from tetrahydropterin (XXII), its rapid autoxidation to pterin (XXIV) is presumed to proceed by a mechanism involving their formation, and the intermediate alcohol (XXIII) has been identified when the oxidation is slowed by the presence of sulphur dioxide[132].

Mager and Berends in a series of papers[134-138] have described hydroperoxide intermediates for tetrahydropteridines and reduced

Figure III Oxidation of Tetrahydropterin Via Hydroperoxide Formation[133].

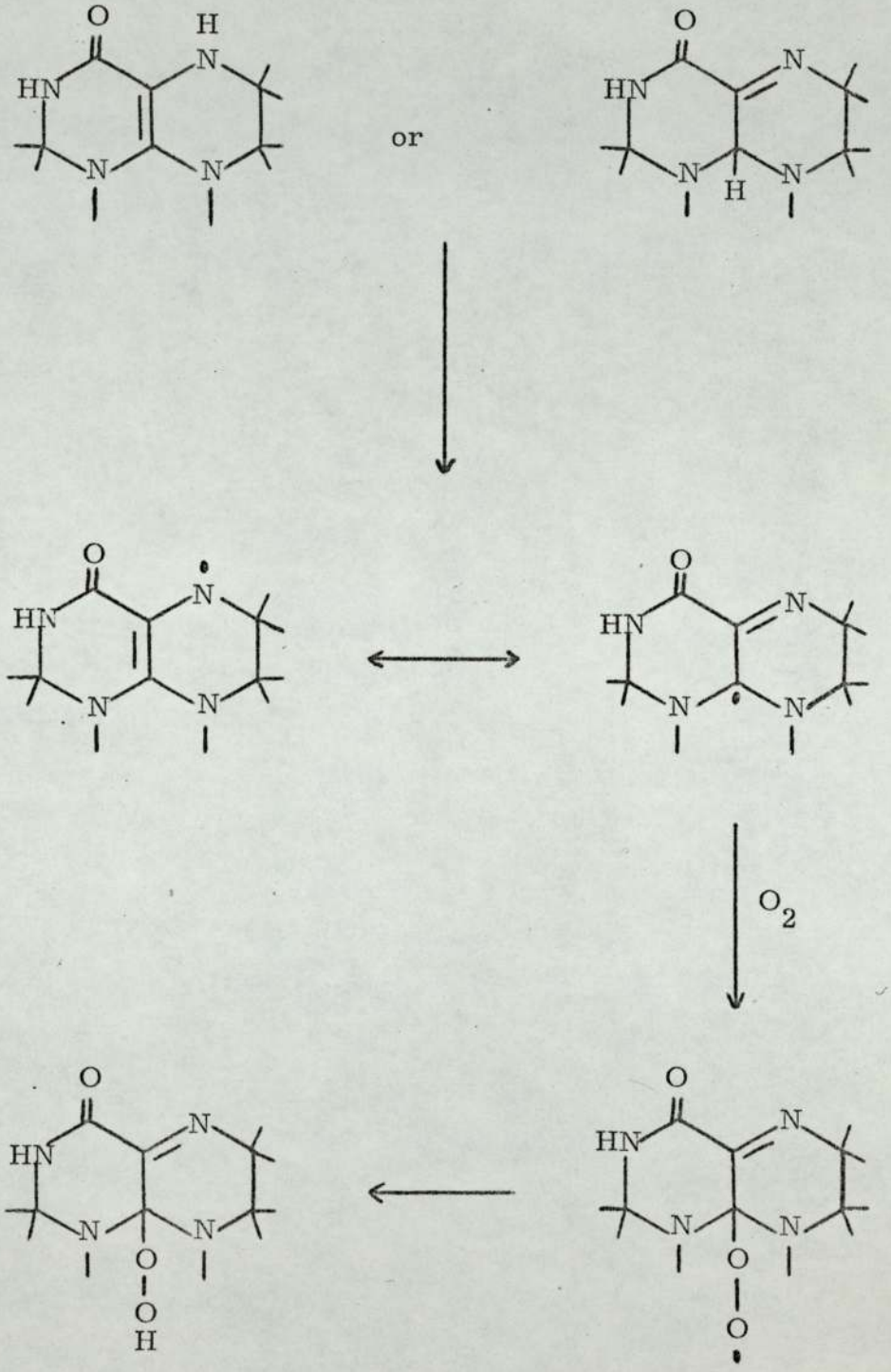


pyrazines, quinoxalines, isoalloxazines and flavins. Their general scheme for hydroperoxide formation during the autoxidation of such compounds is reproduced in Figure IV[135]. According to their theory[135] the hydroperoxide (H_3Pter-OOH), spontaneously produced from the tetrahydropteridine (H_4Pter), undergoes transformation into a hydroxytetrahydropteridine (H_3Pter-OH). A quinonoid dihydropteridine is then produced in a secondary reaction by dehydration of H_3Pter-OH. In the autoxidation of tetrahydropteridines the first step was the rapid uptake of oxygen to yield organic hydroperoxides. Manometric experiments also showed that the rate of oxygen uptake was dependent on the pH[138]. Peroxides were shown by qualitative test-reagents and polarography[135], while the amount of organic and/or hydrogen peroxide present was quantitatively determined manometrically after acidification of the reaction mixture[138]. Reducing agents considered by Kaufman[116], such as reduced nucleotides and mercaptoethanol, may be oxidised by the peroxide intermediate[135]. Mager and Berends[134] have also observed that tetrahydropyrazines, produced by catalytic hydrogenation, form peroxides on exposure to air.

The position with regard to a mechanism can be summarised as follows:- Involvement of radicals has been shown and is accepted. The point of contention is whether quinonoid dihydropterins and/or hydroperoxides are the actual intermediates. The position is further complicated by conflicting interpretations on both sides. On the quinonoid side, Kaufman believes initial attack is at N-5, whereas Archer and Scrimgeour have shown that attack is at C-6. Viscontini is beginning to question the idea of a quinonoid intermediate. Two important objections to the quinonoid dihydropterin scheme are

Figure IV Oxidation of Tetrahydropteridines.

Hydroperoxide Formation According to Mager and Berends[135].



a) that so far an acceptable rôle has never been given to molecular oxygen, and b) the scheme does not satisfactorily explain the presence of compounds such as xanthopterin in the products derived by autoxidation of tetrahydropterins. Objection a) is perhaps surprising when it is considered that Kaufman[139] states that oxygen is essential for phenylalanine tyrosine conversion involving the cofactor, and that in those aerobic hydroxylation systems where the reaction has been studied with ^{18}O it has been shown that the hydroxyl oxygen atom is derived from atmospheric oxygen.

On the hydroperoxide side, Mager and Berends consider a hydroperoxide at position 8-a which eventually produces a quinonoid dihydropterin. Again, this scheme does not explain the oxidation products. Hawkins, on consideration of other people's results, postulates initial hydroperoxide formation at position C-7.

It is clear that the mechanisms discussed above leave many questions unanswered. A mechanism is required that will satisfy experimental observations and correctly explain the formation of products derived by oxidation of reduced pterins and folates.

6. Topics Covered by this Study

The overall objective of this study is the elucidation of a mechanism for the oxidation of reduced pterins; to present a mechanism that will explain all experimental results and observations.

A major topic of this study is the autoxidation of THF in solution. Several reports on the oxidation appear in the literature, but none describes a comprehensive investigation. Also, although the rate of oxidation of reduced pterins is known to be pH and buffer dependent, it is believed that the products of autoxidation of THF are unaffected by pH.

This hypothesis has been tested by an extensive investigation into the autoxidation of THF at pH's 3, 7, 10 and 13. The products, identified by t.l.c., mass spectra, u.v. spectra and fluorescence spectra, are the same for each pH. The experiments performed to estimate the amount of each major pterin, using fluorescence spectra, clearly show the marked affect of pH.

A similar, but smaller, study of DHF and the effect of buffers on its rate of oxidation is described.

Autoxidation of a range of reduced pterins was followed using time-based u.v. spectral studies. Buffer, pH and deaeration effects are reported.

Observations with indophenol and ferricyanide oxidations of reduced pterins have been used to support a mechanism involving quinonoid dihydropterin intermediates. This evidence has been examined in depth and the results obtained are incompatible with such a scheme.

Evidence for the presence of hydrogen peroxide during the

autoxidations of DHF and tetrahydrobiopterin substantiated a belief in hydroperoxide intermediates. All the experimental results and observations to date concerning oxidations of tetrahydropterins have been considered and are explained by schemes involving hydroperoxides.

The mass spectra of a range of pterins are described. The characterisation of these compounds is discussed.

7. Introduction of Proposed Mechanism

A mechanism involving hydroperoxide intermediates is proposed and schemes are presented which explain their involvement.

The quinonoid dihydropterin scheme has been criticised in previous sections because it does not consider molecular oxygen, nor does it explain the oxidation of tetrahydropterins to give products with a new oxygen function in the molecule. Also, examination of certain quinonoid evidence has given results which are inconsistent with a scheme involving quinonoid intermediates (see Chapters VII and VIII).

Mager and Berends consider hydroperoxides at position 8-a which ultimately give quinonoid dihydropterins. Their ideas are therefore open to some of the criticism applied above. Hawkins has proposed a scheme to explain only the results of Viscontini and Weilenmann, and he considers initial attack at C-7. Although hydroperoxide intermediates are a more attractive possibility to explain the involvement of oxygen, it is clear that a completely satisfactory scheme has not appeared in the literature to date.

In this study all the evidence concerning oxidation of tetrahydro -pterins has been considered and is explained in schemes involving hydroperoxides at positions C-6 or C-9, which undergo subsequent rearrangement, etc.[133,140]. The schemes are discussed and presented in the appropriate chapters.

Rembold et al.[180] in their studies on the catabolism of pteridine cofactors report that the in vivo studies show a striking similarity to the in vitro results. The presence of hydroperoxide intermediates could also explain the requirement of a tetrahydropterin cofactor in enzymic hydroxylation reactions. Recent reports on such reactions suggest

the involvement of arene oxide intermediates[146-148]. Stereochemical studies using labelled compounds support these suggestions[149,150].

A possible scheme for the formation of arene oxide intermediates during hydroxylation reactions is one involving the addition of tetrahydropterin hydroperoxide radicals (ROO·) to the substrates aromatic system. Addition of peroxide radicals to benzene has recently been reported[151]. Such a scheme is presented in Chapter XIV.

II Oxidation of Tetrahydrofolic Acid

1. Introduction and Oxidation Procedure

Previous reports on the oxidation of THF have been considered in detail in Chapter I, section 4. Briefly, numerous investigators have attempted to identify the products of oxidation of THF, which is readily oxidised in solution by air, but they report only limited success. p-Aminobenzoylglutamic acid[66, 77, 105, 109], pterin[77, 94, 108], xanthopterin[66, 94, 108] and 6-methylpterin[66] have been observed among the products. Some reoxidation to DHF[77, 106, 107, 110] and folic acid[66, 110] also occurs. In addition, formaldehyde can be detected in the solutions of oxidised THF[108].

THF was prepared by the H_2/PtO_2 catalytic reduction of folic acid, using a modified version of the Hatefi et al.[68] procedure (see Experimental), and stored under nitrogen in the dark.

THF was oxidised by stirring in air a one mg. per ml. solution of THF diacetate in aqueous buffer at pH's 3, 7, 10 and 13. These pH values were chosen after consideration of the dissociation constants for THF, which contains six dissociable groups. The values for the two important groups are: acidic ionisation of the amide at position 4, pKa 10.5, and the N-5 position, pKa 4.82[152].

Initially, THF was oxidised according to the procedure of Zakrzewski[77]. A one mg. per ml. solution of THF was stirred in an open flask for one hour at pH 7, then rapidly adjusted to pH 3 with acetic acid. A transient brown colour was observed, but, unlike Zakrzewski, this acidification did not precipitate DHF in these experiments. Later studies showed that under certain conditions DHF could be precipitated.

Except for experiments to determine the presence of DHF (see Chapter II, section 7), subsequent oxidations were not acidified.

For qualitative investigations the solutions were stirred for 1 - 2 hours, thereby allowing complete oxidation of THF[112]. To ensure total oxidation to the aromatic derivatives, quantitative experiments were stirred for three days. The solutions were then lyophilised. Oxidations in alkaline media were lyophilised immediately after rapid acidification with hydrochloric acid.

The buffer solutions used were:- dilute acetic acid pH 3; 0.1 Molar ammonium acetate pH 7; NaOH - $NaHCO_3$ buffer pH 10 and 0.1N. NaOH pH 13.

At pH's 7, 10 and 13 THF solutions rapidly developed a yellow green colour, whereas at pH 3 the solution became brown on standing.

2. Isolation of the Products

Preparative chromatography was used to separate the products, rather than the usual technique of column chromatography, in the belief that it would be a more convenient method for the scale of the oxidation experiments and the possible number of products.

For qualitative investigations preparative thin-layer chromatography (preparative t.l.c.) was employed. A 1% ammoniacal solution of the lyophilised solid from oxidised THF was applied, by means of a Burkard Streak Applicator, to May and Baker "Chromalay" grooved plates coated with one mm. thick silica gel G. The chromatograms were developed in the solvent system n-propanol/1% ammonia solution, 2 : 1. Under u.v. 365 nm. light the products appeared as bands, since pterins and their dihydro derivatives fluoresce strongly at this wavelength. Removed bands were eluted with 1% ammoniacal solution. T.l.c. using cellulose MN 300 u.v. 254 plates enabled the observation of non-fluorescing compounds, which appeared as dark absorbing spots under u.v. 254 nm. light, while other pterins were observed as fluorescent spots under u.v. 365 nm. light. Lyophilised samples containing a single oxidation product were obtained by re-chromatographing selected eluates. Isolated compounds were identified by comparison with authentic samples using t.l.c., u.v. spectra and fluorescence spectra. Despite considerable effort and time the compounds could not be obtained suitably free of inorganic material, derived from silica gel, to permit the preparation of mass spectra.

To prevent formation of 6-amino pterin it was important to avoid undue contact between the original lyophilised solid and ammonia (see Chapter II, Section 8).

Cellulose could not be used for preparative chromatography because the isolated samples contained impurities which absorbed in the u.v. range and, as with silica gel, were unsuitable for mass spectral analysis.

Preparative paper chromatography was used for the quantitative estimation of the products. Approximately 0.1% ammonia solution was added to the lyophilised solid from oxidised THF and the slurry applied as a streak to Whatman No. 1. paper. The chromatogram, developed in the solvent system n-propanol/1% ammonia, 2:1, was viewed under u.v. 365 nm. light and the bands carefully eluted with 0.1N. NaOH.

Preparative paper chromatography was sometimes used to isolate samples of the oxidation products. To prevent extraneous material in isolated samples, it was important to wash the paper with n-propanol/1% ammonia, 2:1, prior to use. Samples isolated from untreated paper gave mass spectra with additional peaks at all or most of the following m/e values:- 354, 283, 282, 281, 267, 223, 222, 221, 209, 208, 207, 194, 193, with m/e 207 often the dominant peak above m/e 160. The above treatment eliminated or considerably reduced these additional peaks. The weight of impurity derived from Whatman No. 1. paper was examined. 1) 164 cm^2 of untreated paper gave 25 mg. of cream solid when eluted with 50 ml. of 1% ammonia solution. 2) When washed with 0.880 ammonia:water, 1:1, then dried prior to elution, 164 cm^2 gave 3.8 mg. on elution. 3) A sheet of Whatman No. 1. paper was washed with 0.880 ammonia: water, 1:1, dried and then developed in n-propanol/1% ammonia, 2:1, 164 cm^2 bands were removed and eluted with 50 ml volumes of 1% ammonia. The Rf value for the centre of each band and the respective weight of solid isolated (mg) were:- 0.94 (35); 0.81 (34); 0.69 (49); 0.56 (54); 0.44 (40); 0.31 (11); 0.19 (32); 0.06 (8).

3. Identification of the Products.

The products from the oxidation of THF were isolated in a form suitable for analysis by u.v. spectra, t.l.c. and fluorescence spectra, but, unfortunately, not suitable for mass spectral analysis (see previous section). This problem was surmounted by mass spectral examination of the solid obtained by lyophilisation of the oxidised THF solution. Good composite spectra could be obtained and all the pterins present were identifiable by peak matching at the appropriate m/e values. It was possible to identify the components in the composite spectrum, since in this case the fragmentation pattern of any pterin present did not lead to a peak identical to the molecular ion of another pterin.

The lyophilised solid was shown by mass spectra and t.l.c. to contain p-aminobenzoylglutamic acid, pterin, folic acid, dihydro -xanthopterin and xanthopterin, with traces of 6-methyl pterin. Mass spectra also showed traces of 6-methyl-tetrahydropterin.

The presence of p-aminobenzoylglutamic acid as a degradation product of THF has been confirmed by previous investigators[66, 77, 105, 109], so no special effort was put into its investigation, but its presence was further verified during the course of t.l.c. and u.v. studies with isolated pterin.

T.l.c. examination of a freshly prepared 1% ammoniacal or aqueous solution of the lyophilised solid from oxidised THF showed the presence of up to seven fluorescing compounds when the chromatogram was viewed under u.v. 365 nm. light. A mauve (pterin) and a blue-green (dihydroxanthopterin) spot were the major components. Of the remaining spots, one (6-methyl pterin) was small and another was very small with respect to the main two, while the others were only ever observed as

extremely faint spots. Under u.v. 254 nm. light an absorbing spot (p-aminobenzylglutamic acid) was observed. A second absorbing spot (folic acid) was detected when concentrated solutions were examined. Dihydroxanthopterin in solution was readily oxidised solely to xanthopterin. A chromatogram of the ammoniacal solution also contained a green fluorescing spot. T.l.c. showed that with time this became a major component. Formation of this compound, 6-aminopterin, is discussed in Chapter II, section 8.

Pterin, xanthopterin and 6-methylpterin, isolated by preparative t.l.c., were further identified using u.v. spectra at pH's 1 and 13, and fluorescence spectra at pH's 1, 7 and 13. The appearance of the fluorescence spectra and their maxima are both important when identifying pterins. Pterin was also confirmed by t.l.c. in eleven solvent systems, while xanthopterin and 6-methylpterin were confirmed using eight and six solvent systems respectively.

Analytical data is presented in the Experimental.

4. Quantitative Estimation of the Products

Pterin, xanthopterin and 6-methylpterin, isolated by preparative paper chromatography, were quantitatively determined by fluorimetry [183].

Standard solutions of pure compounds were made up in 0.1N. NaOH and used to derive a calibration graph of fluorescence versus concentration for each compound. (Presented in Appendix)

The products from oxidised THF were separated and isolated by preparative paper chromatography, followed by elution of their bands with 0.1N. NaOH. The amount of each compound present was determined by comparison of the fluorescent intensity of the eluate with the calibration graph. The results are presented in the following section.

The efficiency of elution was determined by paper chromatography of known quantities of pterin and xanthopterin, eluting the bands with 0.1N. NaOH under the same conditions as the oxidation products and determining the eluate contents by the above procedure. Recoveries of pterin and xanthopterin were 100% and 97% respectively.

5. Effect of pH on the Oxidation Products

Pterin, xanthopterin and 6-methyl pterin derived from THF oxidations at pH's 3, 7 and 10 were estimated by fluorimetry. 6-Methyl pterin varied little with pH and always accounted for less than 0.5% of the THF used. The reaction products pterin and xanthopterin were pH dependent (see Table II and also Table VI in Experimental).

TABLE II Autoxidation of Tetrahydrofolic Acid

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

The amount of each product, rather than the number of products, varied with these pH values.

Pterin and xanthopterin accounted for 50-60% of the THF used, the balance being mainly folic acid and DHF. These values agree with that of Blakley[66] who observed 52% cleavage of THF after aeration for one hour. His figure is based on a determination of the amount of aromatic amine in solution, which he assumed to be p-aminobenzoyl -glutamic acid.

Since the completion of the above studies, a quantitative investigation of the pterins formed during THF autoxidation has been reported by Rembold et al[94]. THF was oxidised in 0.1 Molar

Tris-HCl buffer, pH 7.5, and after two hours the reaction was boiled. Their ratio for xanthopterin plus dihydroxanthopterin : pterin is 1.81 : 1.00 and these products account for 31% of the THF used.

6. Effect of Metal Ions on the Oxidation

In one experiment THF was stirred for one hour at pH 7 in the presence of Fe^{3+}, as $(NH_4)_2\ SO_4\ Fe_2(SO_4)_3\ 24H_2O$, sufficient salt being added to give approximately one mole of Fe^{3+} per four moles of THF. Examination of the lyophilised solid showed the presence of the usual products.

When THF was oxidised at pH 7 in the presence of four equivalents of Fe^{2+}, using $FeCl_2$, and the amounts of pterin and xanthopterin determined by fluorimetry in the usual manner, the pterin : xanthopterin ratio was 1.46 : 1.00 and accounted for approximately 44% of the THF used.

Chippel and Scrimgeour[111] have recently examined the degradation of THF by ferricyanide under alleged anaerobic conditions. At pH 9 and 24° about 40% of the THF was degraded to p-aminobenzoyl -glutamic acid. Addition of increasing amounts of ferricyanide at pH 9 and 0° produced first DHF, and then folic acid as product. Recoveries from THF reached 70% for DHF (at two equivalents of $K_3Fe(CN)_6$) and 60% for folic acid (at four equivalents of $K_3Fe(CN)_6$). The pterins obtained by oxidation of THF at pH 9 were not examined. At pH 5.6 THF was completely degraded to p-aminobenzoylglutamic acid and several pterin compounds. Recoveries of p-aminobenzoylglutamic acid were in excess of 90% and there was a pterin : xanthopterin ratio of about 1.0 : 3.5.

7. Formation of DHF during the Oxidation

In Chapter II, section 1 mention was made of the fact that during the earlier studies of THF oxidation at pH 7, no DHF was precipitated by the addition of acetic acid. Subsequent investigation showed that DHF was precipitated under certain conditions.

A precipitate appeared at pH 6-7 when hydrochloric acid was slowly added to a one mg. per ml. solution of THF in 0.1N. NaOH, which had been stirred for one hour. Acid addition was continued to give a final solution of pH 1 - 2. By means of t.l.c. and u.v. spectra the precipitate was identified as DHF. U.v. spectra was also used to assay the DHF[79]. The yield of DHF was critically dependent on both the rate of acidification and the acid used. Hydrochloric acid was superior to acetic acid. Rapid adjustment of pH produced a negligible amount of precipitate. For oxidations at pH 13 the precipitated DHF accounted for 40% of the THF used, at pH 9.5 in the presence of sodium bisulphite the figure was 31%, and at pH 7 it was 15%.

When THF was oxidised at pH 7 in the presence of four equivalents of either Fe^{2+} or Sn^{2+}, only trace amounts of DHF were observed.

8. Formation of 6-Aminopterin

When the lyophilised solid from oxidised THF was treated with aqueous ammonia and examined by t.l.c., a green fluorescing spot was present. This spot was never observed when lyophilised solid was dissolved in water. Solids from lyophilised ammoniacal solutions gave mass spectra that contained a major peak at m/e 178. Again, this peak was not present in the absence of contact with ammonia. Peak matching at m/e 178 showed the presence of 6-aminopterin.

The green fluorescing compound, isolated by preparative t.l.c., was further identified by comparison with authentic 6-aminopterin, using u.v. spectra at pH's 1 and 13, fluorescence spectra at pH's 1, 7 and 13, and by t.l.c. in six solvent systems.

Aqueous ammonia is known to react with 7,8-dihydro pteridines to give yellow adducts, which in solution are readily oxidised to the 6-amino compound[52,93,153]. The proposed scheme for autoxidation of THF shows the formation of both dihydropterin (XXXIII) and dihydroxanthopterin (XXXVII) (see Figure VII). A sample of standard dihydroxanthopterin in 0.880 ammonia oxidised to xanthopterin on standing, no additional compound was observed. Formation of 6-aminopterin (XXXVI) is therefore due to the reaction of 7,8-dihydro pterin (XXXIII) with ammonia and subsequent oxidation of the tetrahydro compound (XXXV) (see Figure VII).

9. Discussion of Results

The data suggest a scheme involving autoxidation of THF to form a hydroperoxide, -OOH, at either C-6 or C-9 (see Figures V and VI), which then undergoes decomposition and rearrangement (see Figures VII and VIII). E.s.r. spectra[123,124] of solid THF (XXV), 5-methyl THF, 5-ethyl THF and 5-n-propyl THF show an unpaired electron on a nitrogen atom. As 5-formyl THF does not give a spectrum, this electron may be assigned to N-5 (see XXVII and XXVIIa).

In neutral media oxygen attack occurs at C-6 and C-9. C-6 being a tertiary carbon atom is the favoured point of attack and dihydroxanthopterin (XXXVII) is mainly formed (Table II). In solution, XXXVII is readily oxidised to xanthopterin (XXXVIII). In acid media oxygen attack occurs mainly at C-9 because of protonation of N-5 (pKa 4.82[152]) and pterin (XXXIV) is mainly formed. In alkaline media attack is at C-6. The hydroperoxide is present as the stable ionic species $-O-O^{\ominus}$ (XL) at pH 13, and this anion reacts with the hydroperoxide in bimolecular reaction to form 6-hydroxy-THF (XLII) which then loses water forming DHF (XLIII). Hydroperoxides, even in dilute solution, tend to associate together and predominantly decompose to molecular rather than radical products[154]. This explains the requirement of slow acidification during the precipitation of DHF (See Chapter II, section 7). Hydroperoxide formation at C-9 is preferred over that at C-7 because the formation of a planar free radical is more difficult in the half-chair conformation of the tetrahydropyrazine ring.

(9-^{3}H) THF is oxidised to give tritiated water and formaldehyde[108]. The formation of tritiated water is explained by Figure VI and subsequent exchange of the hydroperoxide proton with that of water. Tritiated

formaldehyde is explained by hydrolysis of CH_2OHR to give formaldehyde plus p-aminobenzoylglutamic acid (IXL) (Figure VII).

A possible scheme for the reaction of THF with Fe^{2+} or Fe^{3+} is one involving a radical cation. Easy formation of such radicals has been predicted[117]. Oxygen attack would then occur preferably at C-9 to form mainly pterin. This postulate is in accord with the experimental results (see Chapter II, section 6). Under aerobic conditions the reaction of THF with Fe^{2+} or Fe^{3+} is expected to be complicated by the fact that the Fe^{3+} ions, by accepting one electron, can induce fission of the O-H bond of hydroperoxides, while Fe^{2+} ions, by electron donation, can induce decomposition of the hydroperoxide at the O-O bond[140]. It follows that the metal ion in either its M^{n+} or $M^{(n-1)+}$ valence state can be used and that a small amount of the ion will suffice to decompose a large amount of hydroperoxide.

Figure V Autoxidation of THF. Radical Formation

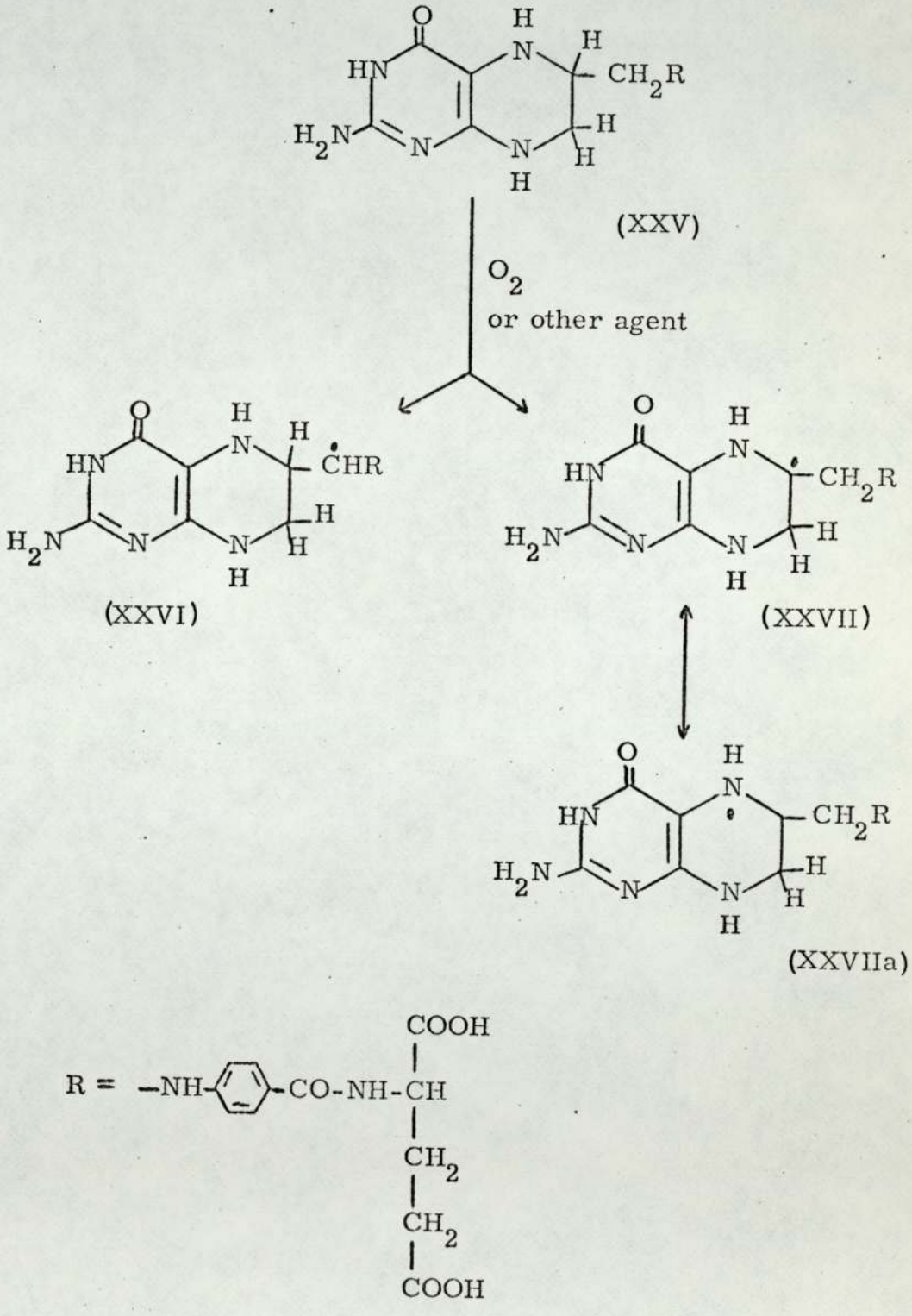
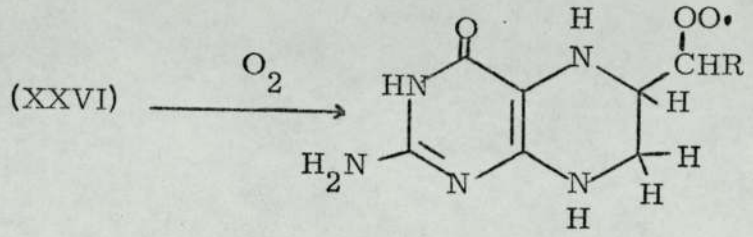
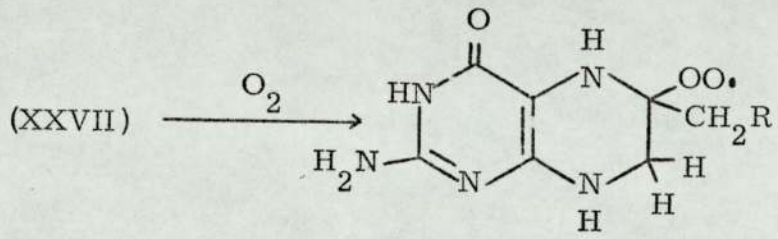


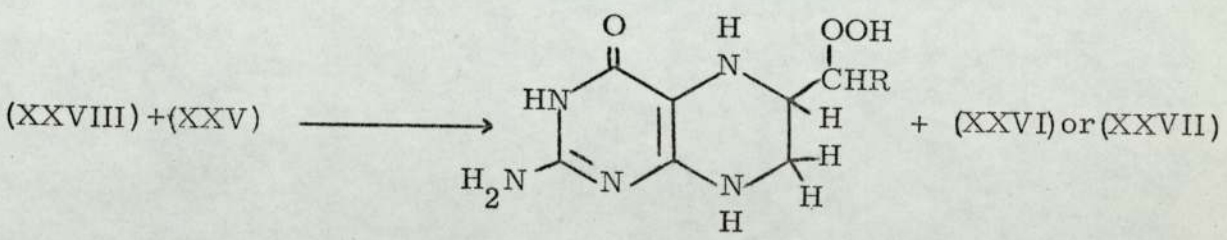
Figure VI Autoxidation of THF (cont.)
Hydroperoxide Formation



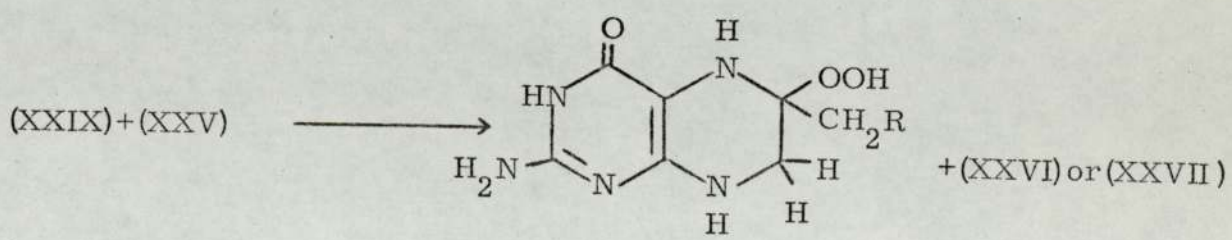
(XXVIII)



(XXIX)



(XXX)



(XXXI)

Figure VII Autoxidation of THF (cont.)
Formation of Pterin, Xanthopterin
and p-Aminobenzoylglutamic Acid

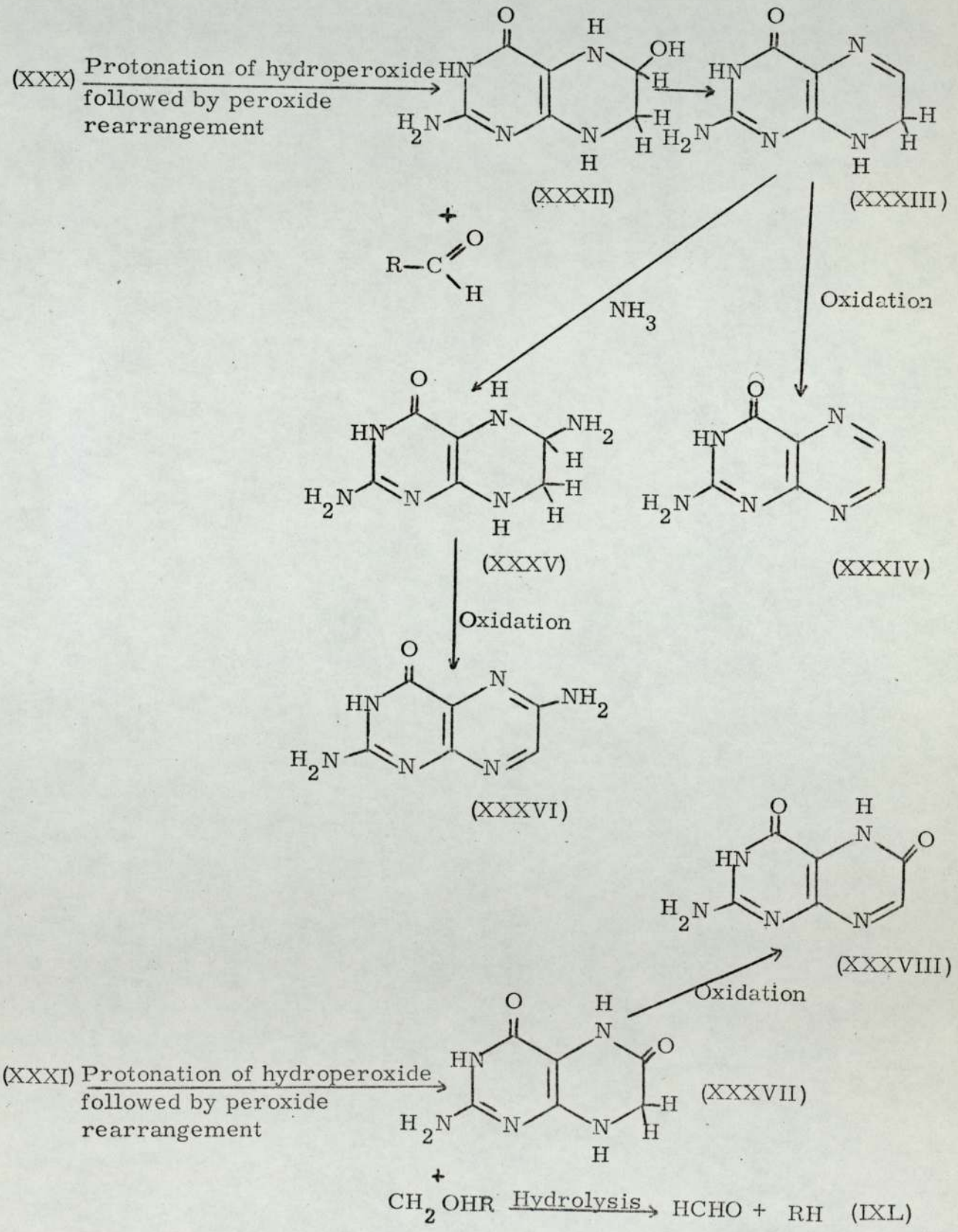
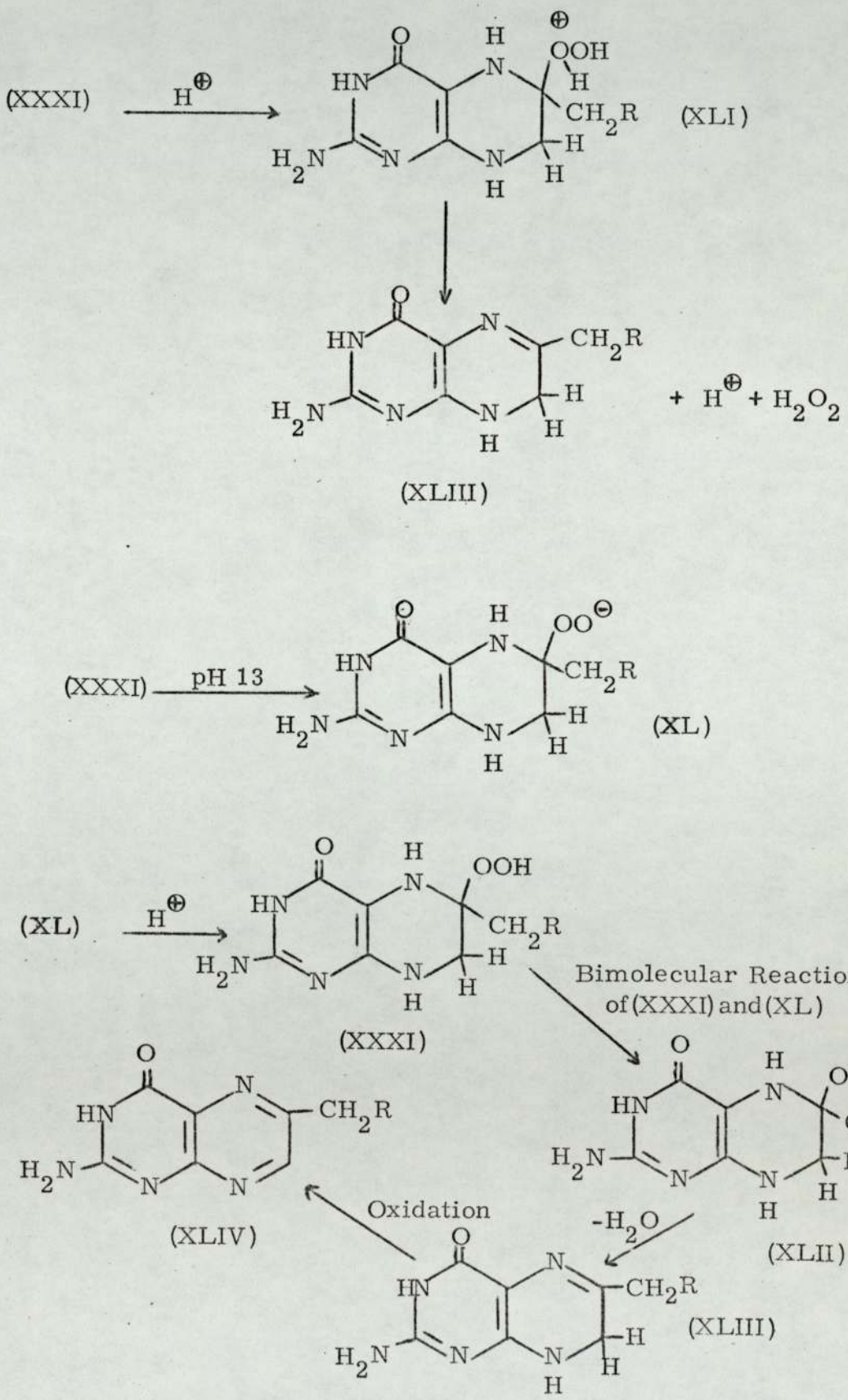


Figure VIII Autoxidation of THF (cont.)
Formation of DHF, Folic Acid
and Hydrogen Peroxide



10. Use of 2-Mercaptoethanol

2-Mercaptoethanol is often used as a convenient antioxidant for solutions of reduced pterins and folates. Some limitations in its use are now reported.

Mass spectral investigation of compounds isolated by preparative t.l.c. had proved to be an unrewarding technique (see Chapter II, section 2). Initially, the silica gel or cellulose was eluted with a 1% ammonia solution containing 2-mercaptoethanol, to prevent further oxidation of any reduced pterin intermediates from the original oxidative degradation. All the spectra of samples isolated by this technique contained a major peak at m/e 154, with no peaks present that corresponded to a pterin. It was found that this peak only occurred in samples eluted in the presence of 2-mercaptoethanol. Peak matching at m/e 154 gave a value of 154.01045. This compares with the corrected m/e value for $C_4H_{10}O_2S_2$ which is 154.01222. The molecular weight of 2-mercaptoethanol is 78. A peak at m/e 154 can be accounted for by its oxidation to β, β -dihydroxydiethyldisulphide (dithio bisethanol).

$$SHCH_2CH_2OH \xrightarrow{\text{oxygen}} HOCH_2CH_2SSCH_2CH_2OH$$

Zakrzewski has postulated the formation of a disulphide during the interaction of THF with 2-mercaptoethanol[110]. Due to its high boiling point, the disulphide would be concentrated during lyophilisation of the eluate.

c.f.	2-Mercaptoethanol[155]	b.pt.	157-8° at 742 mm. Hg.
		or	55° at 13 mm. Hg.
	Dithiobisethanol[156]	m.pt.	26-8°
		b.pt.	141-3° at 0.8 mm. Hg.
		or	106° at 0.015 mm. Hg.

2-Mercaptoethanol in aqueous solution has an absorption spectrum with λ_{max} about 212 nm. U.v. spectral studies showed that in aqueous solution 2-mercaptoethanol was oxidised to give a spectrum with an intense absorbance at about 256 nm. This compares with the values for many organo disulphides which absorb in the region 245 - 260 nm.

III Oxidationof Dihydrofolic Acid

1. Introduction and Results

DHF is oxidised in phosphate buffer (pH 7) to produce a yellow solution ($\lambda_{max.}$ about 270 and 420 nm.) due to the formation of 6-formyl-7,8-dihydropterin and p-aminobenzoylglutamic acid[97,98]. An increased absorbance above 400 nm. is also observed for acid solutions,[99-102] DHF being more stable in alkaline solution[97]. To date, there is no report on whether the dihydro aldehyde is the only pterin isolated from autoxidised DHF, nor has a complete mechanism been proposed for the oxidation. Whiteley et al.[98] report that one mole of oxygen is required for each mole of DHF converted to 6-formyl-7,8-dihydropterin. Although they propose that the oxidation proceeds via attack at the C-9 — N-10 linkage followed by the rapid dissociation of the resultant Schiff's base, no mechanism is presented to explain the formation of a Schiff's base.

DHF was prepared by dithionite reduction of folic acid according to the procedure of Futterman[76], and stored frozen as a suspension in very dilute hydrochloric acid containing 10% ascorbic acid. Before use, DHF was obtained free of ascorbic acid by washing with very dilute hydrochloric acid, followed by acetone, and then dried under vacuum. DHF was oxidised by stirring in air a 0.1% solution of DHF in aqueous buffer. After three days at pH 10, NaOH-$NaHCO_3$ buffer, the products were p-aminobenzoylglutamic acid and dihydroxanthopterin or xanthopterin. After three days in 0.1 Molar ammonium acetate solution, pH 7, the products were p-aminobenzoylglutamic acid, folic acid and dihydro -xanthopterin or xanthopterin. The products were identified by t.l.c.

of the oxidation solutions and by mass spectral and t.l.c. examination of the resultant lyophilised solid. Folic acid, p-aminobenzoyl -glutamic acid and xanthopterin (dihydroxanthopterin is readily oxidised to xanthopterin) were isolated using preparative paper chromatography and further identified by t.l.c., mass spectra and u.v. spectra. (evidence for the products is presented in the Experimental).

When a pH 7 solution of DHF was stirred for a few hours, the products were p-aminobenzoylglutamic acid and 6-formyl-7,8-dihydro -pterin (t.l.c. and u.v. spectral evidence), plus unchanged DHF (t.l.c. evidence). It was not possible to obtain mass spectral evidence for 6-formyl-7,8-dihydropterin. Mass spectra of the lyophilised solid only contained major peaks at m/e 181 and 177, no peak being present at either m/e 193 or 191. 6-Formyl pterin has a mass spectrum with an intense molecular ion peak at m/e 191 (See Chapter XIII). Whiteley et al.[98] do not report isolating the dihydro aldehyde, it being identified by comparison with an authentic sample using t.l.c., u.v. spectra and fluorescence spectra. The oxidation of DHF can be compared to that of 6-hydroxymethyl-7,8-dihydropterin. Rembold et al.[180] have recently observed that 6-hydroxymethyl-7,8-dihydropterin undergoes aerobic oxidation at neutral pH to yield dihydroxanthopterin, xanthopterin and an unidentified yellow compound - possibly 6-formyl-7,8-dihydro -pterin.

The rate of disappearance of DHF at pH 7, as shown by t.l.c., was buffer dependent, being considerably faster in phosphate than in ammonium acetate and very slow in water, pH 6.2.

When the dihydro aldehyde,as lyophilised solid from oxidised DHF, was treated with aqueous ammonia, the products on standing were

xanthopterin and 6-aminopterin (t.l.c. evidence).

After five hours at 78°, DHF in 0.1 Molar ammonium acetate, pH 7, containing 1% ascorbic acid, gave the dihydro aldehyde and p-aminobenzoylglutamic acid. (Shown using t.l.c. and u.v. spectra). Repeating the experiment in the presence of 10% ascorbic acid gave unidentified products and no dihydro aldehyde (t.l.c. evidence).

Addition of hydrogen peroxide solution to DHF in 0.1 Molar ammonium acetate, pH 7, produced four products. T.l.c. examination after one hour showed the major product to have a Rf value similar to xanthopterin. Mass spectra of the lyophilised solid contained large peaks at m/e values 167 - 165, with the major peak at m/e 167. No peaks appeared at the usual pterin values. The products were not identified.

2. Discussion of Results

The results suggest autoxidation of DHF (XLV) to form a hydroperoxide, -OOH, at either C-9 (mainly) or C-7 (minor) which then undergoes decomposition (see Figures IX and XI). Both C-9 and C-7 are secondary carbon atoms, but the acyclic C-9 position is the favoured point of attack since its unrestricted rotation enables formation of a planar free radical whereas at C-7 formation is more difficult because of the half-chair conformation of the reduced pyrazine ring. (C.f. Hydrogen abstraction and radical formation in cycloalkenes is regulated by the geometry of the ring system[195]). The scheme follows the autoxidation of imines[158]. Oxygen attack on an imine is normally at the α-position to the carbon of the C=N function.

e.g. $R\cdot N = CH\cdot CHPh_2 \xrightarrow{O_2} R\cdot N = CH\cdot \underset{\displaystyle HOO}{\overset{\displaystyle |}{C}}Ph_2$

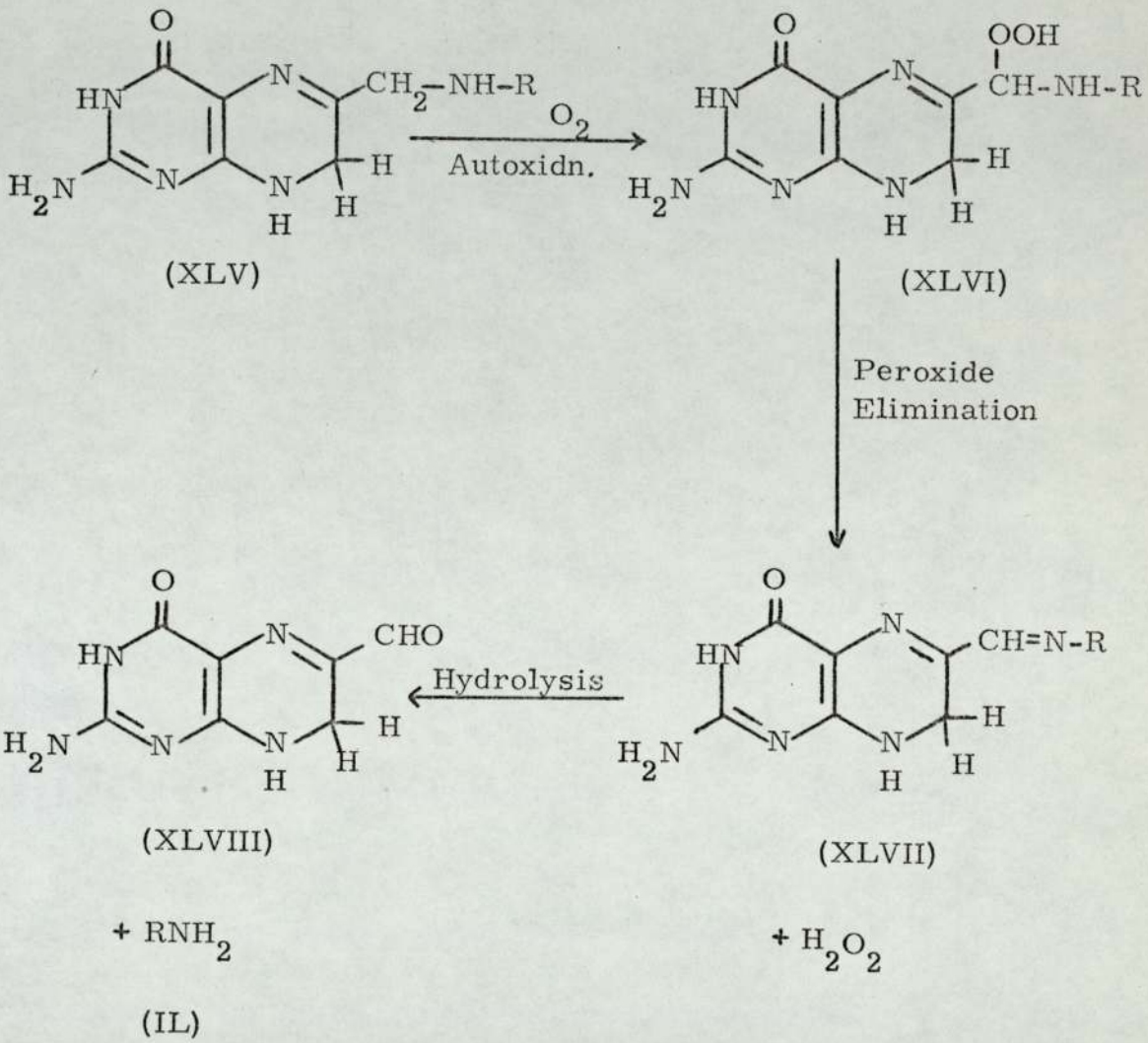
Imines such as $C_6H_{11}N{=}CH\cdot Ph$ or $C_6H_{11}N{=}CPh_2$, having neither a methylene (or methine) group α to the carbon of the C=N nor an activated methylene (or methine)group α to the nitrogen of the C=N are extremely unreactive to normal autoxidation conditions[158].

6-Formyl-7,8-dihydropterin (XLVIII) and p-aminobenzoyl -glutamic acid (IL) are formed via a Schiff's base or anil (XLVII). Anils are readily hydrolysed to the amine and carbonyl compound[159]. Although C-9 is the favoured position for radical formation, some hydroperoxide formation does occur at position C-7 which on peroxide elimination gives folic acid (LIV). Dihydroxanthopterin (LI) and xanthopterin (LII) are formed by autoxidation of the dihydro aldehyde to give a peroxyacid which undergoes peroxide rearrangement. (See

Figure X). Aldehydes can be autoxidised to a peroxyacid which, depending on conditions such as temperature, solvent and the compounds structure, can either lose oxygen to give the normal acid or it can undergo decomposition to give a hydroxy compound[133]. The peroxyacid (L) formed from 6-formyl-dihydropterin undergoes the latter process. Formation of 6-aminopterin (LIX) and xanthopterin is due to the reaction of the 7,8-dihydro aldehyde (XLVIII) with ammonia and subsequent oxidation of the tetrahydro compound (LV)[52,93,153]. (See Figure XII).

Variation in buffer at pH 7 alters the rate of disappearance of DHF rather than the appearance of 6-formyl-dihydropterin. This is due to the buffer anion participating in the autoxidation step by a process of molecule-induced homolysis[160]. Molecule-induced homolysis is most often observed in systems in which the solvent has polarizable electrons and behaves as a nucleophile. In the oxidation of DHF the homolysis is a process of hydrogen abstraction by the buffer anion to yield a radical at either position C-9 or C-7. Formation is expected, and found, to be most rapid in phosphate solution (the most nucleophilic anion).

Figure IX Autoxidation of DHF



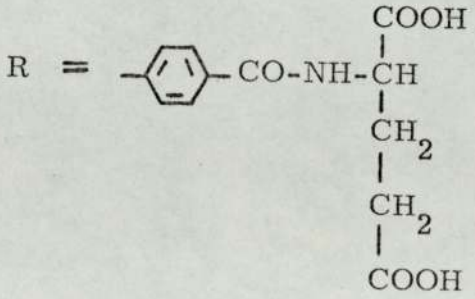


Figure X Autoxidation of 6-Formyl-7,8-Dihydropterin

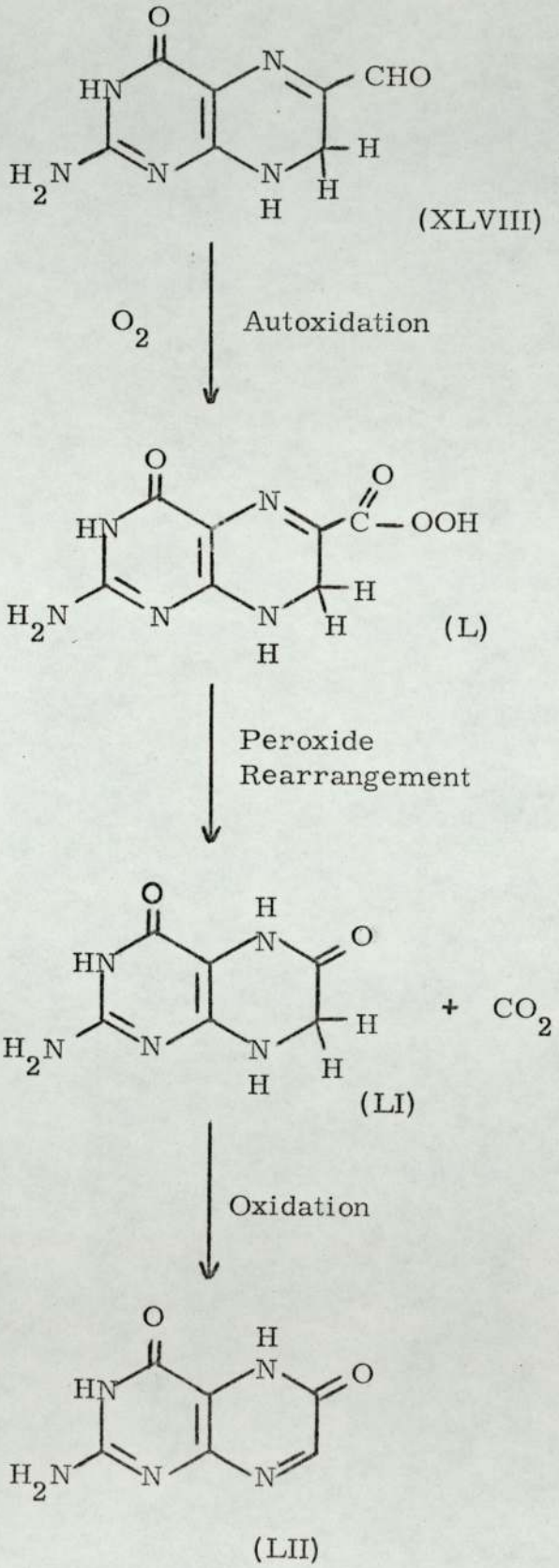


Figure XI Autoxidation of DHF Giving Folic Acid

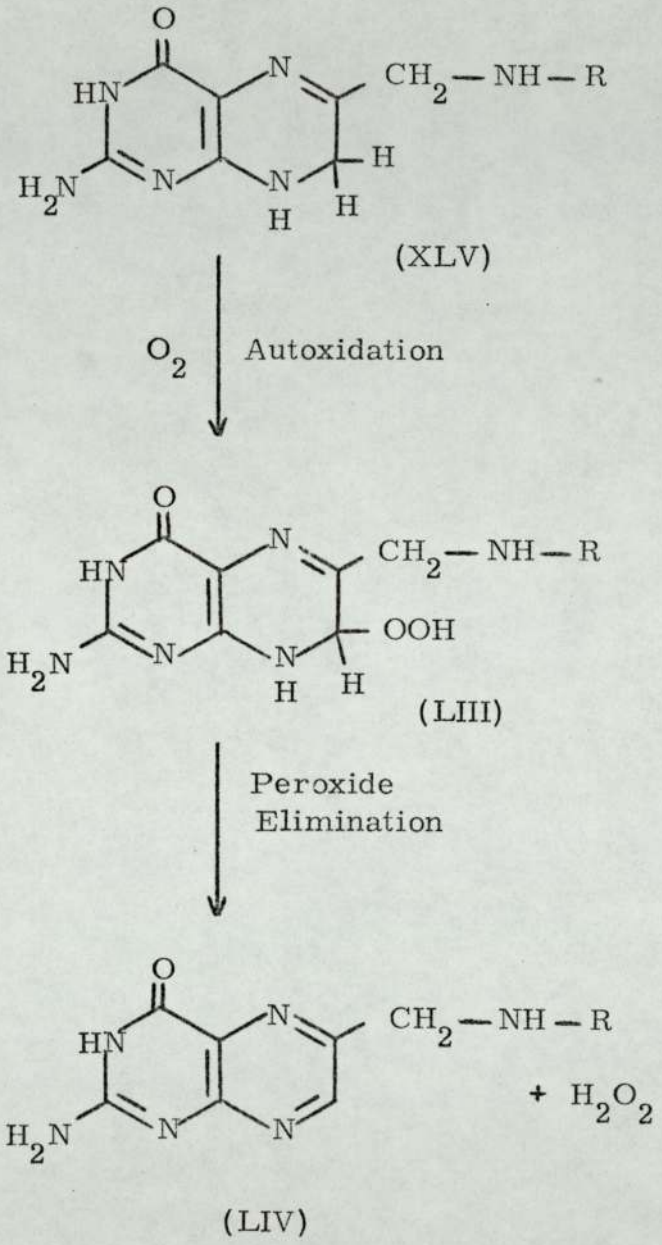
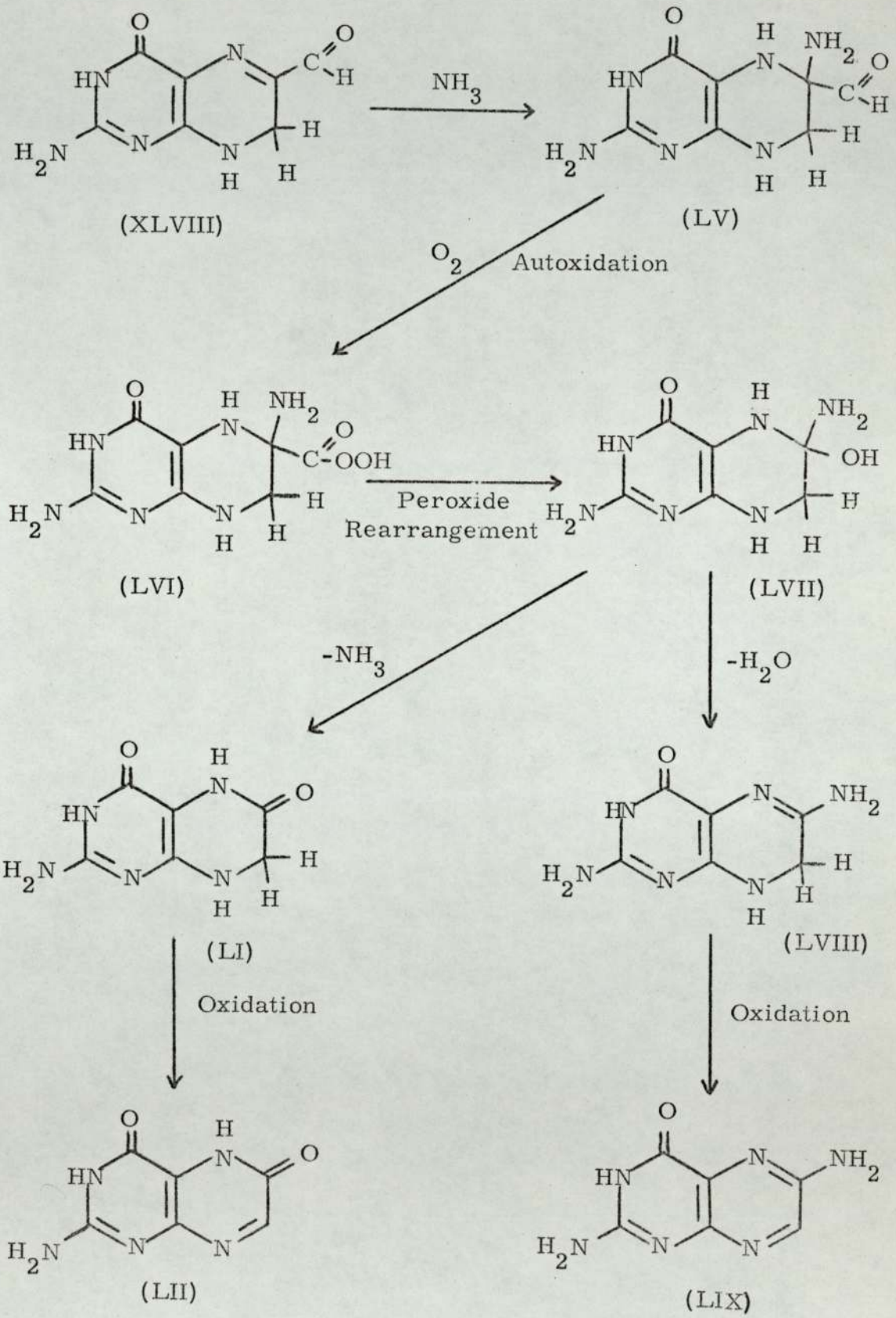


Figure XII Autoxidation of 6-Formyl-7,8-Dihydropterin in the Presence of Ammonia



IV Oxidations of other Tetrahydropterins

This chapter deals mainly with reported oxidations of various tetrahydropterins and the application of schemes involving hydroperoxide intermediates to the interpretation of these results.

When tetrahydrobiopterin was dissolved in phosphate buffer, pH 7, and allowed to stand for 48 hours, a yellow-green solution was obtained. T.l.c. examination showed the major oxidation product to have the same Rf and fluorescence as authentic biopterin. There were traces of several unidentified compounds. (See Experimental)

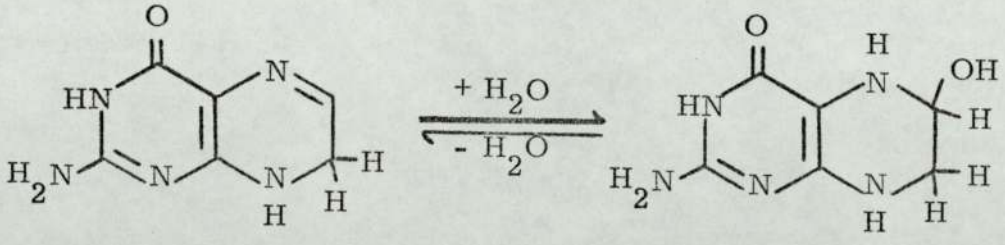
Subsequently, Rembold et al.[61, 94] have reported oxidations of tetrahydrobiopterin, tetrahydroneopterin and tetrahydrolumazine. Oxidation of tetrahydro (2-^{14}C) biopterin (LXb)[61] in 0.05 Molar veronal-acetate buffer, pH 7.5, by boiling in air, gave pterin (LXIX) and dihydroxanthopterin (LXX) as the major products in a 1:1 ratio (see Figures XIII - XVI), whereas oxidation with iodine in 5% acetic acid gave biopterin (LXXVIIIb) as the major product with little pterin and no dihydroxanthopterin, (see Figure XVII). Tetrahydroneopterin (LXc), oxidised in 0.1 Molar Tris-HCl buffer, pH 7.5, and after two hours the solution boiled, gave xanthopterin plus dihydroxanthopterin and pterin in a 1.59:1.00 ratio[94]. Together, these products account for 43% of the oxidised tetrahydroneopterin. Experiments with tetrahydroneopterin having a ^{14}C-labelled side chain show that cleavage of the side chain produces glyceraldehyde (LXXII) (see Figure XV) and is coupled to an oxidative process[61]. Oxidation of tetrahydrolumazine (systematically 2,4-dihydroxytetrahydropteridine) in 0.1 Molar Tris-HCl buffer, pH 7.5 by warming at 37° in air and then boiling, gave 6-hydroxylumazine formed via 6-hydroxy-7,8-dihydrolumazine[94]. Spectrophotometric

evidence indicated the formation of 7,8-dihydrolumazine when tetrahydrolumazine in 0.1 Molar Tris-HCl buffer, pH 7.5, was allowed to stand at room temperature.

Rembold et al.[94] summarize their results in a reaction scheme for the oxidative degradation of hydrogenated pteridine cofactors. Although they could not observe any evidence for quinonoid dihydropterin intermediates, they explain their results using a scheme in which such intermediates are assumed to be present. Tetrahydroneopterin is the example used in the scheme. The scheme centres around dihydropterin, formed as follows:-

Tetrahydroneopterin ⟶ quinonoid dihydro intermediate $\xrightarrow{\text{cleavage of side chain}}$ dihydropterin + glyceraldehyde

The formation of dihydroxanthopterin is explained by a hydration of the 5,6-double bond of dihydropterin to give a hydrated species:-



Oxidation of the hydrated species is postulated to proceed via a quinonoid intermediate which subsequently tautomerizes to give dihydroxanthopterin.

The idea of hydration of the 5,6-double bond in dihydropterin seems unlikely when one considers the reports concerning hydration of pteridines. Albert reports hydration of the 3,4- or 7,8- double bond

in certain pteridines[161], while no hydration has been detected in any species of 4- or 7-hydroxypteridine[162,163]. Reversible covalent hydration is especially strong in 2-hydroxy and 6-hydroxypteridine[161], and polyhydroxypteridines in which these groups are present, such as xanthopterin which adds across the 7,8-double bond[163]. Covalently dihydrated species, two molecules of water adding across the 5,6- and 7,8- bonds, have been reported for 6- and 7- methyl and 6,7-dimethyl derivatives of ethyl pteridine-4-carboxylate[191]. Also, it is reported that 7,8-dihydropterin on aerial oxidation gives pterin[93].

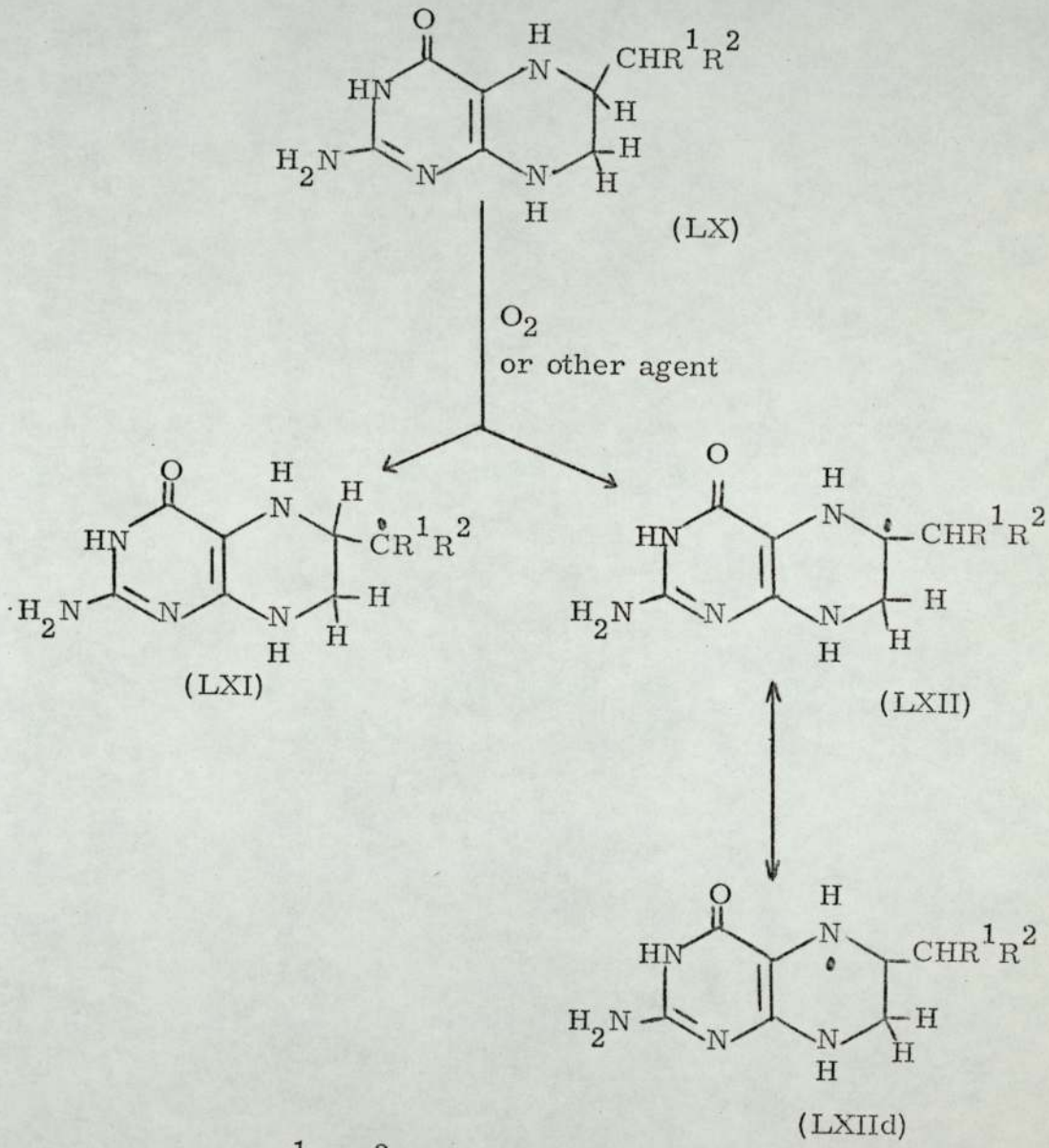
The observed reactions of these compounds are readily explicable in terms of a hydroperoxide intermediate at C-6 or C-9 and its subsequent reactions (see Figures XIII - XVII). The fact that at room temperature the tetrahydro compound is oxidised to the dihydro derivative, whereas higher temperatures yield xanthopterin and pterin as the products, is explained by the effect of heat on the decomposition of the hydroperoxide at C-6 and cleavage of the C-6 — C-9 bond.

The work of Viscontini and Argentini[157] on the aerial oxidation of 6-aminomethyl-6-methyl-tetrahydropterin at pH 6.8 has already been mentioned in Chapter 1, Section 5 and their mechanism reproduced in Figure II. Briefly, the products were 6-methyl-7,8-dihydropterin, formaldehyde and ammonia with yields of 48, 30 and 35% respectively. They propose a mechanism involving oxidation at the carbon atom of the aminomethyl group. Since the pKa values[196] (dissociation constants) for methylamine, ethylamine, n-propylamine and cyclohexylamine are in the range 10.6 - 10.8, while for benzylamine it is 9.33, the nitrogen atom of the aminomethyl group is probably protonated at pH 6.8. Protonation of the amino group enhances oxygen attack at the methyl

group. Formation of formaldehyde is therefore more likely to be derived via a hydroperoxide at the methyl group.

Figure XIII Autoxidation of Tetrahydropterins

a) 6-Methyl-Tetrahydropterin
b) Tetrahydrobiopterin
c) Tetrahydroneopterin

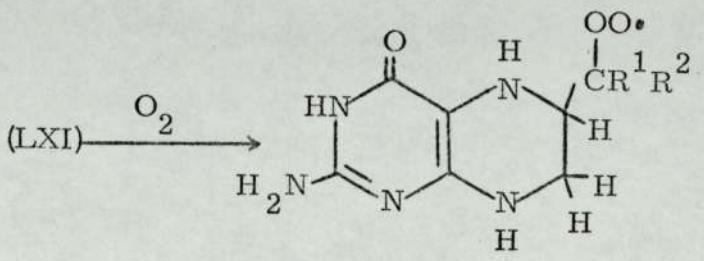


(LXa) $R^1 = R^2 = H$

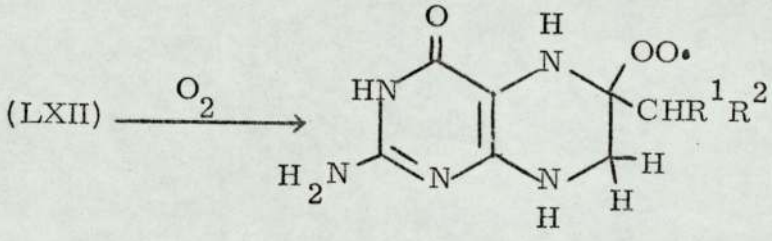
(LXb) $R^1 = OH$, $R^2 = CHOH\text{-}CH_3$

(LXc) $R^1 = OH$, $R^2 = CHOH\text{-}CH_2OH$

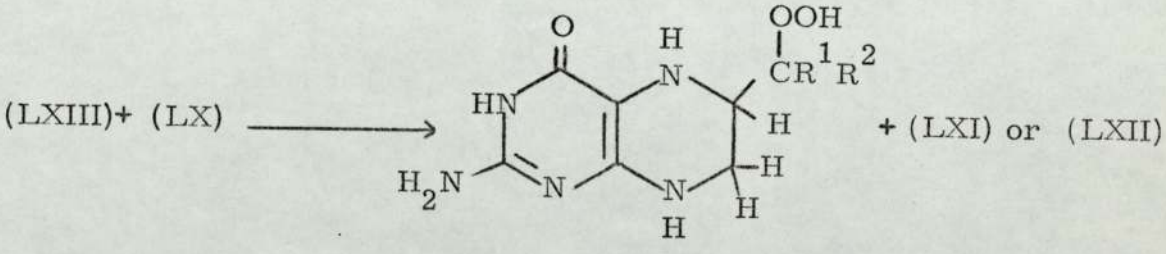
Figure XIV Autoxidation of Tetrahydropterins (cont.)



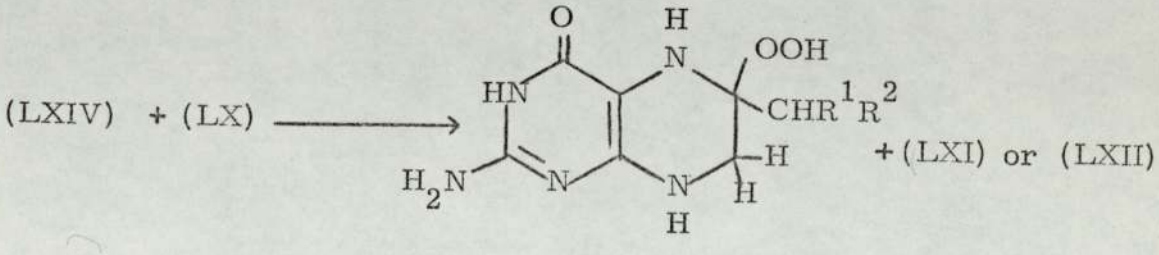
(LXIII)



(LXIV)



(LXV)



(LXVI)

Figure XV Autoxidation of Tetrahydropterins (cont.)

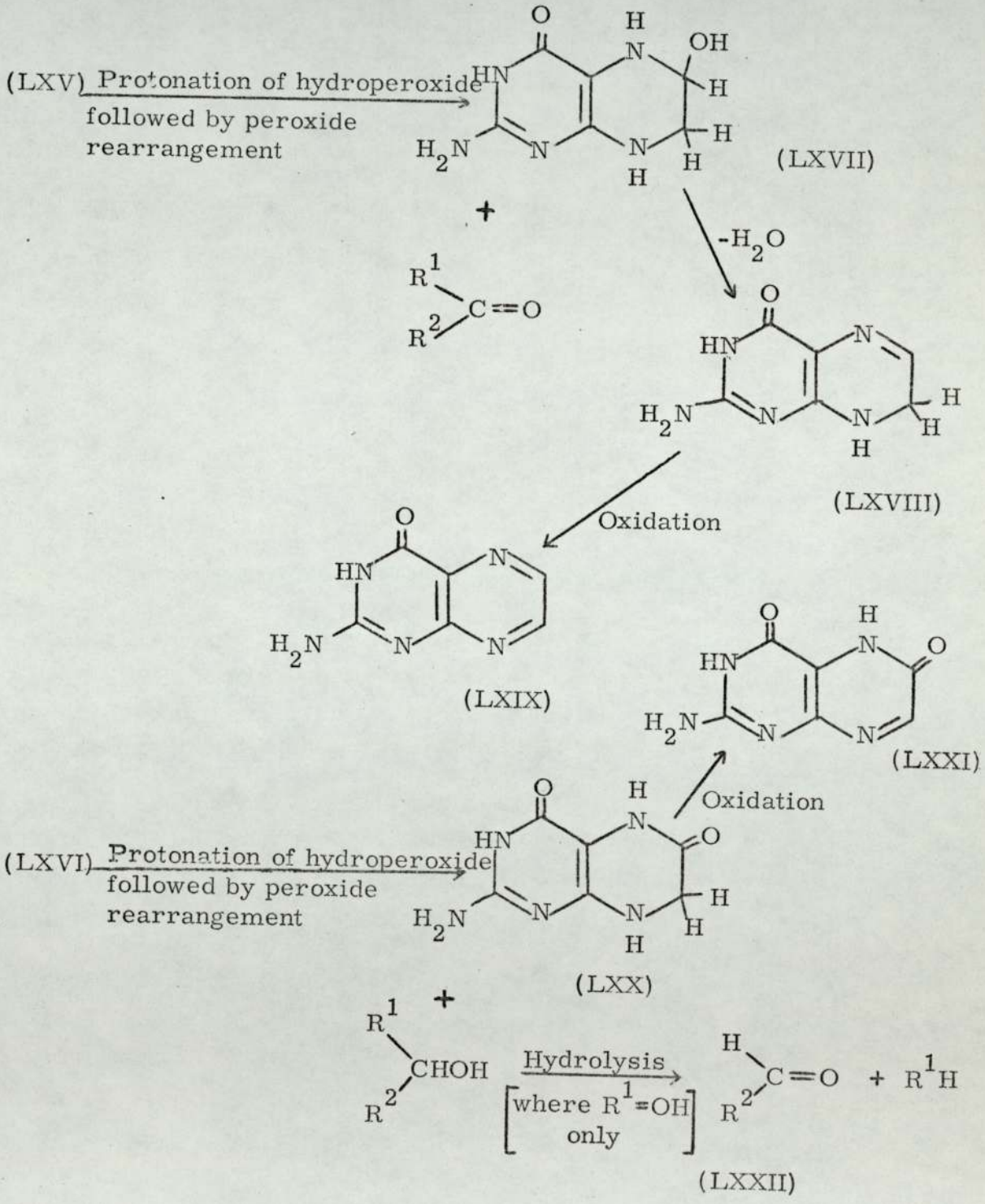


Figure XVI Autoxidation of Tetrahydropterins (cont)

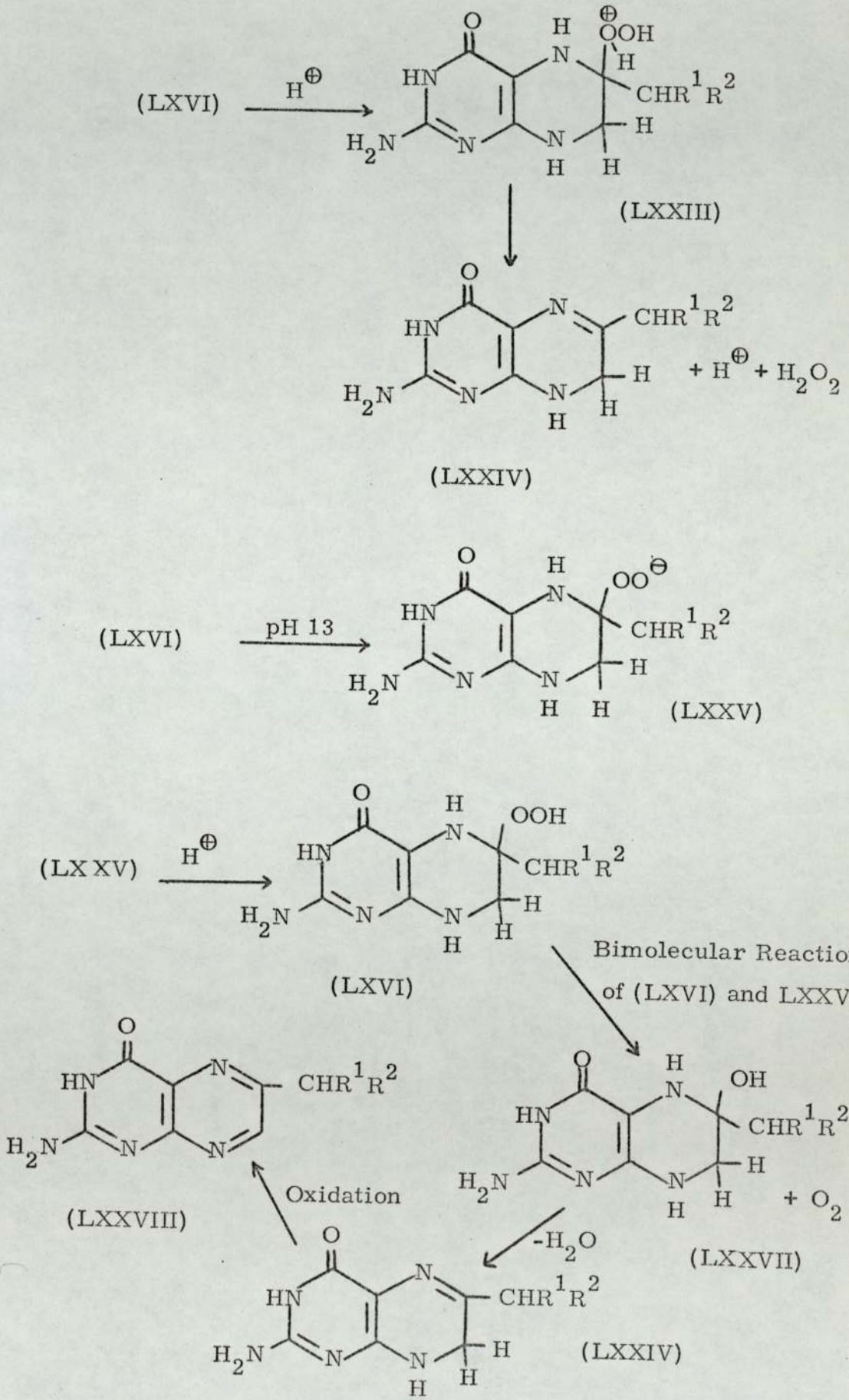
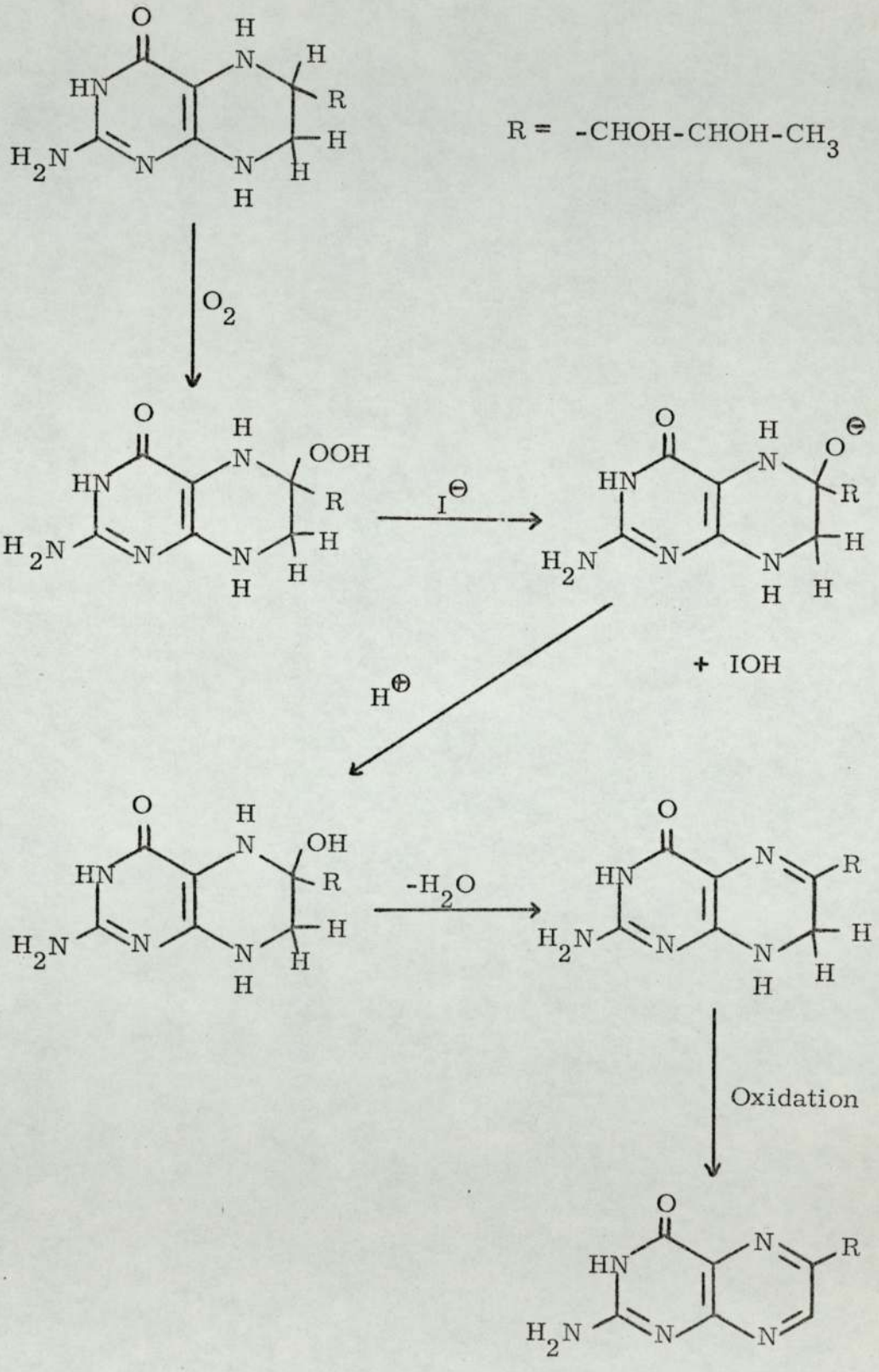


Figure XVII The Iodine Oxidation of Tetrahydrobiopterin



V Time-based U.V. Spectral Studies of Reduced Pterins

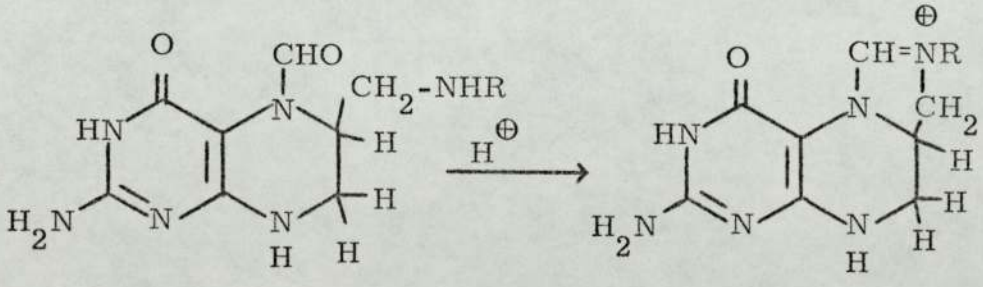
1. Introduction and Results

The oxidation in solution of a variety of reduced pterins was followed using time-based u.v. spectral studies. The effect of pH, buffer and deaeration was investigated and the results are discussed in terms of hydroperoxide intermediates.

Studies with THF at pH's 1, 3, 7, 10 and 13 showed the reaction products to be pH dependent, thereby supporting the results from other investigations (see Chapter II). The most rapid and marked spectral changes were in phosphate buffer solution, pH 7, no THF being observed after ten minutes. This is in agreement with Blakley[112], who measured the rate of change of the spectrum of THF in phosphate buffer, pH 7.2 and observed very little change after about ten minutes. At pH's 1, 3, 7 and 10 the spectra were composite after approximately 30 minutes, whereas at pH 13 a spectrum of DHF was observed. A spectrum of DHF at pH 13 is in accord with the results for DHF isolations recorded in Chapter II, Section 7.

Spectral changes of DHF at pH's 7 and 13 supported the buffer effects recorded in Chapter III. In phosphate buffer, pH 7, a spectrum of 6-formyl-7,8-dihydropterin appeared about ten times more rapidly than in 0.1 Molar ammonium acetate, each having the same composite spectrum on standing for a few days. In water, pH 6.2, there was little change in the DHF spectrum even after 20 hours. At pH 13 DHF was slowly oxidised to give a composite spectrum, the absence of any absorbance above 400 nm. implied that no dihydro aldehyde was formed. Due to the extremely low solubility of DHF in acid solution, no spectra were recorded at pH 1.

5-Methyl THF, barium salt, was effectively stable in solution at pH's 1 (0.1N. HCl), 7 (phosphate buffer) and 13 (0.1N. NaOH). After seven hours there were only small spectral changes at pH's 7 and 13, with none at pH 1. The oxidation of 5-methyl THF in solution at pH's 7 (0.1 molar ammonium acetate) and 10 ($NaOH-NaHCO_3$ buffer) was also followed by t.l.c. Some oxidation had occurred after five hours producing three fluorescent compounds, one of which possessed the same Rf values as pterin. No 5-methyl THF was observed after five days and the major product appeared to be pterin, plus two unidentified fluorescent compounds (t.l.c. evidence). Oxidation of 5-methyl THF in air[184] or with hydrogen peroxide in the presence of peroxidase[83,177-179] yields 5-methyl-5,6-DHF. The above t.l.c.'s did not indicate the presence of this dihydro compound, which would have appeared as a quenching spot[179]. The apparent stability of 5-methyl THF could be due to its oxidation to 5-formyl THF which is stable. U.v. spectra and t.l.c. do not readily distinguish between the two, but 5-formyl THF rearranges in acid media to 5,10-methenyl THF



which has a characteristic u.v. absorption at 355nm. No such absorption was observed when oxidised 5-methyl THF solutions were acidified. Doubt was cast on the apparent stability of 5-methyl THF when mass spectral examination showed the sample in use to be heavily contaminated

by mercaptoethanol. 5-Methyl THF is therefore likely to be less stable than reported above, but insufficient time has prevented further investigation. Recent studies by co-workers[197] have shown that when 5-methyl THF is oxidised in formic acid, 5,10-methenyl THF is obtained in about 5% yield after three hours.

Time based u.v. spectral studies of tetrahydrobiopterin and 6-methyl-tetrahydropterin showed that each was rapidly oxidised in phosphate buffer, pH 7, to their respective 7,8-dihydro-compound (15-20 minutes). Each was stable at pH 1. At pH 13, tetrahydrobiopterin slowly oxidised to dihydrobiopterin whereas 6-methyl-tetrahydropterin oxidised rapidly.

The effect on the rate of oxidation of changing the buffer was investigated for tetrahydrobiopterin and 6,7-dimethyl-tetrahydropterin at pH 7. Tetrahydrobiopterin was examined using phosphate buffer, Tris-HCl buffer and 0.1 Molar ammonium acetate solution. Phosphate showed the most rapid spectral changes, while the slowest were in ammonium acetate. 6,7-Dimethyl-tetrahydropterin, examined using phosphate buffer and Tris-HCl buffer, exhibited a much more pronounced buffer affect. The rate of oxidation again being most rapid in phosphate buffer.

An initial rapid increase then slower decrease in absorbance at 200-210 nm. is sometimes observed during the oxidation. Depending on the buffer and the compound under investigation, this can be either a slight change in a shoulder or a definite absorption peak. It is observed most markedly for 6,7-dimethyl-tetrahydropterin in Tris-HCl buffer, a peak rapidly appears at 203 nm. and then slowly disappears. A possible explanation for this phenomenon is that the absorption is due to peroxide

formation. The -O-O- chromophore is known to absorb at about 200 nm.[165].

Using rigorously deaerated phosphate buffer, pH 7, it was possible to halve the rate of spectral change for THF, 6-methyl -tetrahydropterin and tetrahydrobiopterin. Previous reports on the autoxidation of tetrahydropteridines have shown that there is a rapid oxygen uptake[138]. With 2-mercaptoethanol present tetrahydro compound solutions were stable.

Preparation of a deaerated buffer solution proved to be more difficult than at first believed. No noticeable decrease in the rate of spectral change was observed when using phosphate buffer prepared by addition of the hydrated salt to water that had been boiled and cooled under continuous bubbling of nitrogen or helium. Only by subjecting the buffer solution itself to deaeration, using the above technique, was it possible to halve the rate of spectral change.

The effect of light on the oxidation of tetrahydropterins was investigated using tetrahydrobiopterin in phosphate buffer, pH 7. A solution was stood in daylight and spectra run five minutes after preparation of the solution and then at ten minute intervals for 30 minutes. A solution of the same concentration was prepared using a darkened flask and examined as above. The exclusion of light did not alter the rate of oxidation. An increase in the rate of oxidation was observed when the tetrahydrobiopterin solution was left in the spectrometer during the preparation of time-based spectra. The solution was continually bathed in u.v. light during this procedure and there was also a small increase in temperature.

(A selection of spectra is presented in the Appendix)

2. Discussion of the Results

The autoxidation of reduced pterins by a mechanism involving hydroperoxide can be divided into:-

a) initiation of radicals and hydroperoxide formation

b) decomposition of the hydroperoxides

The spectral results suggest evidence for each section.

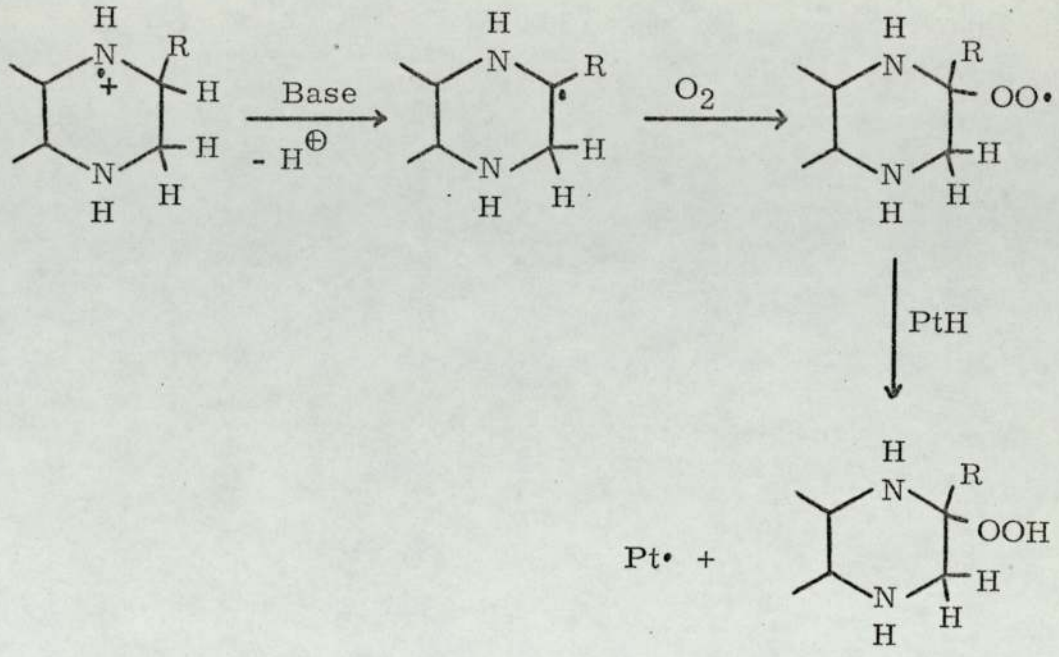
The studies with THF and DHF are in accord with their oxidations discussed in Chapters II and III. The absence of absorption above 400 nm. during the autoxidation of THF at pH 7 supports a mechanism that allows the formation of dihydroxanthopterin and pterin without proceeding via DHF. Blakley[66] observed that THF lost its biological activity for serine synthesis when exposed to air for a short period and this activity returned on rehydrogenation. No biological activity was observed on rehydrogenation after long exposure to air and a diazotisable amine was present. These observations can be explained by hydroperoxide intermediates. Short exposure results in formation of a hydroperoxide which is microbiologically inactive and can be hydrogenated to THF. Long exposure allows decomposition of the intermediate with cleavage of the side-chain to give p-aminobenzoyl -glutamic acid and inactive products which do not give THF on hydrogenation.

Studies with the other reduced pterins are also in accord with the mechanism discussed in Chapter IV.

The results suggest that oxidation in the tetrahydropyrazine ring occurs at position C-6 to give a hydroperoxide. The stability at pH 1 is due to protonation of N-5, preventing oxygen attack at C-6. At pH 13 the peroxide is present as the stable anion (LXXV). A possible

explanation for a slower rate of oxidation of reduced pterins at pH 13 concerns the C=O group at position 4. Bartholomew and Davidson[172] have studied the oxidation of amines and they propose that the primary process in the benzophenone-sensitised photo-oxidation of amines is reaction of triplet benzophenone with the amine to give an α-amino-radical which reacts with oxygen to give a peroxide and, ultimately, the observed products. If the rate of oxidation of reduced pterins is also influenced by excitation of the carbonyl group, then oxidation at pH 13 is expected to be slower since position 4 now contains a $C\text{-}O^{\ominus}$ group. Also, at pH 13 there is expected to be a decrease in the rate constant for proton transfer k_{H^+}[173] leading to formation of a pterin radical. Experiments with 6-deutero-tetrahydropteridines have shown that in the range pH 6.0 - 8.0 cleavage of the C-H bond at position 6 is rate-limiting, with k_{h/k_d} = approximately 11[129]. This agrees with the value for hydrogen abstraction by a peroxy radical, where k_{h/k_d} is 10 - 15[166].

The increase in rate of oxidation observed when the tetrahydrobiopterin solution remained in the spectrometer could be accounted for by the increase in temperature and/or photosensitisation. A photosensitised oxidation is explained by excitation of the C=O group at position 4[172]. The excited molecule undergoes intermolecular reaction with a non-excited molecule, the latter molecule becoming a radical cation. A reaction scheme that accounts for the formation of a peroxide from the radical cation is outlined below (only the pyrazine ring is presented, with R at position 6 and PtH is reduced pterin):



Hydrogen abstraction from the radical cation occurs by reaction with the buffer anion, the latter behaving as a base. This explains the variation in oxidation rate with buffer. Phosphate buffer at pH 7 corresponds to the half-way stage of the reaction $H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$. The dissociation constant, pK value, for the 2nd step of O-phosphoric acid is 7.21, while that for acetic acid is 4.75[196]. Hydrogen abstraction, thereby oxidation, is expected, and found, to be faster in phosphate buffer than ammonium acetate solution. The effect of buffers[129] may also concern decomposition of the peroxide, since the nature of the solvent is known to effect the decomposition of hydroperoxides[167, 168].

The results with 5-methyl THF show it to be more stable to oxidation than the other tetrahydropterins examined, and pterin is a tentative oxidation product. A possible explanation for the slow rate of oxidation of 5-methyl THF is as follows:- 5-Methyl THF forms a

radical cation which, due to the electron donating effect of the methyl group at N-5, does not undergo ready loss of $H^{\oplus}$ from position C-6. The methyl group may also hinder radical formation at C-9. The net effect is for radical formation to occur at position C-7, but because formation of a planar free radical is more difficult in the half-chair conformation of C-7 the process is slow. Subsequent hydroperoxide formation at this position, followed by peroxide elimination, yields 5-methyl-5,6-DHF. Pterin is then formed via a peroxide at C-9 which undergoes rearrangement and demethylation. The 5-methyl derivatives of tetrahydropterin, 6,7-diphenyl-tetrahydropterin and 6,7-diphenyl-5,6-dihydropterin undergo demethylation and oxidation to their aromatic forms[54,88,89].

VI Evidence for a Peroxide

Mager and Berends[135] have reported the formation of peroxides during the autoxidation of 6,7-dimethyl-tetrahydropterin. Peroxide formation was found by qualitative test-reagents and established by polarography. Mager et al.[138] report that 6,7-dimethyl-tetra-hydropterin dissolved in diethylamine is oxidised quantitatively to 6,7-dimethyl-7,8-dihydropterin and hydrogen peroxide could be detected in the solution. They determined the amount of peroxide by adding 2N. H_2SO_4 and MnO_2 to an alkaline solution of the tetrahydropterin and manometrically measuring the rapid oxygen evolution.

Initial experiments to show the presence of a peroxide were inconclusive. The two reagents chosen were p-aminophenol hydrochloride reagent and ferrous thiocyanate reagent[169]. When a chromatogram is sprayed with either of these reagents, hydroperoxides should appear as red spots. Examination of oxidised solutions of DHF and tetrahydrobiopterin by applying large samples to t.l.c. plates and spraying with reagent proved indecisive. Addition of ferrous thiocyanate reagent to a solution of tetrahydrobiopterin in phosphate buffer sometimes produced a transient pink colour. (Addition of a hydrogen peroxide solution to the reagent produced a deep red colour).

Hydrogen peroxide formation was shown using ferric ferricyanide reagent[170], a mixture of equal parts of 0.4% ferric chloride and 0.8% potassium ferricyanide. A more or less intense blue colour or precipitate is formed, depending on the hydrogen peroxide content. Addition of the reagent to a solution of oxidised tetrahydrobiopterin in phosphate buffer produced an immediate dark blue precipitate. A

similar result was obtained when using a DHF solution. The possibility that the blue precipitate was due to chelation of the pterin was discounted by subsequent experiments using various reduced pterins.

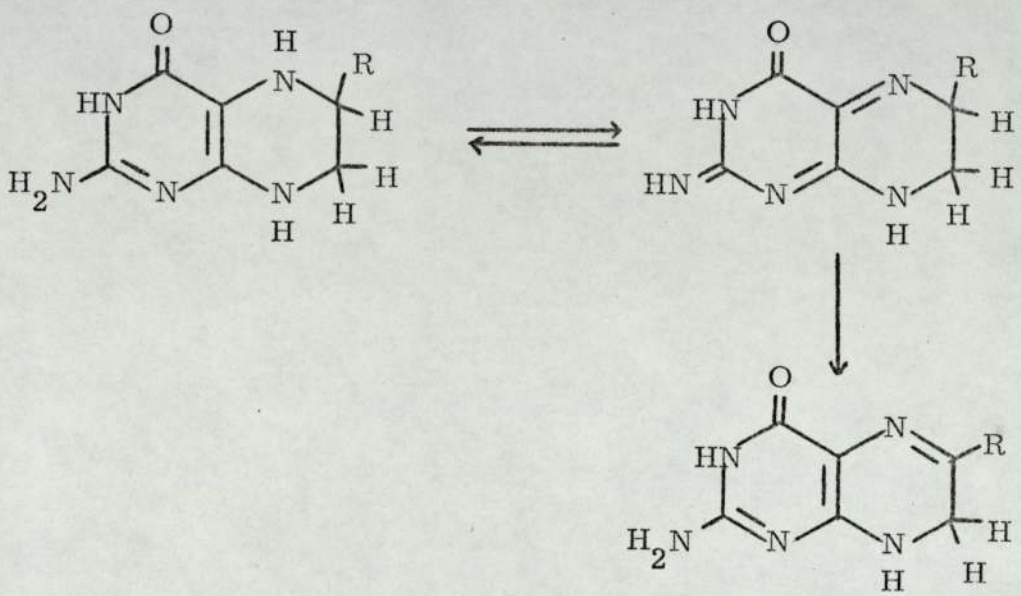
Tetrahydrobiopterin was dissolved in water, solution about pH 4.8, and after standing for 30 minutes the solution was distilled. When a sample of the distillate was added to an equal volume of the ferric ferricyanide reagent, a blue-green solution and a small quantity of dark blue precipitate were produced. A blank, prepared by mixing equal volumes of reagents and water, was a clear yellow solution. When tetrahydrobiopterin was dissolved in phosphate buffer, pH 7, and the solution distilled, the distillate gave a positive test on treatment with ferric ferricyanide reagent, but the blue-green colour was less intense than for the above distillate. This is not unexpected, since the stability of hydrogen peroxide in solution at 100° rapidly decreases above about pH 5.7[171]. Distillation of a solution of DHF in phosphate buffer, pH 7, also gave a distillate that contained peroxide.

These results confirm the presence of peroxide during the autoxidation of DHF and tetrahydrobiopterin. The formation of hydrogen peroxide is explained in Figures IX, XI and XVI.

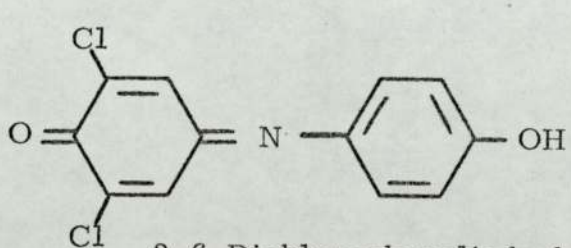
In Raman spectra of peroxides the O-O stretching frequency appears as a fairly intense band at 11.3 to 11.4 μ,[140] but results with THF were negative (fluorescence inhibits Raman spectra). Using n.m.r., Johnson[219] studied 6-methyl-tetrahydropterin. If the proton at C-6 is replaced by -OOH, the methyl group will appear as a singlet instead of a doublet. This change was observed in one spectrum.

VII Studies with 2, 6-Dichlorophenolindophenol

Recent work postulates the formation of a quinonoid dihydro intermediate from the tetrahydro compound by a reversible step, which irreversibly changes to the dihydro compound:



The oxidation of tetrahydropteridines by 2, 6-dichlorophenolindophenol has been cited as evidence for such an intermediate[116].



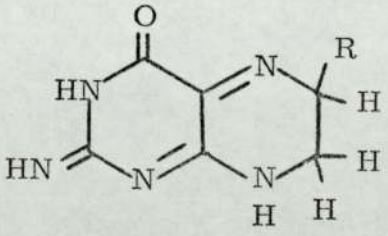
2, 6-Dichlorophenolindophenol

6, 7-Dimethyl-tetrahydropterin, oxidised by 2, 6-dichlorophenol -indophenol and the dye then removed by ether extraction, has been shown to give a u.v. spectrum with $\lambda_{max.}$ about 227, 280 and 305 nm.[116]. The spectrum then changes to give a spectrum of 6, 7-dimethyl-7, 8 -dihydropterin. The absorbance maximum at 305 nm. is attributed

to the presence of a quinonoid dihydropterin intermediate.

The observations with 2,6-dichlorophenolindophenol have been re-examined and investigated more fully.

Normal and deaerated solutions of 2,6-dichlorophenolindophenol in phosphate buffer, pH 7, were immediately decolourised by equimolar amounts of THF and tetrahydrobiopterin and less rapidly by 5-methyl-THF, but not by dihydroxanthopterin, xanthopterin, folic acid, DHF and 5-formyl-THF. Under an atmosphere of nitrogen, a 5:1 molar ratio of DHF : dye required 90 minutes to decolourise the solution. A solution of the dye in water, pH 6.8, was immediately decolourised by an equimolar amount of tetrahydrobiopterin. The observations with 5-methyl-THF, dihydroxanthopterin and xanthopterin are not in accord with the quinonoid dihydropterin scheme. This scheme involves hydrogen abstraction from the N-5 position of the reduced pyrazine ring to give a quinonoid intermediate. Kaufman[116] regards decolourisation



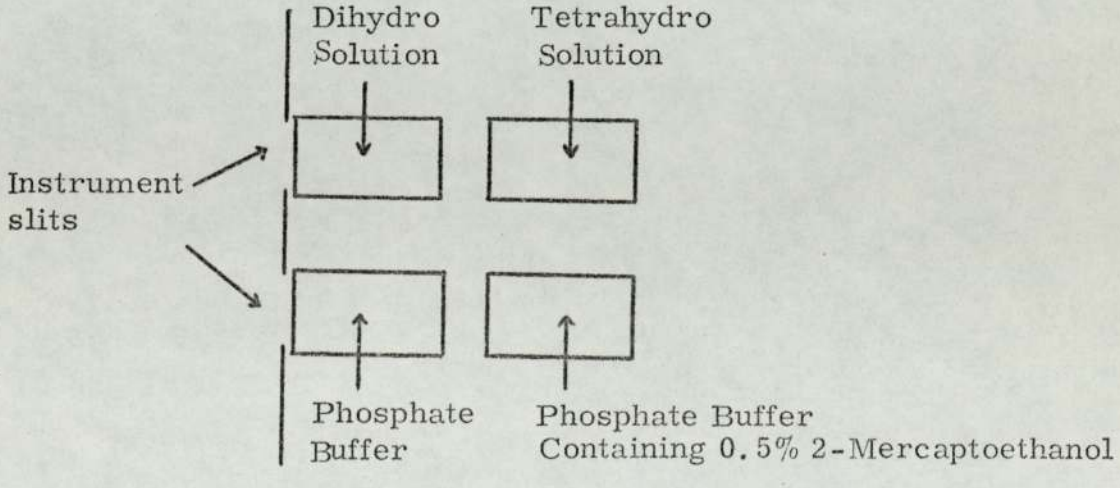
of the indophenol as evidence for such an intermediate. On this basis there should be no reaction with 5-methyl THF, while dihydroxanthopterin and xanthopterin, being in the oxo-form with a hydrogen at N-5, should decolourise the dye.

Time based u.v. spectral studies were carried out on the oxidation of tetrahydrobiopterin by 2,6-dichlorophenolindophenol,

equimolar ratio, without removal of the dye in phosphate buffer, pH 7. There was an immediate appearance of the spectrum of the 7,8-dihydro compound and no peak at 303-305 nm. and thus no evidence of a quinonoid dihydro intermediate. A solution of the dye in phosphate buffer, pH 7 had a spectrum with $\lambda_{max.}$ 210, 271, 308 and 600 nm. Partial decolourisation of the dye solution by the addition of ascorbic acid gave $\lambda_{max.}$ 208, 271 and 600 nm., while the completely decolourised solution had $\lambda_{max.}$ 207 and 270 nm. Ascorbic acid in phosphate buffer, pH 7, had an absorption at $\lambda_{max.}$ 267 nm. Spectra with $\lambda_{max.}$ 303-305 nm. were observed when using a tetrahydrobiopterin : 2,6-dichlorophenol -indophenol ratio of 2:1 and the spectral changes were similar to those described by Kaufman[116]. Superimposing u.v. spectra of dihydro- and tetrahydrobiopterin verified that the spectral changes were summation spectra of the dihydro and slowly oxidising tetrahydro compounds.

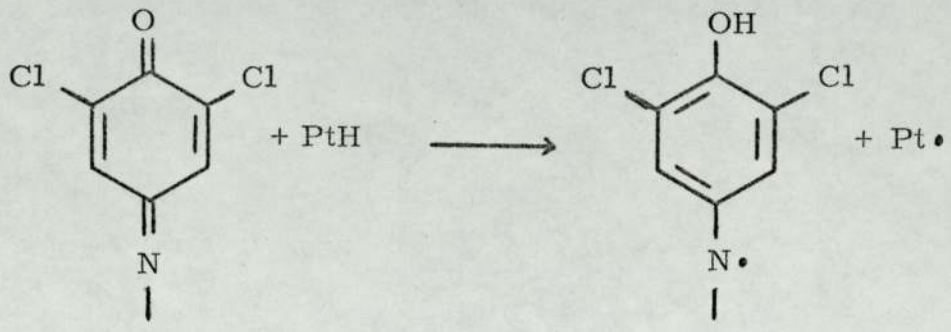
(Spectra are presented in the Appendix)

Dihydrobiopterin was prepared by allowing a solution of the tetrahydro compound in phosphate buffer, pH 7, to stand for 30 minutes. A standard tetrahydrobiopterin solution was prepared using phosphate buffer containing 0.5% 2-mercaptoethanol. For the superimposed spectra, two pairs of cells were used and arranged as shown below:



Summation spectra for various ratio's of dihydrobiopterin : tetrahydro -biopterin were prepared using solutions of appropriate concentration.

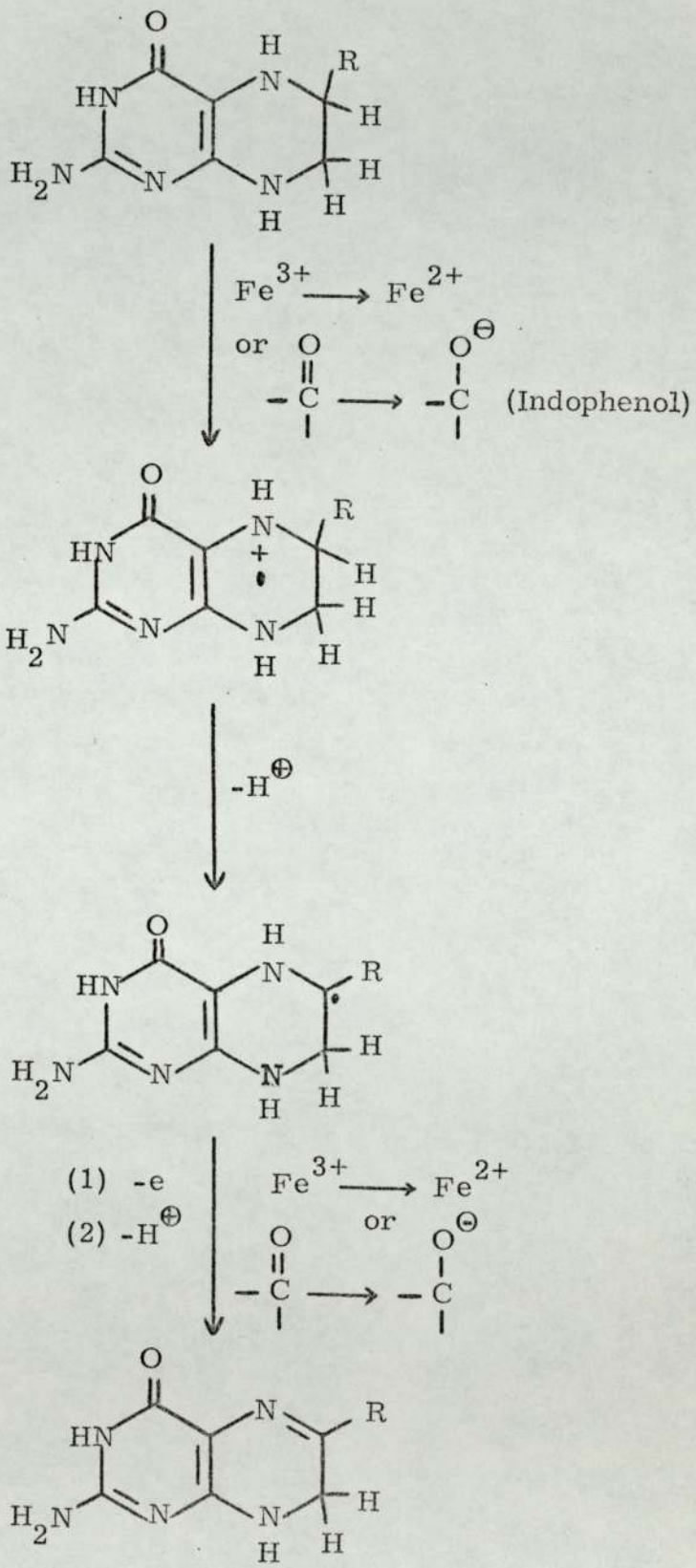
The rapidity with which tetrahydropterins decolourised the dye solution can be explained by a mechanism in which the primary reaction is abstraction of hydrogen from the pterin by the dye[173].



The results suggest that oxidation of reduced pterins by indophenol requires the easy abstraction of a hydrogen from position C-6, rather than from the N-5 position. A possible scheme for the reaction of tetrahydropterins with 2, 6-dichlorophenolindophenol is one involving a radical cation (see Figure XVIII). Easy formation of such radicals has been predicted[117], since THF appears to be an excellent electron donor and not an electron acceptor.

Figure XVIII

Oxidation of Tetrahydropteridines
By Fe^{3+} or Indophenol



VIII Ferricyanide Oxidation of Tetrahydropterins

The appearance of a transient peak at $\lambda_{max.}$ 303 nm. when a tetrahydropterin is oxidised by ferricyanide is another observation that has been attributed to a quinonoid dihydropterin intermediate[129]. 6,7-Dimethyl-tetrahydropterin was oxidised by addition to two equivalents of potassium ferricyanide solution and the oxidation was following using time based u.v. spectra[129]. The reference cell contained an equivalent amount of potassium ferrocyanide. All solutions were deaerated by continuous bubbling with pure nitrogen, the cells were filled with an atmosphere of nitrogen and the conditions were regarded as anaerobic. The spectrum at 5° immediately after addition of 6,7-dimethyltetrahydropterin contained a peak with $\lambda_{max.}$ 303 nm. whereas at 25° there were two peaks with $\lambda_{max.}$ about 282 and 305 nm. On the basis of the evidence reported in Chapter V concerning the difficulty of deaerating aqueous solutions, it would seem most likely that small amounts of oxygen were still present.

This evidence has been re-examined using tetrahydrobiopterin.

When tetrahydrobiopterin was oxidised by ferricyanide in phosphate buffer, pH 7, there was an immediate appearance of the spectrum of the 7,8-dihydro compound and no peak at 303 nm. It was observed that ferricyanide in phosphate buffer, pH 7, has a spectrum with $\lambda_{max.}$ 210, 261, (286), 303, (320) and 423 nm., that the peak at 303 nm. is unchanged when the reference cell contains an equimolar amount of ferrocyanide, and that this peak disappears when the ferricyanide is reduced by the gradual addition of tetrahydrobiopterin. Ferrocyanide in phosphate buffer, pH 7, has a spectrum with $\lambda_{max.}$ 219 nm. (Spectra are presented in the Appendix)

The transient peak observed at 303 nm[129] is better attributed to the last disappearing traces of ferricyanide than to a possible quinonoid dihydropterin intermediate. Differences in spectra with temperature[129] is explained by a temperature affect on the rates of (a) oxidation by ferricyanide and (b) admixing of the pterin and ferricyanide solutions in the cuvette. A possible scheme for the reaction of tetrahydropterins with Fe^{3+} is one involving a radical cation (see Figure XVIII). Easy formation of such radicals has been predicted[117].

IX Addition of Hydrogen Peroxide to a Dihydro Pterin

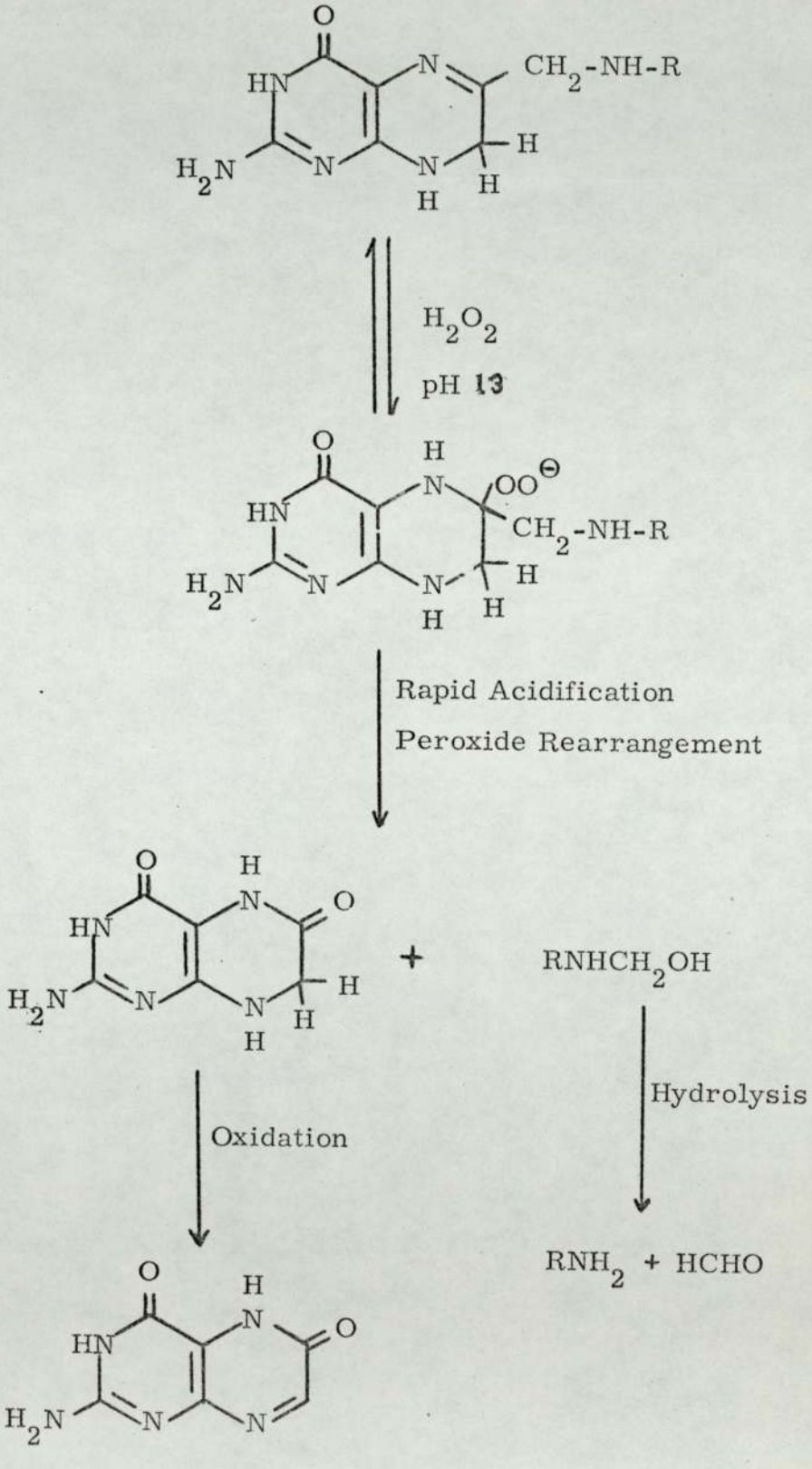
Nucleophiles are known to react with the pyrazine ring of pteridines and dihydropteridines[52,93,132,153,174], addition also occurs with DHF and folic acid[175,176]. It seemed likely that hydrogen peroxide addition could occur with formation of a 6-hydroperoxide and that this would provide information relevant to the mechanism of oxidation of tetrahydropterins.

DHF in 0.1N. NaOH solutions containing a large excess of sodium peroxide gave u.v. spectra similar to those of THF, thereby indicating nucleophilic addition of peroxide anion (see Figure XIX). Similar results were obtained when using 6-methyl-7,8-dihydropterin.

When a solution of DHF in 0.1N. NaOH containing a large excess of sodium peroxide was rapidly acidified by addition to concentrated hydrochloric acid, cooled to 5°, xanthopterin was the only pterin produced, as shown by t.l.c. (see Figure XIX).

Formation of xanthopterin via a 6-hydroperoxide is in complete agreement with the proposed mechanism for the oxidation of tetrahydropterins. (See Chapters II and IV)

Figure XIX Hydrogen Peroxide Addition to DHF



X Oxidation of Reduced Pterin

Tetrahydropterin is very rapidly oxidised in solution under air and attempts to isolate the solid yield a complex mixture of oxidation products[56,87,132]. 7,8-Dihydropterin on aerial oxidation gives pterin[93].

Pterin was reduced using a modified version of a general borohydride procedure of Albert and Matsuura[49]. Pterin, suspended in potassium carbonate solution, was reduced by sodium borohydride. After 17 hours the reaction mixture was warmed to 40 - 45°, producing a cloudy yellow solution, and adjusted to about pH 4 with acetic acid. The resultant buff coloured precipitate, which accounted for 75% of the starting material, was shown by t.l.c. and mass spectra to be pterin. The mother liquor was shown by t.l.c. to contain five fluorescent compounds, two having similar Rf values to pterin and xanthopterin. The three remaining compounds were not identified.

Pterin was also reduced by catalytic hydrogenation. Pterin was dissolved in aqueous ammonia and hydrogenated using platinum dioxide. When hydrogen uptake had ceased, 0.880 ammonia was added via a side-arm and air was then introduced into the flask. After standing for three days the catalyst was removed by filtration to leave a yellow-orange solution. T.l.c. examination showed the solution to contain a large amount of pterin, plus xanthopterin and 6-aminopterin. 6-Aminopterin was isolated, by means of column chromatography and preparative paper chromatography, and identified using mass spectra, u.v. spectra and fluorescence spectra (see Experimental).

The products obtained are exactly those expected from the

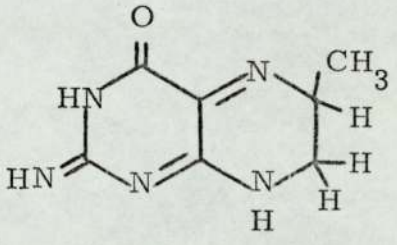
studies with other reduced pterins (see appropriate pages). Xanthopterin formation is explained by reduction of pterin to the tetrahydro derivative, oxygen attack at C-6 to form a hydroperoxide and subsequent peroxide elimination to give dihydroxanthopterin which oxidises to xanthopterin. 6-Aminopterin is formed by reaction of 7,8-dihydropterin with ammonia and subsequent oxidation of the tetrahydro compound. These explanations have been discussed in detail in previous chapters.

XI Heat of Formation of Quinonoid Dihydro versus Dihydro Compound

Schemes involving quinonoid dihydro intermediates require that the tetrahydro compound is reversibly oxidised to the quinonoid dihydro derivative, which then irreversibly forms the 7,8-dihydro compound. The possibility of the second step was examined.

Heats of formation of quinonoid dihydro-6-methylpterin and 6-methyl-7,8-dihydropterin were calculated from Pauling's bond energy constants, with the bond energy E in k. cal/mole[181].

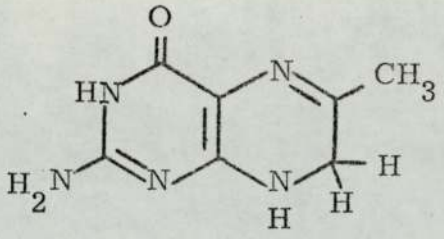
Quinonoid Dihydro-6-Methylpterin



Bond	E	Number of bonds	Total E
N-H	84	3	252
N=C	94	3	282
N-C	49	6	294
C-C	59	4	236
C=O	150	1	150
C-H	87	6	522

Total is 1736 k. cals.

6-Methyl-7, 8-Dihydropterin



Bond	E	Number of bonds	Total E
N-H	84	4	336
N=C	94	2	188
N-C	49	7	343
C-C	59	3	177
C=O	150	1	150
C-H	87	5	435
C=C	100	1	100

Total is 1729 k. cals.

As the quinonoid dihydro derivative is 7 k. cal more stable than the 7, 8-dihydro compound, a scheme involving its rearrangement to the latter is unlikely.

The difference in entropy between these two molecules is largely dependent on the group at position 2. The =NH group has no rotation, unlike the $-NH_2$ group, so the quinonoid dihydro form is expected to have a slightly smaller entropy.

Heats of formation indicate that the dihydropterin to quinonoid dihydropterin rearrangement is the favoured direction, while entropy considerations appear to favour the opposite direction.

Possible rearrangement of a dihydropterin to the quinonoid dihydro form, followed by hydrolysis of the imino group, is suggested by the presence of lumazines in the products from oxidations of reduced pterins[61].

This may also explain the formation of unknown products when a DHF solution containing 10% ascorbic acid was heated (see Chapter III section 1.).

XII Polarography

Certain polarographic results and observations have been cited as evidence for the existence of a quinonoid dihydro intermediate in the oxidation of tetrahydropterins[125,128]. It also happens that polarography is probably the most commonly used instrumental method of analysis of peroxides, which are reduced at the dropping mercury electrode[140,182]. Hydroperoxides, peroxy esters and diacyl peroxides have been determined using this method.

Vonderschmitt and Scrimgeour[125] have oxidised tetrahydropterin with Fe^{3+} to give an intermediate that they regard as being a 'quinonoid' dihydropterin. The intermediate was characterised by its having a polarographic wave similar to that obtained when tetrahydropterin was oxidised with indophenol according to the method of Kaufman[30]. Therefore, the evidence is based on the criterion that Kaufman is correct in assuming the intermediate to be a quinonoid dihydropterin.

Archer and Scrimgeour[128] have determined the reduction potentials of tetrahydropterins using polarography under alleged anaerobic conditions. They also report the standard reduction potential for the quinonoid dihydropterin - tetrahydropterin couple.

The results can be explained by the involvement of hydroperoxide intermediates. Metal ions such as Fe^{3+} are known to react with hydroperoxides (see Chapter II, Section 9) and the half-wave potentials quoted[128] are of the same order as those expected for hydroperoxide intermediates[140].

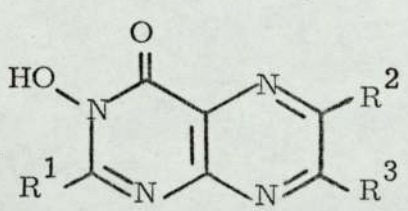
XIII Mass Spectra of Pterins and Folates

The presence of the amino- and oxo- substituents in the pteridine ring leads to strong intermolecular hydrogen bonding and in consequence the naturally occurring pterins are infusible, difficult of elemental analysis and depend principally on differences in behaviour in partition chromatography for their characterisation. Although mass spectrometry requires only small amounts and gives characteristic spectra, it is only recently that mass spectra have been reported in the analysis of pteridines.

The mass spectra of simple pteridines[185, 186, 191, 192] and of the acetates of some naturally occurring pteridines[187] have been described. Recent papers have described the mass spectra of some pteridines and their trimethylsilyl derivatives[188, 189]. Lumazine, various methyl substituted lumazines and 8-substituted lumazine derivatives have also been examined[193, 194]. Blair and Foxall[190] have reported the mass spectra of pterin, xanthopterin, isoxanthopterin, leucopterin and 6-hydroxymethylpterin. These spectra were determined on an A.E.I. MS9 spectrometer by direct insertion on the inlet probe at 70 ev. and an ion source temperature of 250-300°. This technique avoids the necessity for prior conversion to the acetate or trimethyl silyl derivative. The work of Blair and Foxall[190] has been continued and expanded to cover a variety of pterins and their reduced derivatives, as well as more complex pterins such as folates.

The spectra were determined by direct insertion on the inlet probe as described above. The pterins examined were:- pterin, xanthopterin, dihydroxanthopterin, 6-methylpterin, 6-methyl-7,8 -dihydropterin, 6-methyl-tetrahydropterin, 6-aminopterin,

6-formylpterin, biopterin and tetrahydrobiopterin. These mass spectra were characterised by a sparse fragmentation pattern and a molecular ion peak which was frequently the dominant peak in the spectrum. An exception was biopterin which, unlike tetrahydrobiopterin, did not yield a molecular ion peak. High resolution studies on the fragment ions showed that the major processes were either the loss of a carbon monoxide fragment or the loss of an HCN fragment[190]. The spectra recorded by Yamakami et al.[187] (pterin, xanthopterin and isoxanthopterin each as acetate) all have small molecular ion peaks and an intense peak representing the free pteridine. The fragmentation patterns after allowing for the acetate group are similar to those obtained by direct insertion of the free pteridine. Clark and Smith[192] from their studies of some 3-hydroxypteridin-4-ones



report that fragmentation occurs mainly by successive loss of NO, CO, HCN and HCN, with initial loss from the oxygen containing ring. Methyl substitution produced corresponding changes in the m/e values. Fragmentation of 6-unsubstituted and 6-methyl substituted-2, 4, 7-tri -hydroxypteridines begins with loss of HOCN, followed by loss of CO[189]. Similar fragmentation patterns are observed with lumazines [193, 194].

Folic acid, DHF and THF also yielded mass spectra when

examined using the direct insertion technique. These spectra had no molecular ion peak, but yielded fragments characteristic of the pterin and p-aminobenzoylglutamic acid moieties.

The mass spectra of various pterins and folates, with the relative abundance for peaks above m/e 100, are given below:

Pterin.	Mol. Wt. 163								
m/e	108.	109.	120.	121.	122.	135.	136.	163.	164
Relative abundance	6	10	5	6	20	8	5	100	9

Xanthopterin.	Mol. Wt. 179						
m/e	109.	110.	134.	151.	152.	179.	180
Relative abundance	30	20	10	10	33	100	8

Dihydroxanthopterin	Mol. Wt. 181									
m/e	109.	110.	111.	126.	133.	134.	135.	151.	152.	153.
Relative abundance	8	15	5	5	5	5	24	5	68	12
m/e	179.	180.	181.	182						
Relative abundance	14	6	100	9						

6-Methylpterin.	Mol. Wt. 177									
m/e	106.	107.	108.	109.	122.	133.	134.	135.	136.	150.
Relative abundance	7	13	11	11	10	5	7	7	11	15
m/e	177.	178								
Relative abundance	100	11								

6-Methyl-7,8-dihydropterin. Mol. Wt. 179

m/e	108.	119.	133.	136.	147.	150.	161.	164.	165.
Relative abundance	5	5	8	5	5	5	6	50	7
m/e	166.	177.	178.	179.	180.	181.			
Relative abundance	20	42	53	100	13	26			

6-Methyl-tetrahydropterin. Mol. Wt. 181

m/e	107.	108.	109.	110.	111.	113.	121.	124.	135.
Relative abundance	16	6	6	7	20	32	9	16	7
m/e	136.	138.	149.	152.	163.	164.	165.	166.	167.
Relative abundance	32	7	16	8	32	6	15	100	8
m/e	177.	178.	179.	180.	181.	182.			
Relative abundance	15	5	5	11	95	9			

6-Aminopterin. Mol. Wt. 178

m/e	100.	106.	107.	108.	109.	110.	111.	112.	115.
Relative abundance	7	6	7	29	30	17	7	7	6
m/e	122.	123.	124.	133.	134.	135.	136.	137.	151.
Relative abundance	9	7	9	6	7	9	21	7	11
m/e	152.	178.	179.						
Relative abundance	21	100	15						

6-Formylpterin. Mol. Wt. 191

m/e	107.	108.	109.	120.	121.	122.	135.	136.	137.
Relative abundance	9	11	14	11	5	14	9	35	6
m/e	149.	150.	162.	163.	190.	191.	192.		
Relative abundance	5	11	14	26	5	100	10		

Biopterin. Mol. Wt. 237

m/e	119.	122.	123.	136.	147.	148.	162.	163.	164.
Relative abundance	7	8	6	14	18	12	8	12	22
m/e	175.	177.	191.	192.	193.	194.			
Relative abundance	10	15	30	30	100	9			

Tetrahydrobiopterin. Mol. Wt. 241

m/e	105.	107.	109.	110.	111.	112.	119.	121.	122.
Relative abundance	10	11	10	100	22	11	8	10	7
m/e	141.	142.	147.	149.	163.	164.	165.	166.	167.
Relative abundance	11	8	7	8	19	25	28	79	10
m/e	176.	177.	202.	203.	204.	205.	207.	241.	
Relative abundance	7	8	18	12	7	19	7	10	

p-Aminobenzoylglutamic acid. Mol. Wt. 266

m/e	120.	121.	137.	248.
Relative abundance	100	11	43	12

Folic acid. Mol. Wt. 441

m/e	101.	106.	107.	108.	109.	118.	120.	121.	122.
Relative abundance	7	13	13	6	7	30	100	11	5
m/e	129.	130.	136.	137.	138.	150.	177.		
Relative abundance	18	6	12	91	8	6	36		

DHF. Mol. Wt. 443

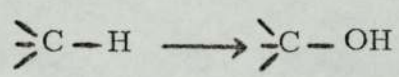
Mass Spectrum similar to folic acid.

THF Mol. Wt. 445

m/e	104.	105.	106.	107.	108.	109.	119.	120.	121.
Relative abundance	19	24	19	18	10	8	8	100	19
m/e	122.	124.	125.	129.	130.	133.	134.	135.	136.
Relative abundance	13	10	12	6	7	6	6	8	15
m/e	137.	138.	147.	148.	149.	150.	151.	152.	163.
Relative abundance	48	8	10	18	49	44	8	14	19
m/e	164.	165.	166.	167.	177.	178.	179.	180.	181.
Relative abundance	21	31	88	14	60	13	11	17	13
m/e	191.	202.							
Relative abundance	8	6							

XIV Biochemical Application of Hydroperoxide Intermediates.

Many biosynthetic and metabolic reaction sequences include hydroxylation at a carbon atom.



as an important biochemical step. The reaction is generally carried out by a mixed function oxidase working in conjunction with oxygen and a reducing agent[198,199]. Tetrahydropterins are important cofactors in enzymatic processes involving molecular oxygen and their role in several hydroxylation reactions has been outlined in Chapter I, Section 2.

Recent reports on enzymic hydroxylation reactions requiring a tetrahydropterin cofactor suggest the involvement of arene oxide intermediates[146-148]. Stereochemical studies using labelled compounds support these suggestions[149,150]. All the data are in agreement with a process involving, in effect, the direct insertion of an oxygen atom (an "oxene" or "oxenoid" mechanism)[199,200]. Studies using phenylalanine and tyrosine labelled with ^{3}H or ^{14}C, have shown that 6,7-dimethyl-tetrahydropterin, in large molar excess and in the presence of ferrous ion, is an effective, non-specific hydroxylating agent for aromatic amino acids[201].

Rembold et al.[180] in their studies on the catabolism of pteridine cofactors report that the in vivo studies show a striking similarity to the in vitro results. The deposition of xanthopterin in many insects is explained by degradation of pterin cofactors such as THF and tetrahydrobiopterin[94].

The reported reactions of molecular oxygen and tetrahydropterins with substrates may be rationalised in terms of attack by peroxy radicals.

A possible scheme for enzymic oxidations is one involving a radical cation[117] which loses $H^{\oplus}$ to give a radical that reacts very rapidly with oxygen (diffusion controlled[166]) to give a peroxy radical (see Figure XX). The tetrahydropterin peroxy radical (ROO·) then undergoes addition to the substrates aromatic system to give an arene oxide intermediate (LXXIX), this then rearranges to give the hydroxylated derivative (LXXX) and a RO· radical (see Figure XXI). Evidence for a scheme involving arene oxide intermediates is as follows:- Addition of peroxide radicals to benzene has recently been reported[151]. In order to qualify as intermediates, deuterated arene oxides must retain their label on rearrangement to hydroxylated metabolites; they must undergo the "NIH shift" (National Institute of Health shift[216]). An example of the "NIH shift" is the conversion of toluene-4-^{2}H to 4-hydroxy-toluene-3-^{2}H[146]. It has been shown that when toluene -4-^{2}H is hydroxylated either chemically or enzymatically, migration and retention of deuterium takes place to a comparable extent and hydroxylation occurs at the para-position[146]. m-Halogenotyrosine constitutes 90% of the product when p-bromo- or p-chlorophenylalanine is hydroxylated enzymatically[220]. Since migration and retention of halogen and deuterium take place to a comparable extent, a mechanism involving ionic species[220-222] is unlikely, rather, the results suggest a scheme involving radicals. Formation of 1,2-naphthalene oxide from naphthalene by liver microsomes has also been shown[147].

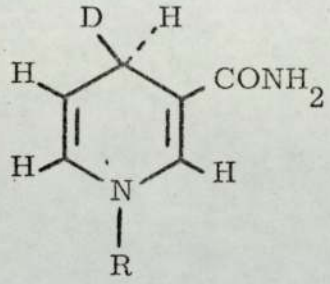
Kaufman[51,116] has shown the participation of tetrahydropterins in the enzymatic conversion of phenylalanine to tyrosine. During the hydroxylation reaction the tetrahydropterin is oxidised to an intermediate that can be reduced back to the original tetrahydropterin by NADPH, a

reaction catalysed by sheep liver enzyme. In the absence of NADPH and enzyme, the intermediate is converted to the 7,8-dihydropterin. These observations are explained by consideration of the above RO· radical, which in solution forms ROH. When NADPH and enzyme are present, ROH underoges reductive elimination of the hydroxyl group to give the original tetrahydro compound[116]. In their absence, ROH undergoes loss of H_2O to give the inactive dihydro compound.

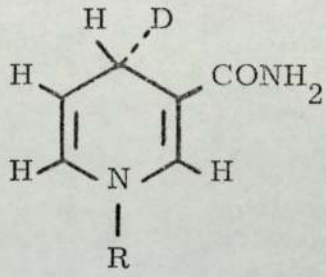
From studies on the reduction of the oxidised pterin intermediate by $NADP^3H$, Kaufman[51] concluded that the primary site of oxidation of tetrahydropterins is N-5 rather than C-6. In these experiments, the reduction with $NADP^3H$ was followed spectrophotometrically and on completion the $NADP^3H$ and $NADP^+$ were separated from the reaction mixture, which was then lyophilised. The lyophilised sample was regarded as being the tetrahydropterin and, since it showed no radioactivity, no tritium was incorporated into a stable linkage in the tetrahydropterin. A mechanism involving hydroperoxide intermediates should result in tritium incorporation in the tetrahydro compound. Kaufman's results do not disprove the presence of peroxide intermediates because his studies with $NADP^3H$ are open to questioning on several points.

1) No figure is given for the isotopic purity of his $NADP^3H$.

2) Reduction by dithionite in a D_2O medium forms two stereoisomers of the reduced coenzyme[223,224]. These are given below, showing only the pyridine ring.



Isomer A



Isomer B

Some stereospecificity in the reduction is shown, for a greater percentage of the product is the A isomer[224, 225]. Kaufman does not appear to have considered a tritiated A : B ratio.

3) Only one of the two isomers is active, since enzymes are stereospecific in that they catalyse transfer of hydrogen from one particular side of the pyridine ring (Group B type is larger than the A type)[226].

4) Isotope discrimination is well known (an example is aromatic nitration).

5) The amount of tritium released was considerably less than that expected from the quantity of NADP^{3}H oxidised.

6) No tetrahydropterin was isolated nor was its presence in the lyophilised sample confirmed. If the tetrahydropterin underwent oxidation during handling prior to lyophilisation to yield the dihydro derivative, then tritium would be eliminated as tritiated hydrogen peroxide and lost during lyophilisation. When these points are considered, it is perhaps not too surprising that no tritium was observed.

In association with the above studies, Kaufman[51] also used N-alkyl substituted-tetrahydropterins. For compounds such as 2-dimethylamino-6, 7-dimethyl-tetrahydropterin, they can give the

o-quinonoid structure but not the p-quinonoid. Alkylation of either N-8 or the 2-amino group decreased the rate of reduction of the intermediate by NADPH. As the electron-donating effect of the alkyl group(s) will increase the electron density of the pyrazine ring, the reduction, which involves a hydride ion-mechanism[227,228], is expected to be slower.

It has recently been reported that 6-methyl-tetrahydropterin is produced in small amounts by the degradation of THF during catalytic reduction[217]. Also that the major part of the hydroxylation cofactor activity of THF is not due to THF itself, but rather to its contamination with the highly active 6-methyl-tetrahydropterin. This difference in activity between THF and 6-methyl-tetrahydropterin is explained by considering the stability of their respective peroxide intermediates. THF forms an unstable intermediate which readily undergoes decomposition and cleavage of the side-chain. The 6-methyl-tetra -hydropterin peroxide intermediate is more stable and can enter the hydroxylation cycle described above, i.e. PtH $\longrightarrow$ PtOO· $\longrightarrow$ PtO· $\longrightarrow$ PtOH $\longrightarrow$ PtH, where PtH represents the tetrahydro compound.

Pterins are common natural products, occurring in groups with either xanthopterin and leucopterin or pterin and isoxanthopterin[202]. (^{14}C) Folic acid administered to Drosophila gives (^{14}C) isoxanthopterin[203]. A possible route for this conversion would be reduction of folic acid to THF, peroxide formation at C-9, rearrangement to pterin and further oxidation to isoxanthopterin by the action of xanthine oxidase[204]. Xanthopterin is derived by formation of the 6-hydroperoxide and subsequent rearrangement and oxidation. Rembold et al.[61,94] have studied the aerobic oxidation of various tetrahydropterins, with and

without the presence of xanthine oxidase. There is a high molecular oxygen consumption during the overall reaction. Isoxanthopterin is present in the products when xanthine oxidase is present, whereas pterin is obtained in the absence of xanthine oxidase. Xanthine oxidase is also known to convert xanthopterin, lumazine and 6-hydroxylumazine to leucopterin, 7-hydroxylumazine and 6,7-dihydroxylumazine respectively[205-207].

The occurrence of pterins and the reactions of tetrahydropterin cofactors are therefore explained in terms of hydroperoxide intermediates and their subsequent reactions.

Figure XX Enzymatic Oxidation

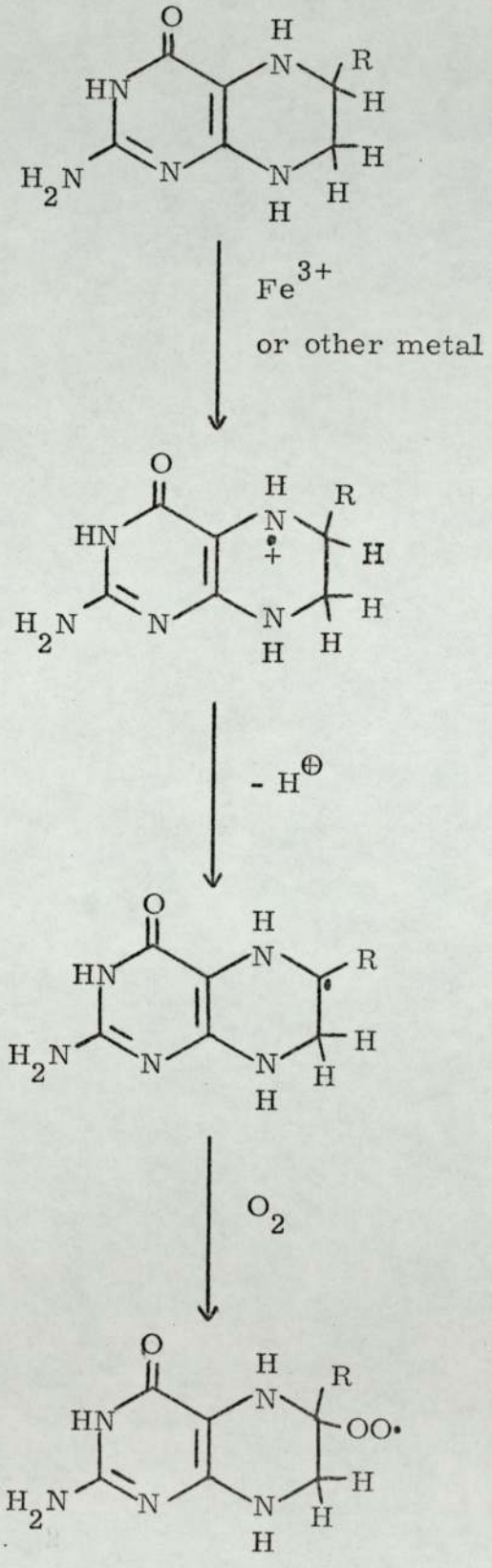
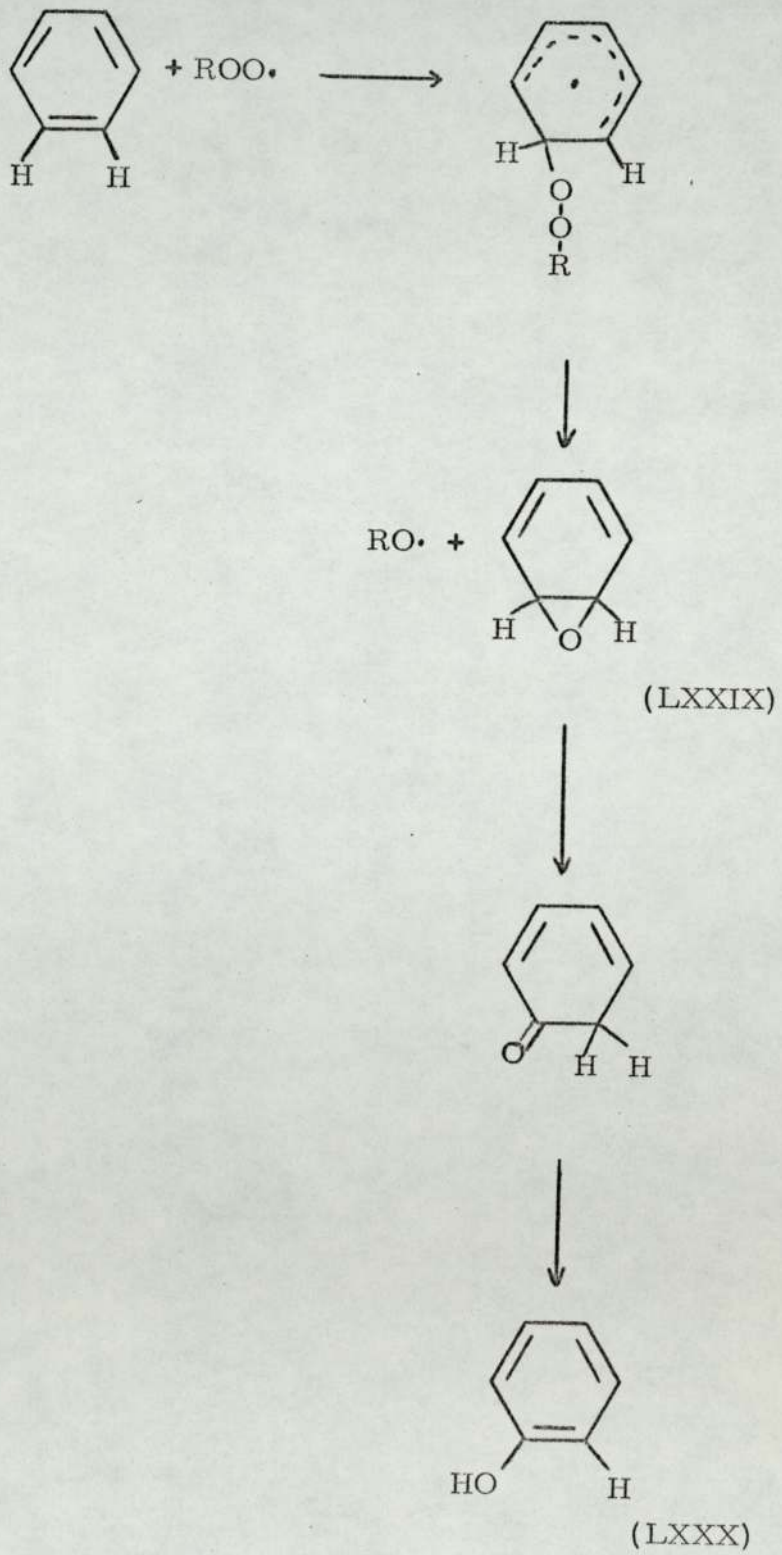


Figure XXI A possible mechanism for Hydroxylation Reactions



XV The Mechanism of the Autoxidation of Tetrahydropterins

Experimental observations discussed in previous chapters have been presented as support for a mechanism involving hydroperoxide intermediates, with the mechanism developing in depth as more evidence is considered. A probable mechanism is now presented in which all the previous discussions are combined.

The mechanism can be divided into two sections:-

1. Hydroperoxide formation
2. Reactions of the hydroperoxide.

1. Hydroperoxide Formation

Autoxidation commences by the production of a free radical that is capable of combining directly with free oxygen.

Radicals may be formed by homolysis of a C-H bond. Homolytic abstraction of hydrogen is facilitated by polar substituents in the molecule which favour the movement of electrons towards the CH group. Light frequently initiates autoxidation due to the absorption of enough energy to effect the homolysis. Ions, particularly of the transition metals, which readily undergo a single electron transfer, can also catalyse radical formation.

A chain reaction involving homolysis, with PtH representing a tetrahydropterin, is as follows:-

$$PtH \longrightarrow Pt\cdot + (H\cdot)$$

$$Pt\cdot + O_2 \longrightarrow PtOO\cdot$$

$$PtOO\cdot + PtH \longrightarrow PtOOH + Pt\cdot$$

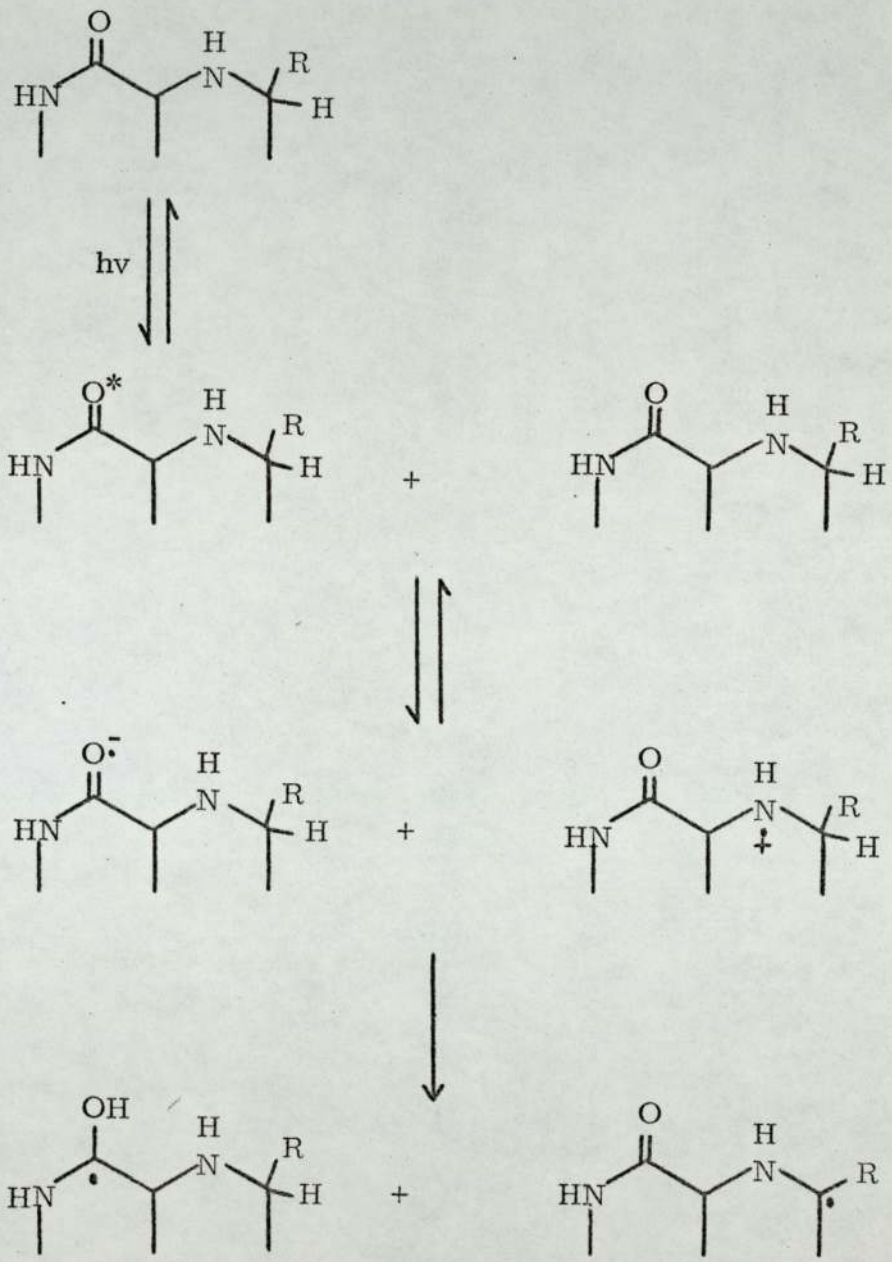
Although the above scheme is often presented in reports on autoxidation, it does not explain certain observations for tetrahydropterins. Neither

the effect of pH on the oxidation (see appropriate chapters) nor the decrease in rate of oxidation with increase in the concentration of tetrahydropterin[229], are explicable in terms of homolytic radical formation. On the basis of the above scheme, an increase in concentration of tetrahydropterin should speed up the rate of oxidation. It is therefore evident that a different mechanism is required.

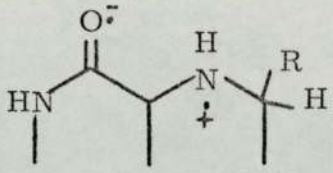
Although there are no reported studies on the effect of light on the oxidation of tetrahydropterins, many workers often exclude light from their experiments, e.g. during column chromatography, and samples are usually stored in the dark. (An experiment to observe any effect is described in Chapter V).

Carbonyl compounds have been shown to sensitise the photo-oxidation of amines[172,173, 218]. The oxygen in the carbonyl group is excited to the triplet state. The reactions occur by abstraction of hydrogen from the amines by the triplet sensitiser, to give radicals which react with oxygen giving peroxides and, finally, the observed products. From studies of the effect of solvent polarity on these reactions it has been proposed that in polar solvents reaction via radical ions can occur[218]. This scheme can be applied to the oxidation of tetrahydro -pterins, involving photo-excitation of the carbonyl group at position 4, reaction occurring by electron transfer, followed by a proton transfer. A scheme for the intermolecular reaction is outlined in Figure XXII. The peroxide is then formed by the rapid reaction of LXXXI and oxygen. An intramolecular reaction is also possible, but this is unlikely since it would involve proceeding via a high energy intermediate radical (LXXXII).

Figure XXII Tetrahydropterin C-6 Radical Formation



(LXXXI)



(LXXXII)

The rate of oxidation of tetrahydropterins varies with buffer (see Chapters III and V) suggesting that photo-excitation results predominantly in formation of a radical cation, with the charge centred at N-5. Hydrogen abstraction from the radical cation then occurs by reaction with the buffer anion, the latter behaving as a base. The more nucleophilic is the buffer anion, the faster is the rate of oxidation. Experiments with 6-deutero-tetrahydropteridines have shown that in the range pH 6.0 - 8.0 cleavage of the C-H bond at position 6 is rate limiting[129]. This supports the above scheme in which the rate determining step is hydrogen abstraction from the radical cation to yield a radical at C-6. The next step, which is addition of oxygen to this radical, is extremely fast and is probably diffusion controlled in many instances[166].

A decrease in rate of oxidation with increase in concentration of the tetrahydropterin[229] is explained by quenching of the excited carbonyl group due to molecules colliding.

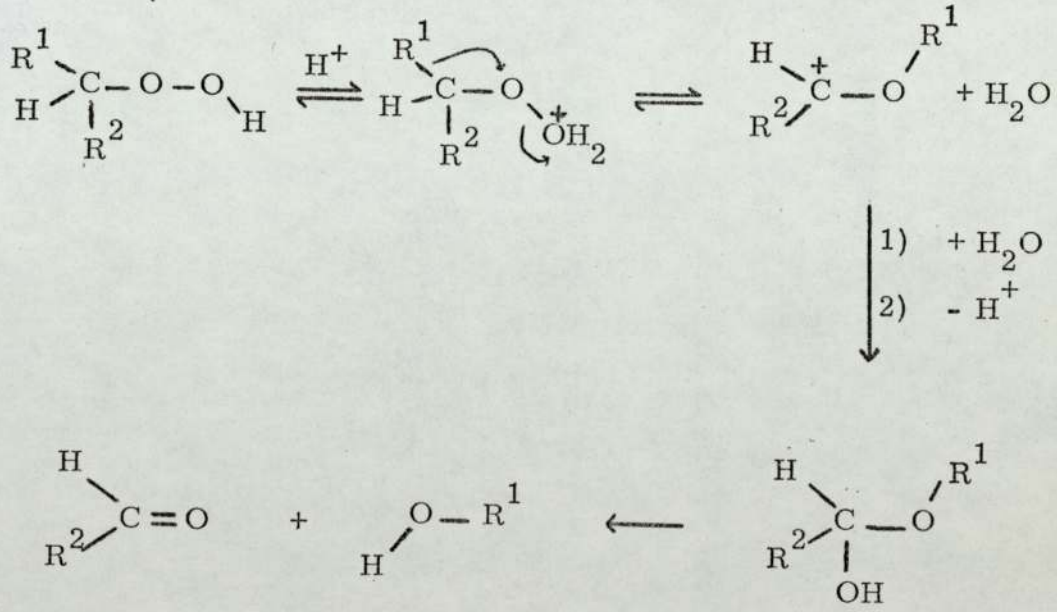
Tetrahydropterins substituted at N-5 with an electron-donating group e.g. 5-methyl THF, form radical cations, whereas those substituted with an electron-withdrawing group e.g. 5-formyl THF, do not[123,124]. 5-Formyl THF is stable to oxidation. 5-Methyl THF

is less readily oxidised than THF because the electron-donating effect of the methyl group reduces the ease of H^+ abstraction from C-6 of the radical cation. Oxidation is slow since attack now occurs at the unfavourable C-7 position (see Chapter V).

2. Reactions of the Hydroperoxide

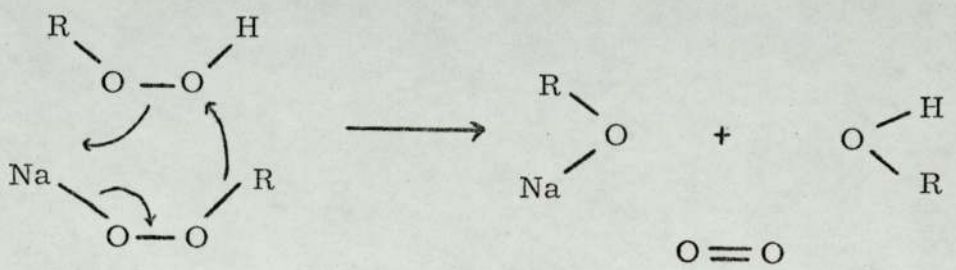
Organic hydroperoxides are polarized molecules and consequently there is an inherent tendency for their weak O-O bonds to break heterolytically. They undergo acid and base catalysed decompositions with either rearrangement or elimination[154].

Hydroperoxides are able to add a proton to the hydroxyl group. The molecule then becomes so strongly polarized that O-O fission occurs, together with a concerted molecular rearrangement that yields a carbonium ion.



The greater the nucleophilicity of R^1, the more readily will it migrate. Heating also favours rearrangement.

The anions of hydroperoxides are reasonably stable in cold dilute alkali, but many tertiary hydroperoxides undergo bimolecular (S_N2) hydrolysis with oxygen evolution.



Hydroperoxides may also undergo solvolysis (S_N1) to the corresponding hydroxy compound with elimination of hydrogen peroxide.

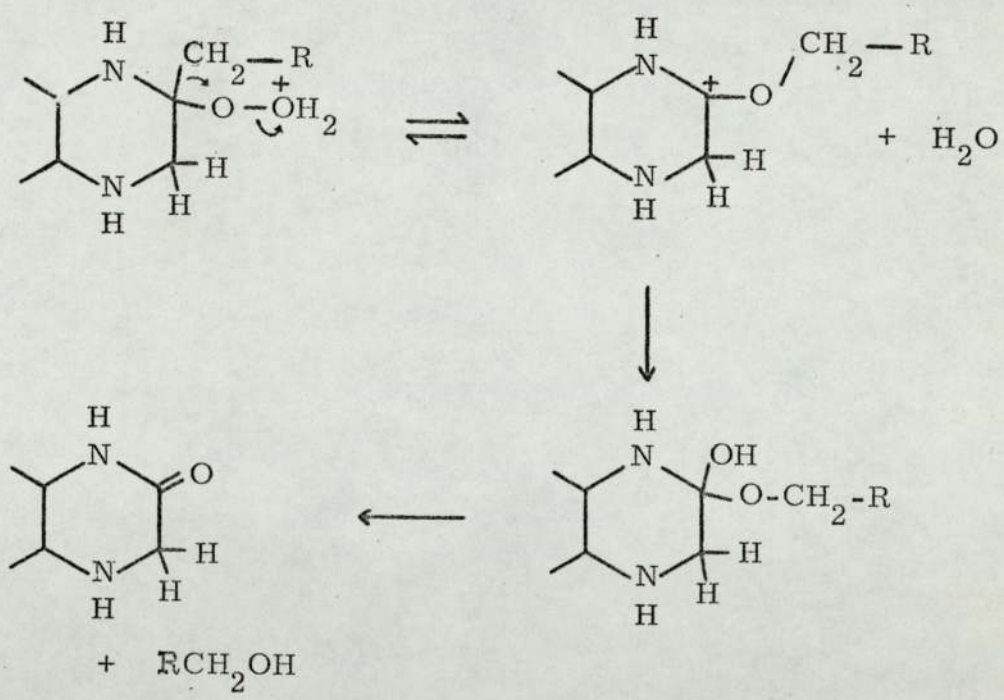
$$HO\text{-}O\text{-}CR_3 \rightleftharpoons HO\text{-}O^- + {}^+CR_3 \xrightarrow{H_2O} HO\text{-}OH + HO\text{-}CR_3$$

Water may subsequently be eliminated from the hydroxy compound.

The above reaction schemes can be applied to the hydroperoxides formed during the autoxidation of tetrahydropterins.

Studies with 6,7-dimethyl-tetrahydropterin have shown the oxidation to be acid-base catalysed[129]. Tetrahydrobiopterin, tetrahydroneopterin, tetrahydrolumazine[61,91], 6-methyl-tetrahydropterin (see Chapter V) and 10-formyl THF[230] are oxidised to their 7,8-dihydro derivative at room temperature. THF at room temperature and pH 7 is rapidly oxidised to xanthopterin and pterin, plus a small amount of DHF (See Chapter II). At acid pH THF is protonated at N-5, loss of H^+ from C-6 is now difficult, so favouring peroxide formation at C-9 to yield pterin. At alkaline pH H^+ abstraction

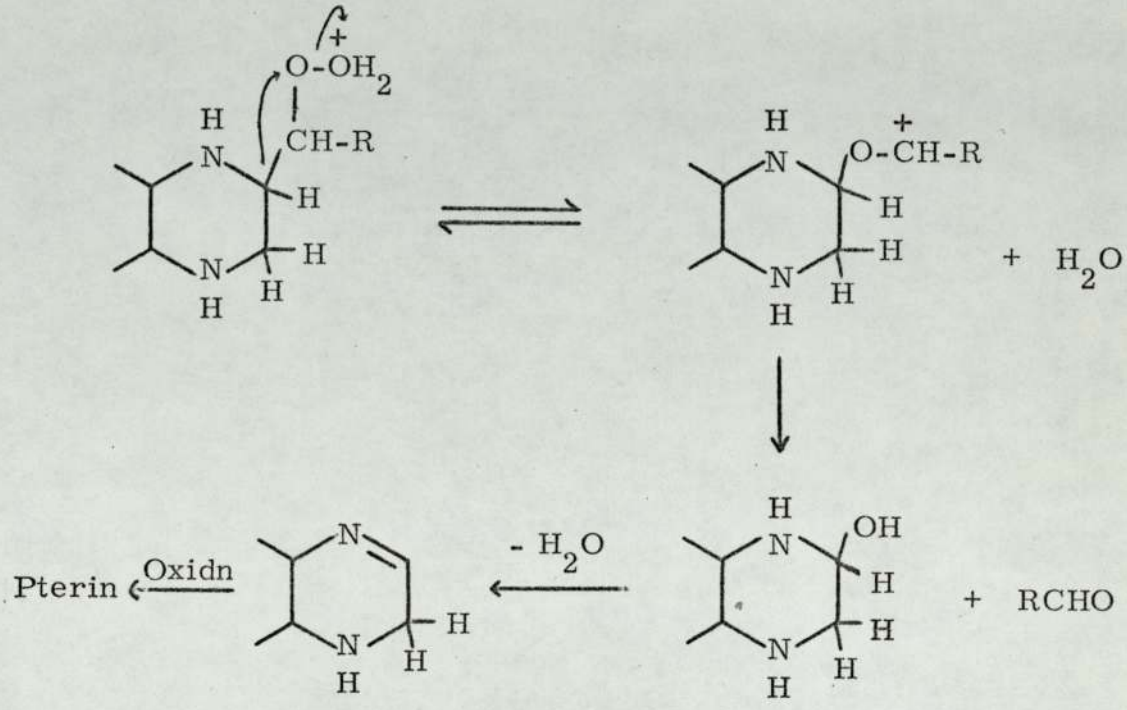
from C-6 is facilitated, thereby enhancing the formation of C-6 peroxide and, ultimately, xanthopterin. (See Chapter II). With the exception of pterin formation, derived from peroxide formation at C-9, the above observations are explicable in terms of peroxide formation at C-6. The side-chain of THF is more nucleophilic than that of any of the other pterins mentioned above, consequently the C-6 hydroperoxide of THF readily undergoes protonation and molecular rearrangement. A scheme for the rearrangement is outlined below in which only the pyrazine ring is considered.



The 7,8-dihydro derivatives are formed by hydrogen peroxide elimination from the C-6 hydroperoxide (see above).

At higher temperatures tetrahydrobiopterin and tetrahydro-neopterin yield xanthopterin and pterin[61,94], while 10-formyl THF yields xanthopterin[230]. This is in accord with the fact that increasing temperature favours rearrangement. 10-Formyl THF only yields xanthopterin because the electron-withdrawing effect of the formyl group

at N-10 prevents peroxide formation at C-9. Therefore only the C-6 peroxide is formed, which rearranges to yield xanthopterin. Pterin is formed by rearrangement of the C-9 peroxide as shown below:



At pH 13 DHF is formed from THF by bimolecular hydrolysis between the C-6 peroxide and its anion to yield two molecules of 6-hydroxy THF and oxygen (see beginning of this section). Elimination of water from the 6-hydroxy THF then yields DHF. This explains why DHF formation is favoured by oxidation at pH 13, followed by slow acidification (see Chapter II section 7).

DHF is oxidised to a C-9 peroxide that undergoes hydrogen peroxide elimination to yield a Schiffs base. Hydrolysis of the Schiffs base produces 6-formyl-7,8-dihydropterin. (See Chapter III).

The schemes presented in this chapter comprise a mechanism that explains all the experimental results and observations concerning the autoxidation of reduced pterins.

Experimental

Ultra-Violet (u.v.) spectra were recorded on a Unicam SP-800 spectrometer, fluorescence spectra on an Aminco-Bowman fluorescence spectrophotometer, mass spectra on an A.E.I. MS9 and nuclear magnetic resonance spectra (n.m.r.) on a Perkin-Elmer R10 or R14 spectrometer.

Folic acid, pterin, xanthopterin, florisil (60/100 mesh) and 2-mercaptoethanol came from Koch-Light Laboratories Ltd. T.l.c. material came from either Camlab or May and Baker. Adams platinum oxide catalyst came from either Hopkins and Williams Ltd or Johnson Matthey and Co. Ltd. Biopterin and tetrahydrobiopterin, were kindly supplied by Dr. R.F. Long (Roche Products Ltd). 5-Methyl THF, as the barium salt, was provided by Mr. A. Robb.

T.l.c. solvent systems are presented, where applicable, with the v/v ratio of the individual components, unless shown otherwise.

1. Synthesis of THF

Folic acid is reduced to the tetrahydro compound by low-pressure catalytic hydrogenation over platinum oxide. The method of preparation is a modification of that described by Hatefi et al.[68].

Two grams of platinum oxide and two grams of folic acid were suspended in 50 ml. of dry glacial acetic acid in the hydrogenation vessel. Reduction was carried out at 20° and atmospheric pressure until no more hydrogen was absorbed. Reduction of catalyst in the presence of the folic acid sometimes caused the production of colloidal platinum, which hindered filtration. On completion of the hydrogenation the apparatus was flushed with nitrogen. A solution of 0.3 ml. of 2-mercaptoethanol in 10 ml. of dry glacial acetic acid was introduced into the flask, care being taken to prevent contact of the solution with air. For the first two experiments a "dry-box" continuously flushed with nitrogen was used, in which the contents of the flask were filtered through a glass-sinter directly into a round-bottomed flask immersed in a dry ice-acetone bath. An alternative to the "dry-box" was used for subsequent experiments. For this, the flask was modified to allow the contents to be filtered, under nitrogen pressure, through a glass-sinter attached to a side-arm. By the controlled application of a vacuum to the immersed receiver, the contents were filtered without exposure to air. The flask with its frozen contents was then attached to a lyophilisation apparatus and taken to dryness. The product, a fluffy white powder, was stored under nitrogen in sample bottles wrapped in metal-foil to prevent exposure to light. The bottles were kept under an atmosphere of nitrogen in a desiccator. Solid THF became brown on exposure to air[69,81].

U.v. spectra of deaerated solutions containing 0.5% v/v of 2-mercaptoethanol. $\lambda_{max.}$ (nm.) pH 13, 294; pH 7, 297; pH 1, 270, 295. In view of the sensitivity of THF to aerial oxidation it is difficult to determine its purity accurately, the best estimate was 96% at pH 7, using $\varepsilon_{max.}$ (mol) x 10^{-3} = 29.1[152]. (ε = 32 has been reported[208], but this is an average of results with ε values from 25.6 - 39).

N.m.r. 100 MHz spectrum of solution in trifluoroacetic acid p.p.m. values 1.8, 1.89, 2.32, 2.4 (benzene ring); 4.82 (α CH in glutamic acid); 5.47 (CH_2 at C-7); 5.88 (CH_2 at C-9); 7.19, 7.4 (CH_2's in glutamic acid).

T.l.c's were prepared using anaerobic conditions. 1, 2 and 5 µl samples of a solution of 5 mg. THF per ml. of a 1% ammonia solution, containing 0.5% v/v 2-mercaptoethanol, were applied to cellulose MN 300 U.V.$_{254}$ plates impregnated with 1% of 2-mercaptoethanol. The plates were developed in darkened tanks, well purged with nitrogen. A single absorbing spot, sometimes with only traces of any fluorescent impurities, was discerned on the chromatograms (see Table III).

TABLE III Rf values of THF

Solvent system	Rf value
n-Propanol/1% ammonia solution, 2:1.	0.46
n-Butanol/acetic acid/water, 4:1:5	0.56
Phosphate buffer, pH 7.	0.81
0.1N. Borax solution, pH 9.2	0.98

2. Synthesis of DHF

DHF was prepared by dithionite reduction of folic acid according to the procedure of Futterman[76]. N KOH solution was slowly added to a suspension of 200 mg. folic acid in 20 ml. of water until solution was complete. The solution was then carefully adjusted to pH 6 by the addition of 2N. HCl. A small amount of a fine suspension appeared. 50 ml. of potassium ascorbate solution (100 mg. per ml), adjusted to pH 6 with 2N. HCl was added, followed by 2 g. of sodium dithionite. After five minutes the solution was cooled to about O° and adjusted to pH 2.8 with 2N. HCl. The resultant white solid was removed by centrifuging, redissolved in 50 ml. potassium ascorbate (100 mg. per ml.) at pH 6 and reprecipitated with 2N. HCl. After centrifuging, the solid was washed twice with an ascorbic acid solution (10 mg. per ml) at pH 2.8. The product was stored frozen as a suspension in very dilute hydrochloric acid containing 10% ascorbic acid. Before use, DHF was obtained free of ascorbic acid by washing with very dilute hydrochloric acid, followed by acetone, and then dried under vacuum.

U. v. spectra[20,79], $\lambda_{max.}$ (nm.) pH 13, 221 (239) 285 (306); pH 7, 227, 282 (303).

T. l. c. data are presented in Table VII.

3. Synthesis of Dihydroxanthopterin

a) A slurry of 1.14 g. of sodium borohydride in ten ml. of water was added to a stirred solution of 0.50 g. of xanthopterin in 200 ml. of Tris buffer, pH 7.8. After 20 minutes excess sodium borohydride was destroyed and the solution adjusted to pH 7-8 by the addition of 6 ml. of a 1:1, acetic acid : water solution, giving a buff precipitate. The

reaction medium was lyophilised to leave about 10 ml. of a slurry, this was dissolved in 100 ml. of 0.1N NaOH solution and the solution adjusted to pH 4-4.5 with glacial acetic acid. The precipitated product was removed by centrifuging, washed twice with dilute acetic acid and dried. Yield 0.41 g.

b) This method is based on a general procedure by Albert and Matsuura[49].

0.18 g. of sodium borohydride was added to a suspension of 0.36 g. of xanthopterin in 25 ml. of 0.6N potassium carbonate solution. On stirring, the contents of the flask changed in colour from deep yellow to cream. After six hours a further 0.18 g. of sodium borohydride was added and stirring was continued overnight. The reaction medium was adjusted to pH 5 with glacial acetic acid and cooled to 5°. The suspension was removed by centrifuging, washed with 25 ml. of water and dried to yield 0.33 g. of product.

U.v. spectra[58], $\lambda_{max.}$ (nm.) pH 13, 224, 279 (310); pH 7, 224, 275, 311; pH 1, 279, 310.

Fluorescence spectra, $\lambda_{max.}$ (nm.) pH 13, excitation 390, emission 475; pH 7, 345, 420; pH 1, 350, 410.

M^+ 181.0596. $C_6H_7N_5O_2$ requires 181.0600.

N.m.r.[58] spectrum in trifluoroacetic acid, sharp singlet at 5.46 ppm (CH_2 at C-7), broad, diffuse absorption around 2 ppm (protons bound to nitrogen).

4. Synthesis of 6-Methylpterin[45,209].

3 g. of folic acid and 60 g. of zinc dust in 3 litres of 0.4N -H_2SO_4 were stirred for one hour. After standing for 15 minutes the excess zinc was removed by filtration. 60 g. of manganese dioxide

was added, the filtrate stirred for 20 minutes and refiltered. Zn^{2+}, precipitated as zinc phosphate by the addition of 4.8 litres of molar Na_2HPO_4 solution was removed by filtration and the filtrate adjusted to pH 6 with concentrated H_2SO_4. After standing for 66 hours at 5°, which produced only a very small amount of precipitate, the filtrate was acidified to pH 2.2 with concentrated H_2SO_4 and run onto a Florisil, 60/100 mesh column. The column was eluted with water, followed by very dilute ammonia solution. 6-Methylpterin was isolated by combining and lyophilising appropriate fractions.

U.v. spectra[210], $\lambda_{max.}$ (nm.) pH 13, 253, 364; pH 1, 247, 325.

Fluorescence spectra, $\lambda_{max.}$ (nm) pH 13, excitation 265, emission 455 - 460; pH 7, 355, 450; pH 1, 325, 480.

M^+ 177.0646, $C_7H_7N_5O$ requires 177.0651.

T.l.c. data are presented in Table V.

5. Synthesis of 6-Aminopterin.

Pterin is reduced to the tetrahydro compound by low pressure catalytic hydrogenation over platinum oxide. In the presence of ammonia the tetrahydro compound is oxidised mainly to pterin, plus 6-aminopterin and xanthopterin

1 g. of platinum oxide and 1 g. of pterin were suspended in 50 ml. of dilute ammonia solution. Reduction was carried out at 20° and atmospheric pressure until no more hydrogen was absorbed. On completion of the hydrogenation, 30 ml. of 0.880 ammonia solution was added and the atmosphere of hydrogen was replaced by air. After standing for three days at 20° the platinum catalyst was filtered off and washed with water.

T.l.c., using n-propanol/1% ammonia, 2:1, of the filtrate gave:- very large mauve (0.48), small absorbing (0.31), large green (0.27) small blue-green (0.17). (cf. pterin (0.47), 6-aminopterin (0.27) xanthopterin (0.17)).

Lyophilisation of the yellow-orange filtrate gave a brown-orange solid. Half of this material was chromatographed on a Florisil, 60/100 mesh column, giving a product suitable for spectroscopic and t.l.c. analysis, but, due to a large amount of inorganic impurity, unsuitable for mass spectral examination. A purer product was prepared from the brown-orange solid by preparative paper chromatography. Whatman No. 1. paper was used and the chromatogram was developed ascendingly in the solvent system n-propanol/1% ammonia solution, 2:1.

U.v. spectra[58,153] $\lambda_{max.}$ (nm.) pH 13, 260, 392; pH 1, 270, 375.

Fluorescence spectra, $\lambda_{max.}$ (nm.) pH 13, excitation 390, emission 480; pH 7, 390, 475; pH 1, 375, 460.

M^+ 178.0581, $C_6H_6N_6O$ requires 178.0603.

T.l.c. data are presented in Table V.

6. Autoxidation of THF.

The procedure used is described in Chapter II, Section 1. Isolation and identification of products, plus the formation of DHF and 6-aminopterin, are discussed in Chapter II. The products were identified by comparison with authentic samples, the analytical data for which is presented below. Recovered ..... refers to an isolated product, while pure ..... refers to an authentic sample.

a) Examination of the solid from lyophilised oxidised THF solution.

A mass spectrum contained major peaks, above m/e 160, at m/e values 181, 177, 163. High resolution studies showed m/e 181 to be a doublet. When the solid was treated with aqueous ammonia, an additional peak was observed at m/e 178. Peak matching gave:- 181.0609 (major) and 181.0974 (minor). Dihydroxanthopterin ($C_6H_7N_5O_2$) requires 181.0600; 6-methyl-tetrahydropterin ($C_7H_{11}N_5O$) requires 181.0964.

178.0574. 6-Aminopterin ($C_6H_6N_6O$) requires 178.0603.

177.0648. 6-Methylpterin ($C_7H_7N_5O$) requires 177.0651.

163.0493. Pterin ($C_6H_5N_5O$) requires 163.0494.

Since dihydroxanthopterin is oxidised to xanthopterin, a peak at m/e 179 was also observed. Only a very small quantity of 6-methylpterin was present (See Chapter II, Section 4), consequently the peak at m/e 177 was mainly derived from folic acid (See Chapter XIII). T.l.c. examination using the solvent systems a) n-propanol/1% ammonia solution, 2:1, and b) n-butanol/acetic acid/water, 4:1:5, showed a fresh aqueous solution of the lyophilised solid to contain dihydroxanthopterin (Rf: a) 0.23; b) 0.39) among the products. On standing, it oxidised to xanthopterin (Rf: a) 0.17; b) 0.53). When using concentrated solutions, folic acid was observed. (Rf: a) 0.19; b) 0.00).

b) Recovered Products

T.l.c. data of pure and recovered compounds are presented in Tables IV and V. The yields of pterin and xanthopterin from autoxidised THF, estimated by fluorimetry, are given in Table VI.

Pterin

U.v. spectra, $\lambda_{max.}$ (nm.) pH 13, 253, 363; pH 1 (240) 316. (cf. pure pterin - pH 13, 252, 363; pH 1 (244) 312).

Fluorescence spectra, $\lambda_{max.}$ (nm.) pH 13, excitation 360, emission 450 - 455; pH 7, 355, 445-450; pH 1, 320, 470. (cf. pure pterin, pH 13, 360, 450 - 455; pH 7, 355, 445-450; pH 1, 320, 470).

Xanthopterin.

U.v. spectra, $\lambda_{max.}$ (nm.) pH 13, 258 (276) 398; pH 1 232, 262, 358. (cf. pure xanthopterin, pH 13, 258 (276) 398; pH 1, 232, 262, 360).

Fluorescence spectra, $\lambda_{max.}$ (nm.) pH 13, excitation 385, emission 475; pH 7, 390, 465; pH 1, 350, 445. (cf. pure xanthopterin, pH 13, 390, 475, pH 7, 390, 465; pH 1, 350, 445).

6-Methylpterin

U.v. spectra, $\lambda_{max.}$ (nm.) pH 13, 253, 364; pH 1, 247, 324. (cf. pure 6-methylpterin, expt. 4).

Fluorescence spectra, $\lambda_{max.}$ (nm.) pH 13, excitation 365, emission 455-460; pH 7, 355, 450; pH 1, 325, 475-480. (cf. pure 6-methylpterin, expt. 4).

6-Aminopterin

U.v. spectra, $\lambda_{max.}$ (nm.) pH 13, 260, 390; pH 1, 271, 375. (cf. pure 6-aminopterin, expt. 5).

Fluorescence spectra, $\lambda_{max.}$ (nm.) pH 13, excitation 390, emission 480; pH 7, 390, 475; pH 1, 375, 460. (cf. pure 6-amino -pterin, expt. 5).

TABLE IV Rf values of Pterin

Solvent System	Rf of	
	Recovered Pterin	Pure Pterin
n-Propanol/1% ammonia, 2:1	0.46	0.46
3% w/v Ammonium chloride	0.56	0.56
0.1N-Potassium carbonate	0.50	0.50
5% Acetic acid	0.72	0.72
n-Butanol/acetic acid/water, 4:1:5	0.49	0.49
i-Propanol/2% w/v Ammonium acetate, 1:1	0.55	0.55
2% w/v Ammonium Acetate/Pyridine, 59:5.	0.75	0.75
Pyridine/Ethyl acetate/water, 4:3:3.	0.72	0.72
1% w/v Citric acid.	0.69	0.69
5% w/v Tartaric acid	0.77	0.77
n-Butanol/Ethanol/Water, 15:30:35.	0.48	0.48

The solvent systems were chosen from those available in the literature[211-214].

TABLE V Rf values of Pterins

	Solvent System *							
	1	2	3	4	5	6	7	8
Recovered Xanthopterin	0.16	0.44	0.65	0.55	0.52	0.47	0.39	0.46
Pure Xanthopterin	0.17	0.44	0.65	0.55	0.53	0.47	0.39	0.47
Recovered 6-Methylpterin	0.55	0.53	0.60	0.68	0.60	0.50		
Pure 6-Methylpterin	0.56	0.53	0.60	0.67	0.60	0.50		
Recovered 6-Aminopterin	0.26	0.27	0.40	0.52	0.32	0.27		
Pure 6-Aminopterin	0.27	0.27	0.40	0.52	0.31	0.28		

* Solvent systems:-

1. n-Propanol/1% ammonia, 2:1
2. 3% w/v ammonium chloride
3. 0.1N-Potassium carbonate
4. 5% Acetic acid
5. n-Butanol/Acetic acid/water, 4:1:5
6. 0.1 Molar Na_2HPO_4
7. 4% w/v Sodium Citrate
8. n-Butanol/5N-acetic acid, 2:1

The solvent systems are a selection from those available in the literature[215].

TABLE VI Autoxidation of THF
Yields* of Pterin and Xanthopterin

pH of Oxidation	Pterin %	Xanthopterin %	Ratio of Pterin:Xanthopterin		Total %
10	2.68	48.54	1.00	18.08	51.22
10	2.91	48.64	1.00	16.67	51.55
7	22.38	31.84	1.00	1.42	54.22
7	17.68	36.55	1.00	2.07	54.23
7	20.78	40.86	1.00	1.97	61.64
3.	52.70	4.48	11.76	1.00	57.18
3.	48.75	3.85	12.66	1.00	52.60
3	53.93	3.98	13.54	1.00	57.91

* The yields of pterin and xanthopterin in the products are expressed as a % of the THF used.

c) Formation of DHF

31.5 mg. of THF diacetate in 32 ml. of 0.1N NaOH, pH 13, was stirred for one hour, then 0.4 ml. of 2-mercaptoethanol added. The solution was adjusted to about pH 1, by the slow, dropwise addition of 0.4 ml. of concentrated HCl. The precipitate was removed by centrifuging and dried under vacuum. Yield : 15.8 mg.

T.l.c. evidence for the precipitate being DHF is presented in Table VII. U.v. spectrum: 1.6 mg. of precipitate in 100 ml. of phosphate buffer, pH 7, containing 0.5% 2-mercaptoethanol, gave a spectrum with $\lambda_{max.}$ (nm.) 285 (303) and absorbance 0.64. The precipitate therefore contained 62% DHF (using $\varepsilon_{max.}$ (mol) x 10^{-3} = 28.4[79]), which accounted for 40% of the THF used.

TABLE VII Rf values of DHF

Solvent System	Rf of	
	Recovered DHF	Pure DHF
n-Propanol/1% ammonia, 2:1	0.06	0.06
n-Butanol/acetic acid/water, 4:1:5	0.45	0.45
0.1 Molar-Na_2HPO_4	0.27	0.27
3% w/v Ammonium chloride	0.13	0.13
0.1N-Potassium Carbonate	0.35	0.36

7. Autoxidation of DHF

The procedure used is described in Chapter III, Section 1. Solutions of DHF at pH 7 in a) phosphate buffer and b) 0.1 Molar-ammonium acetate, were examined by t.l.c. using the solvent system n-propanol/1% ammonia, 2:1. After one hour, t.l.c.'s showed (with Rf values):- a) small absorbing (0.52), large yellow (0.30), small blue (0.05). b) faint yellow (0.30), large blue (0.06). (cf. p-Aminobenzoylglutamic acid (0.53) and DHF (0.06)). T.l.c. examination after two hours showed a slight decrease in the DHF of a), while there was a small increase in the yellow spot of b). The solutions were lyophilised. Mass spectra of the lyophilised solid from a) contained major peaks, above m/e 160, at m/e's 181 and 177. Peak matching gave:- 181.0580. Dihydroxanthopterin ($C_6H_7N_5O_2$) requires 181.0600. 177.0641. 6-Methylpterin ($C_7H_7N_5O$) requires 177.0651.

U.v. spectra of the solid from oxidation in phosphate buffer, $\lambda_{max.}$ (nm.) pH 13, 221, 275, 450; pH 7, 218, 275, 432; pH 1, 217, 275, 416. (Absorption above 400 nm. is characteristic of 6-formyl-7,8-dihydropterin [97,98]).

T.l.c. evidence for the formation of xanthopterin and 6-aminopterin from 6-formyl-7,8-dihydropterin, when 1% ammonia solution is added to lyophilised solid from the DHF oxidation in phosphate buffer, is presented in Table VIII.

TABLE VIII. T.l.c. Evidence for the formation of Xanthopterin and 6-Aminopterin from 6-Formyl-7,8-Dihydropterin.

Solvent System	Rf of		
	Ammoniacal solution	Xanthopterin	6-Amino-pterin
5% Acetic acid	0.52; 0.47	0.52	0.47
0.1 Molar-Na_2HPO_4	0.41; 0.21	0.41	0.21
n-Propanol/1% ammonia, 2:1	0.17; 0.26	0.17	0.26
3% w/v Ammonium chloride	0.42; 0.24	0.42	0.24
0.1N-Potassium Carbonate	0.60; 0.33	0.60	0.33

A solution of DHF in NaOH-$NaHCO_3$ buffer, pH 10, was stood for three days. T.l.c., using the solvent system n-propanol/1% ammonia, 2:1, showed the major components at Rf values 0.55, 0.22, 0.18. (cf. p-aminobenzoylglutamic acid (0.54), dihydroxanthopterin (0.23), xanthopterin (0.17)). The lyophilised solid gave a mass spectrum with the major peak, above m/e 160, at m/e 181. U.v. spectra of xanthopterin isolated by preparative paper chromatography were:- $\lambda_{max.}$ (nm.) pH 13, 256, (274), 390; pH 7, 233, 276, 385; pH 1, 228, 260, 352, (cf. pure xanthopterin pH 13, 258 (276) 398; pH 7, 234, 276, 389; pH 1, 232, 262, 360).

After three days, a solution of DHF in 0.1 Molar-ammonium acetate, pH 7, gave a t.l.c.. using n-propanol/1% ammonia, 2:1, with compounds at Rf's 0.55, 0.30, 0.24, 0.20, 0.18. (cf. p-amino

-benzoylglutamic acid (0.54), 6-formyl-7,8-dihydropterin (0.30), dihydroxanthopterin (0.23), folic acid (0.19), xanthopterin (0.17)). The lyophilised solid gave a mass spectrum with peaks, above m/e 160, at m/e's 248, 181 and 177 (signifying p-aminobenzoylglutamic acid, dihydroxanthopterin and folic acid respectively). Xanthopterin was isolated by preparative paper chromatography. M^+ 179.0432 . $C_6H_5N_5O_2$ requires 179.0443. U.v. spectra $\lambda_{max.}$ (nm.) pH 13, 256, (274) 392; pH 7, 234, 276, 389; pH 1, 227, 260, 355 (cf. pure xanthopterin above). A second sample contained p-aminobenzoyl -glutamic acid, xanthopterin and folic acid, (T.l.c. and mass spectra) and its u.v. spectra were: $\lambda_{max.}$ (nm.) pH 13, 223, 256 (275) 374; pH 7, (217) (235) 279, 364; pH 1, (215) (253) (267) 294, (333) (357). (cf. pure xanthopterin above; p-aminobenzoylglutamic acid pH 13, 218, 274; pH 7, 212, 274; pH 1, 225, 267; folic acid pH 13, 256, 284. 364; pH 7 217, (235) 282, 346; pH 1, (249) 296).

8. Effect of Dissolving DHF in Na_2O_2/H_2O.

7.8 g. of Na_2O_2 was dissolved in 200 ml. of water. 16 mg. of DHF was dissolved in 7 ml. of the peroxide solution and slowly added, with stirring, to 10 ml. of concentrated HCl at about 5°. After stirring for 15 minutes at 5° the solution was warmed to 20°. 20 ml. of water was added and the solution lyophilised. The lyophilised solid was shown by t.l.c. to contain xanthopterin. (Rf values were: n-propanol/1% ammonia, 2:1, 0.17; 3% w/v ammonium chloride 0.47; 5% acetic acid 0.51; respective values for xanthopterin were 0.17; 0.47; 0.51).

9. Oxidation of Dihydroxanthopterin in the presence of Ammonia

Dihydroxanthopterin was dissolved in 0.880 ammonia. After about six weeks the solution was lyophilised. The dominant peak in the mass spectrum was at m/e 179, with a small peak at 181. T.l.c. showed the product to be xanthopterin. There was no evidence of any 6-aminopterin.

10. Autoxidation of Tetrahydrobiopterin

Tetrahydrobiopterin was dissolved in phosphate buffer, pH 7. After 48 hours the solution was examined by t.l.c. using n-propanol/1% ammonia, 2:1. The major component had the same Rf value (0.49) and fluorescence as standard biopterin.

11. Oxidation of Reduced Pterin

The borohydride reduction of pterin and oxidation in air is described in Chapter X. The precipitate, which accounted for 75% of the starting material, was pterin. M^+ 163.0505. Pterin ($C_6H_5N_5O$) requires 163.0494. Rf of 0.45 in n-propanol/1% ammonia, 2:1 (cf. pure pterin 0.47).

T.l.c. using n-propanol/1% ammonia, 2:1 of the mother liquor gave the following (with Rf values):- mauve (0.45), mauve (0.37), green (0.23), blue (yellow in visible light) (0.21), blue-green (0.17). cf. pterin (0.47) and xanthopterin (0.17).

The green fluorescing compound was isolated by preparative paper chromatography. U.v. spectra $\lambda_{max.}$ (nm.) pH 13, 260 (277) 393; pH 1 271, 376.

The catalytic hydrogenation of pterin and subsequent oxidation in the presence of ammonia is describe in Chapter X and experiment 5.

12. Autoxidation of 5-Methyl THF

5-Methyl THF, barium salt, was oxidised by stirring in air for five hours a 0.1% solution in 0.1 Molar ammonium acetate, pH 7, and NaOH-$NaHCO_3$ buffer, pH 10. The solution was lyophilised and the resultant solid examined by t.l.c. Some oxidation had occurred producing three fluorescent compounds, one of which possessed the same Rf values as pterin (see Table IX).

TABLE IX Autoxidation of 5-Methyl THF

Solvent System	Rf values of			
	pH 7	pH 10	pterin	5-Methyl THF
n-Propanol/1% ammonia, 2:1	0.45	0.44	0.46	
	0.43	0.40		0.43
	0.21	0.21		
	0.00	0.00		
3% w/v Ammonium Chloride	0.75	0.75		0.75
	0.53	0.51	0.53	
	0.27	0.41		
	0.00	0.00		
0.1 Molar-Na_2HPO_4	0.84	0.82		0.84
	0.53	0.51	0.53	
	0.25	0.37		
	0.00	0.00		

5-Methyl THF in 0.1 Molar-ammonium acetate, pH 7, was examined by t.l.c., using 3% w/v ammonium chloride, after one and five days. No 5-Methyl THF was observed and the major product appeared to be pterin. T.l.c. after five days:- large mauve (0.51), small green (0.23), small yellow (0.00). (cf. 5-methyl THF (0.73) and pterin (0.52)). Small amounts of p-aminobenzoylglutamic acid were observed at each pH.

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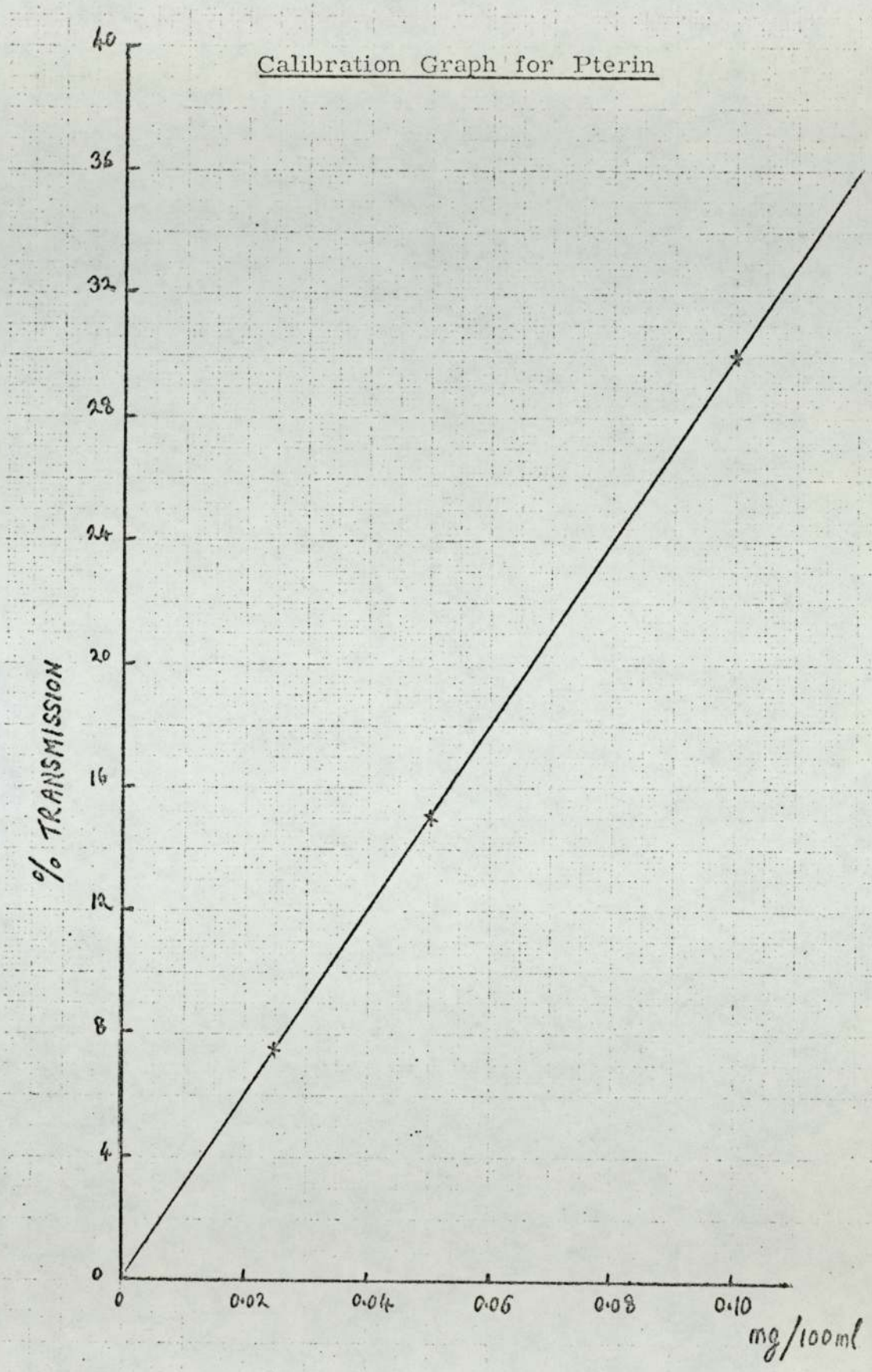
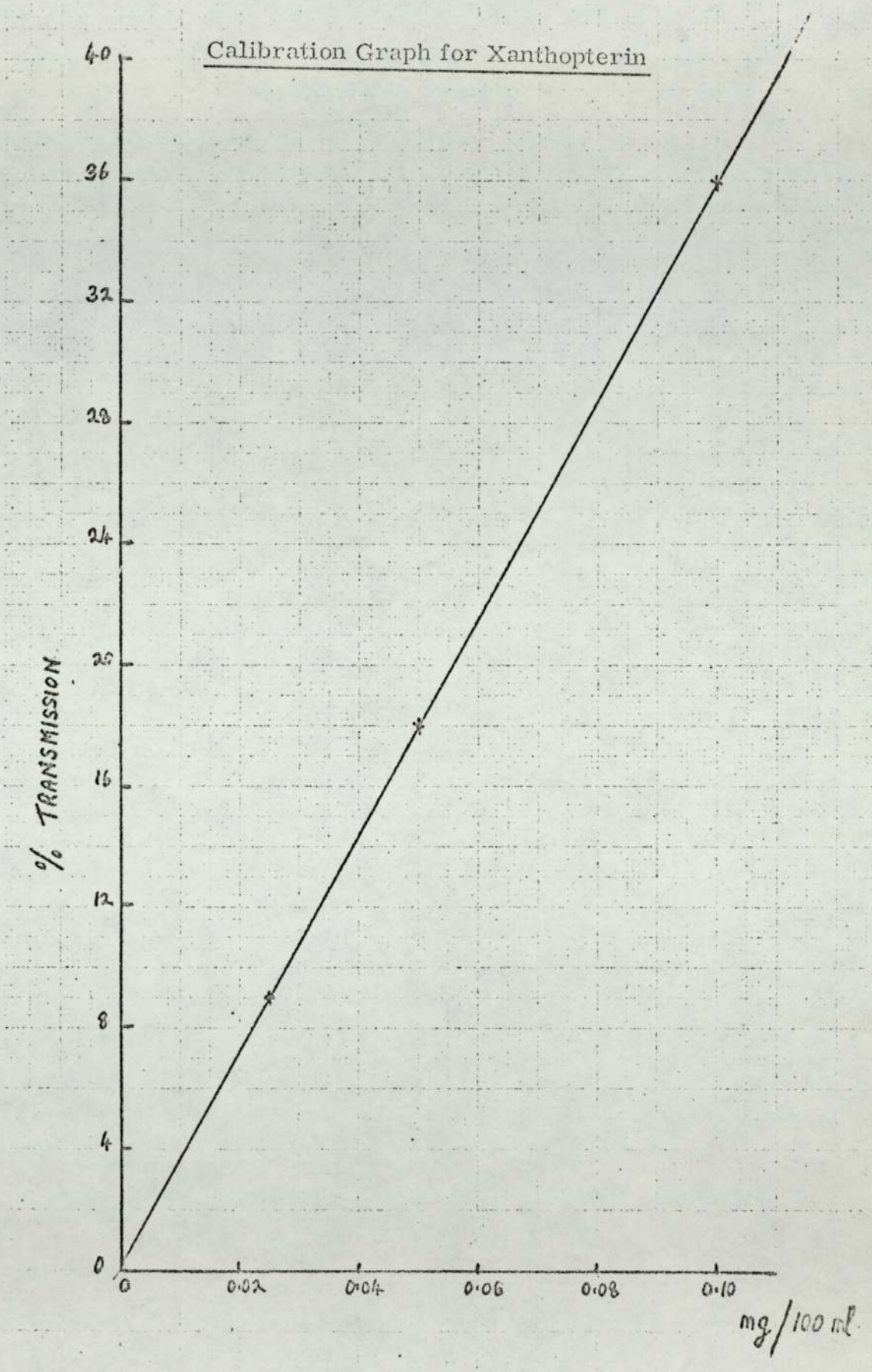
APPENDIX

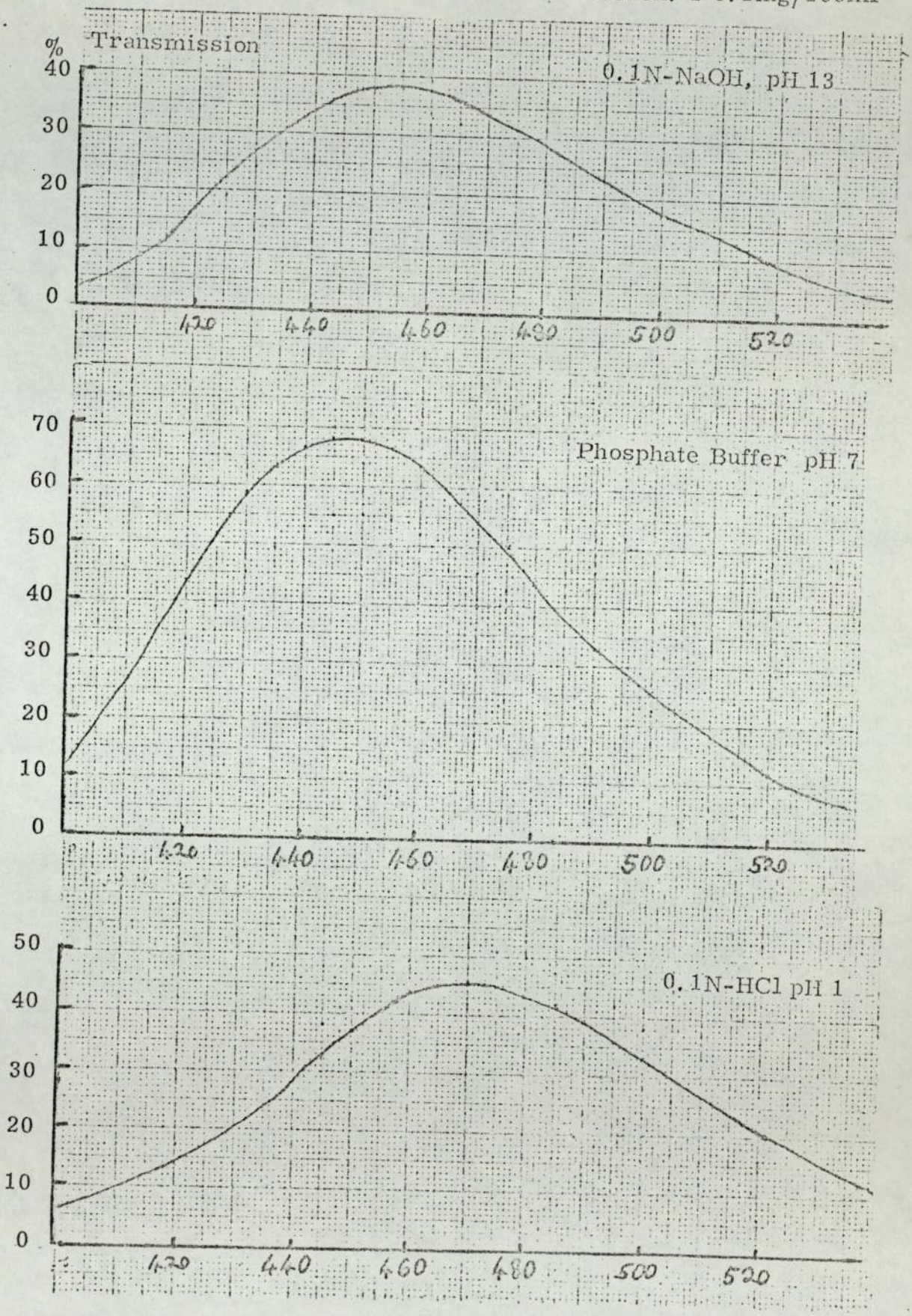
Index


Calibration Graph for Pterin
% TRANSMISSION
40
36
32
28
24
20
16
12
8
4
0
0
0·02
0·04
0·06
0·08
0·10
mg/100ml


Calibration Graph for Xanthopterin
% TRANSMISSION
40
36
32
28
24
20
16
12
8
4
0
0
0·02
0·04
0·06
0·08
0·10
mg/100 ml

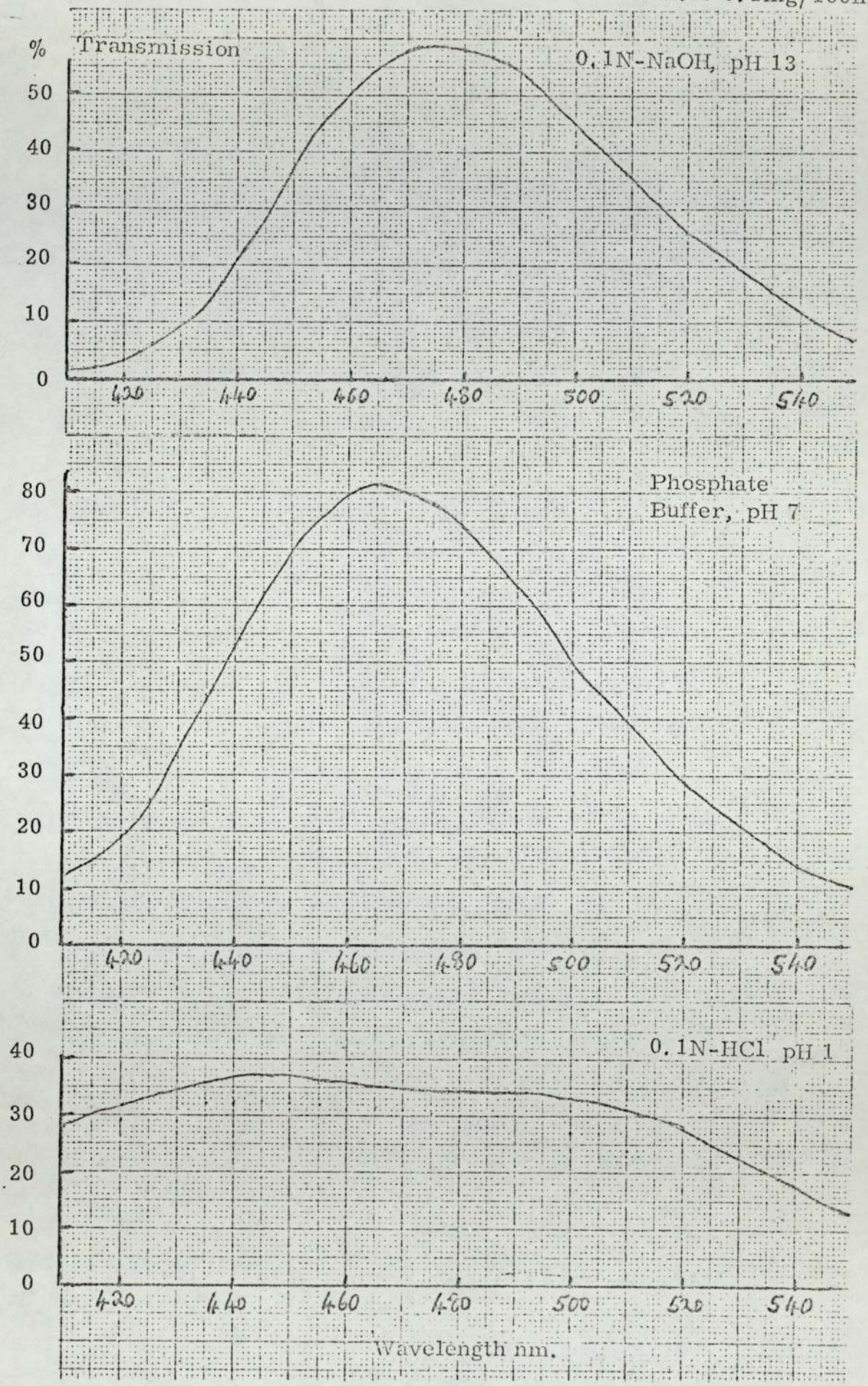
Emission Spectra of Pterin

concn.'s 0.1mg/100ml



Emission Spectra of Xanthopterin

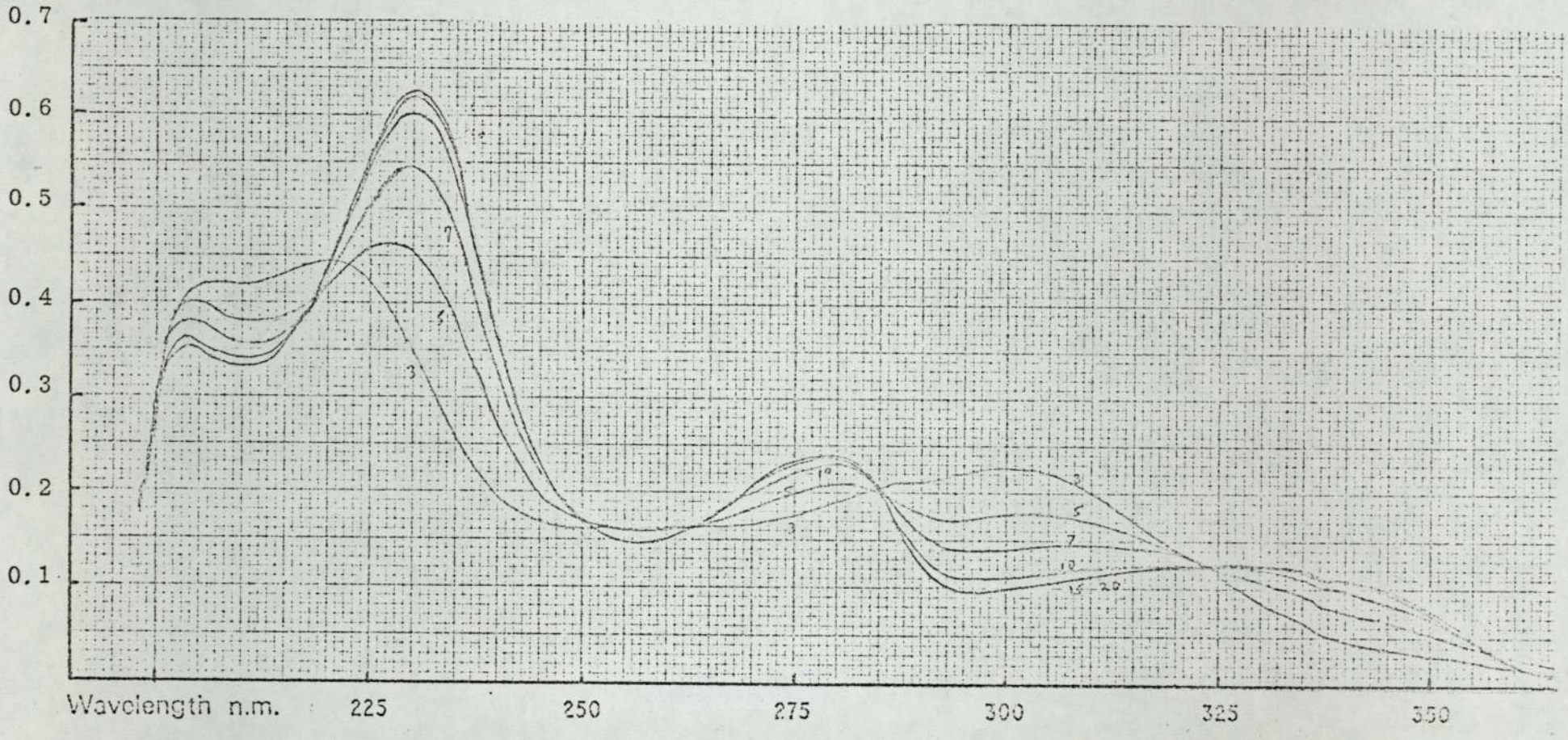
concn.'s 0.1mg/100ml

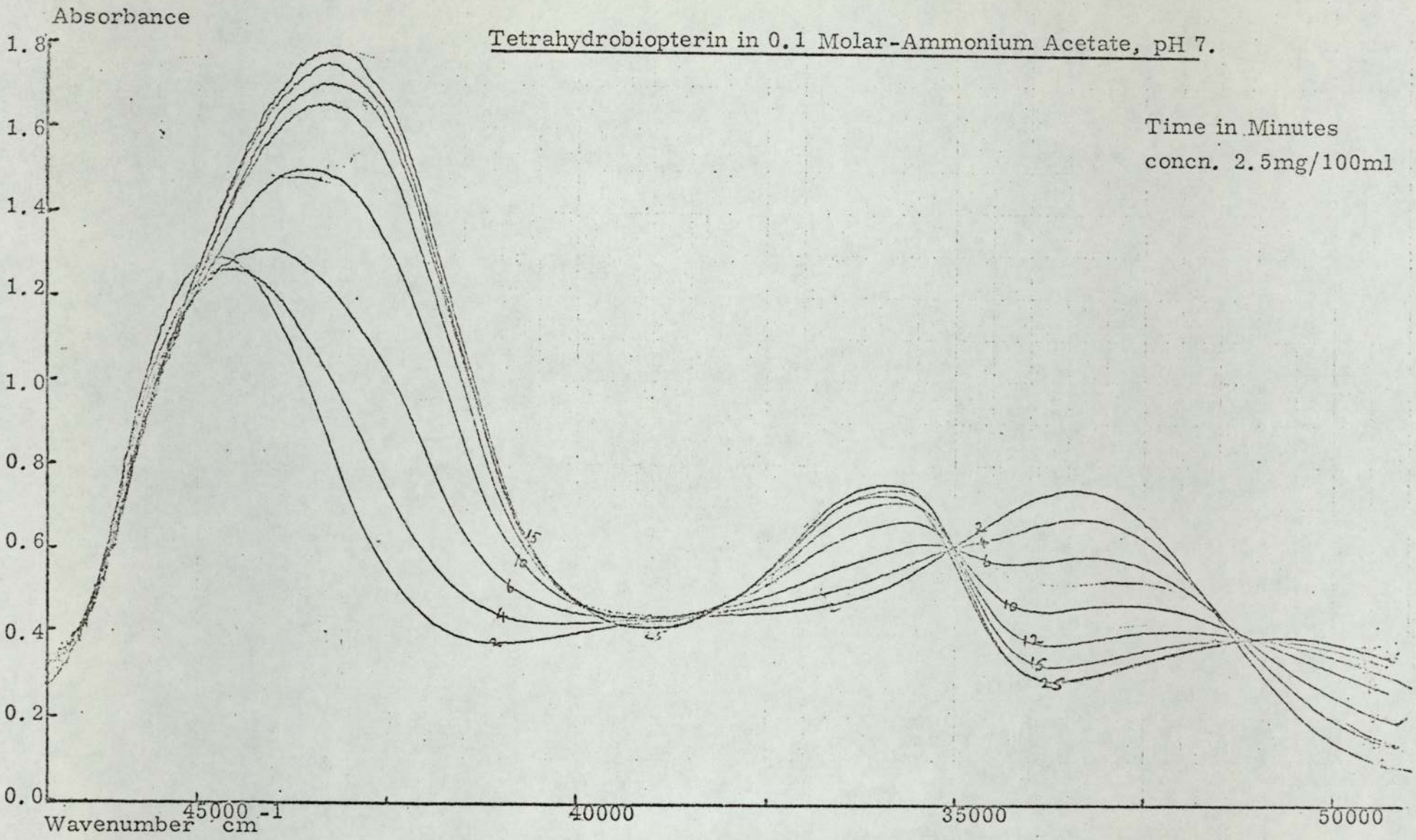


Tetrahydrobiopterin in Tris Buffer, pH 6.9

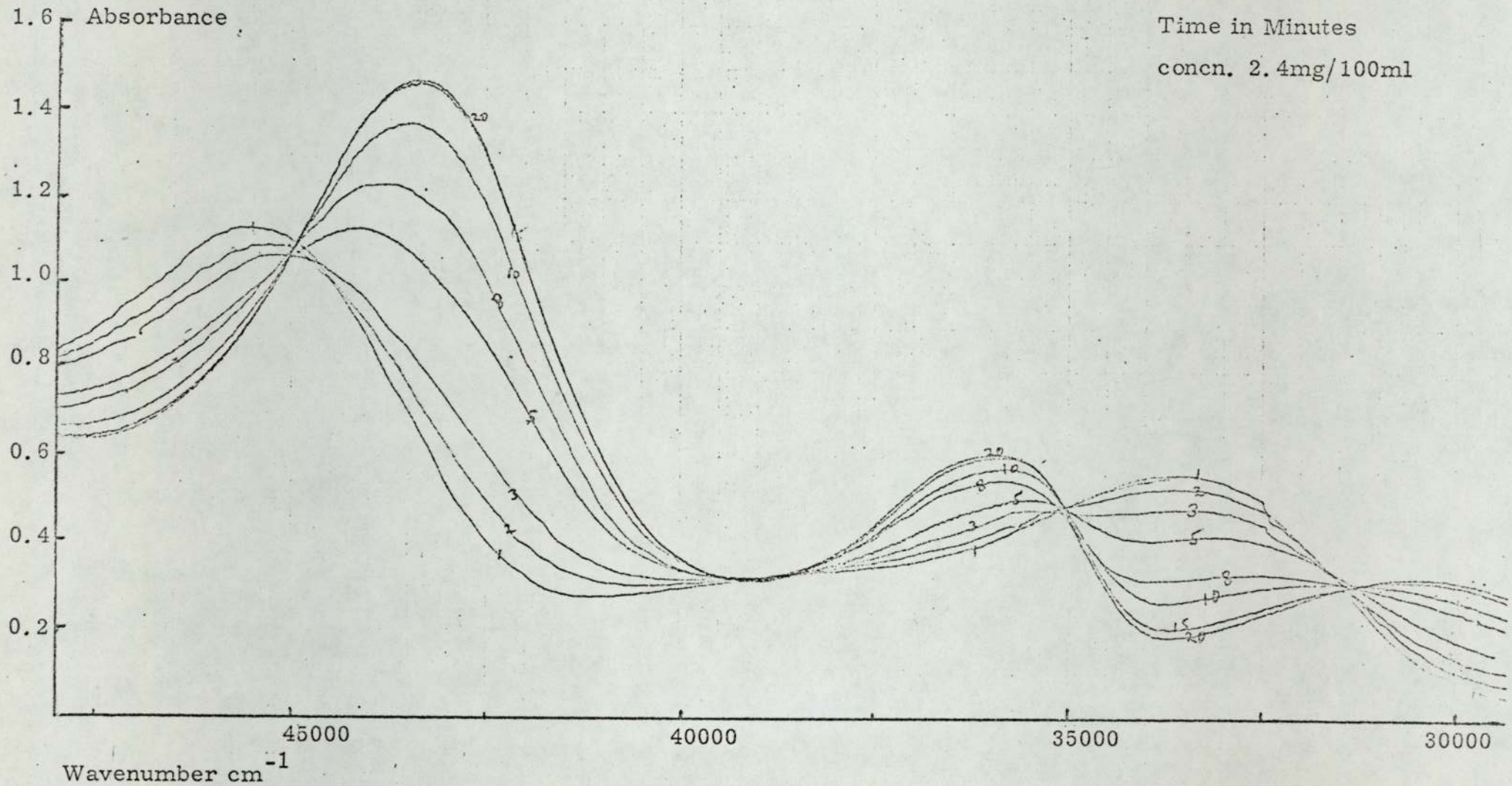
Time in Minutes

concn. 1 mg/100ml



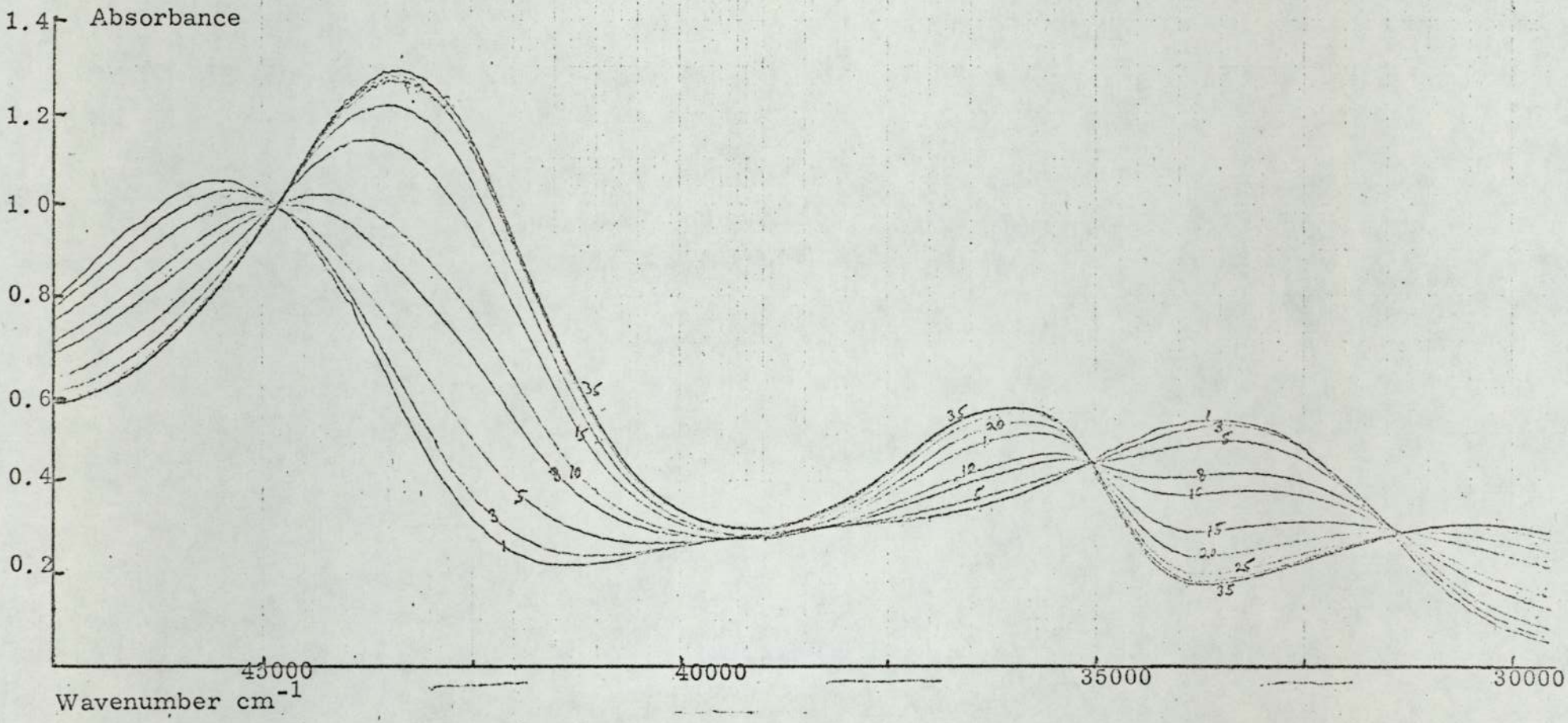

Absorbance
Tetrahydrobiopterin in 0.1 Molar-Ammonium Acetate, pH 7.
Time in Minutes
concn. 2.5mg/100ml
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
45000
40000
35000
50000
Wavenumber cm -1

Tetrahydrobiopterin in 0.1 Molar Phosphate Buffer, pH 7.



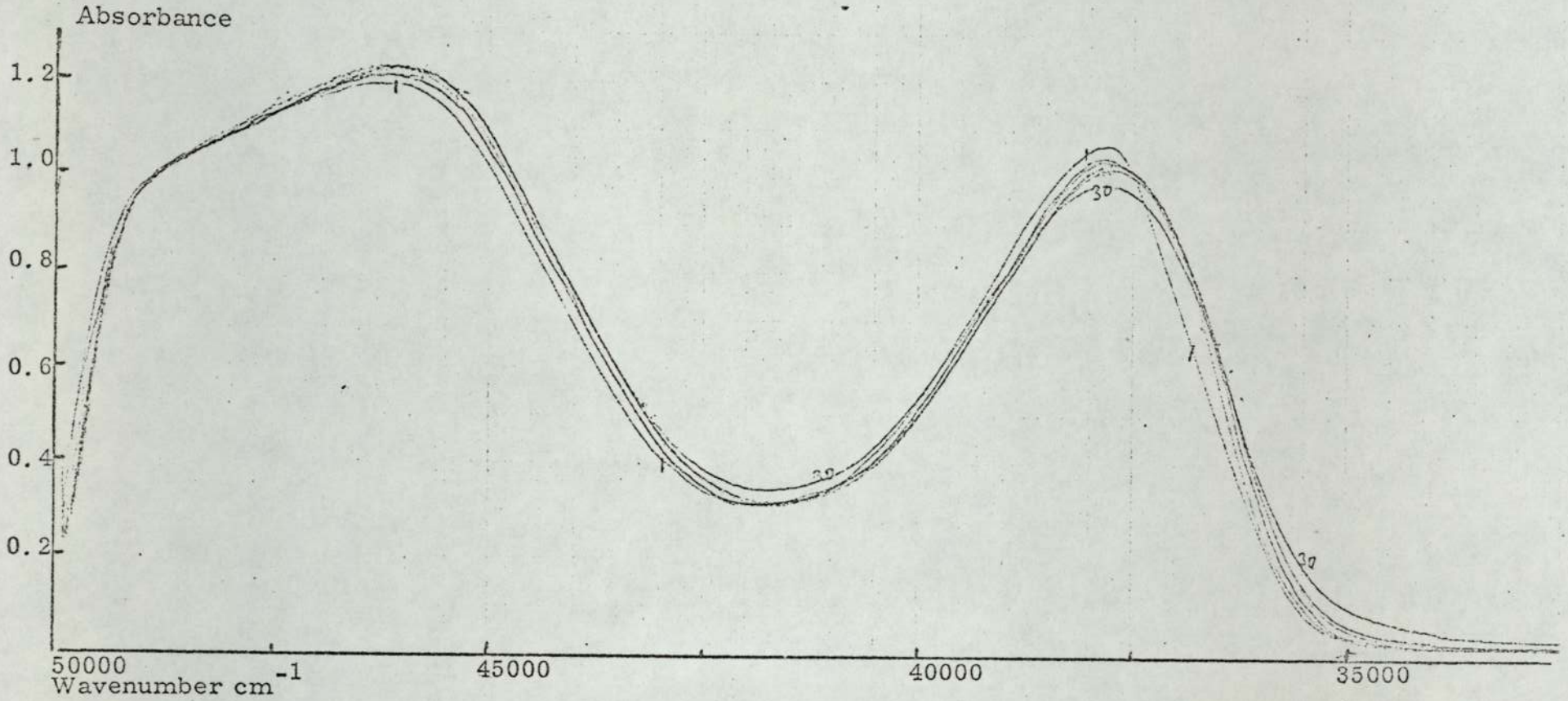
Tetrahydrobiopterin in Deaerated Phosphate Buffer, pH 7.

Time in Minutes
concn. 2.5mg/100ml



Tetrahydrobiopterin in 0.1N-HCl pH 1.

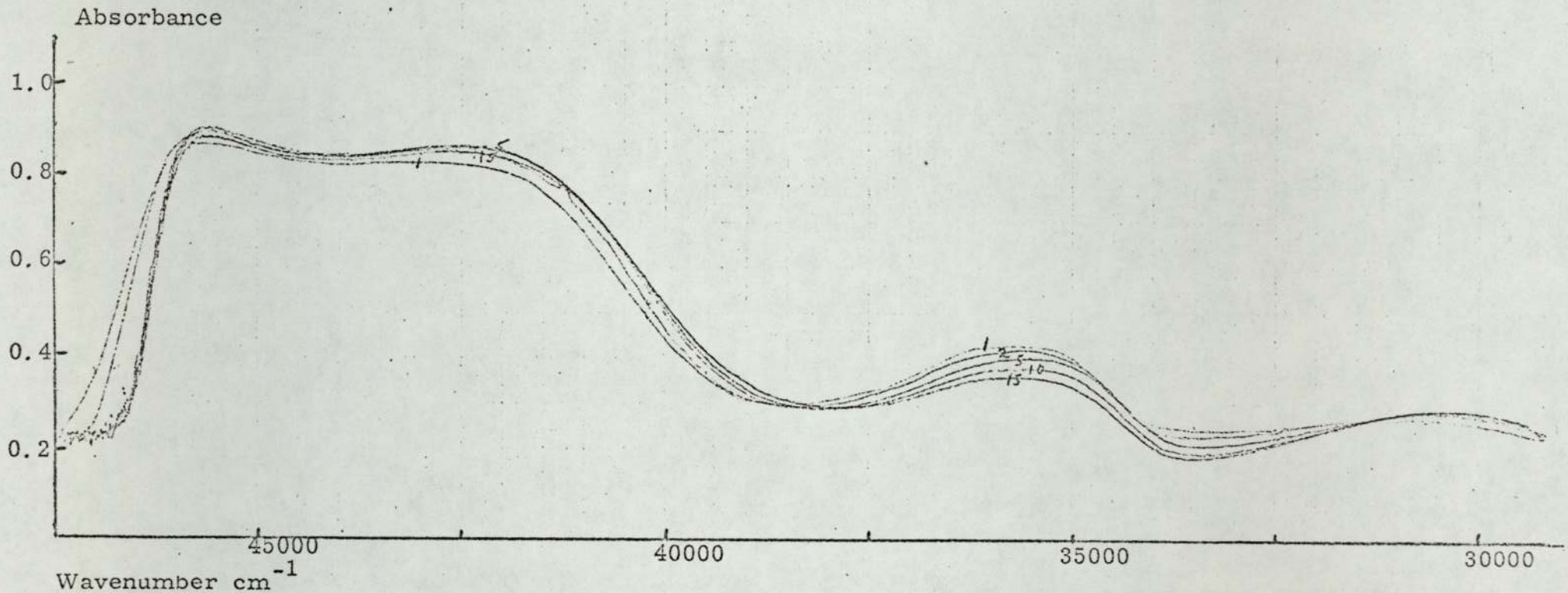
Time in Minutes.
concn. 2.7mg/100 ml

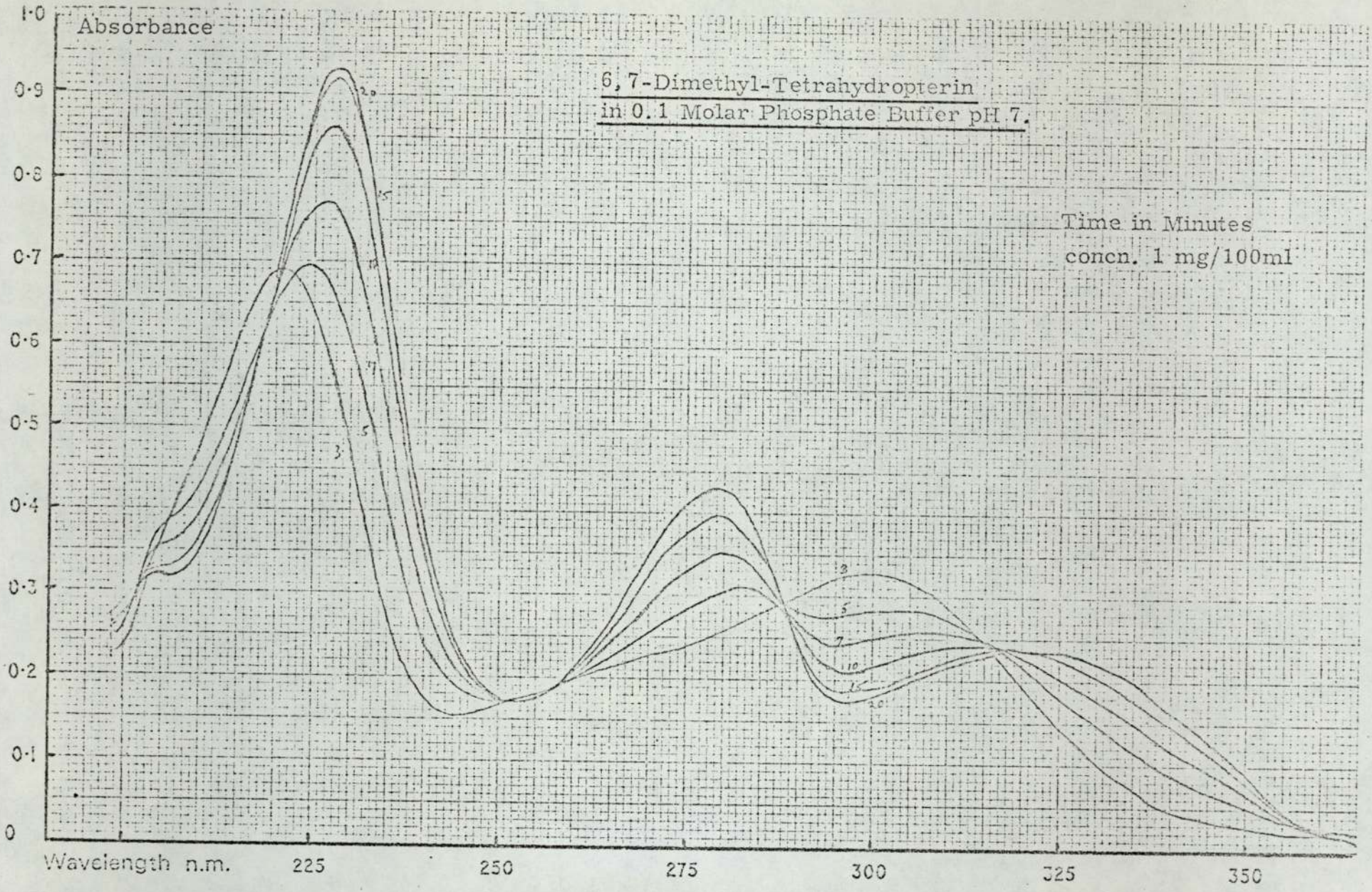


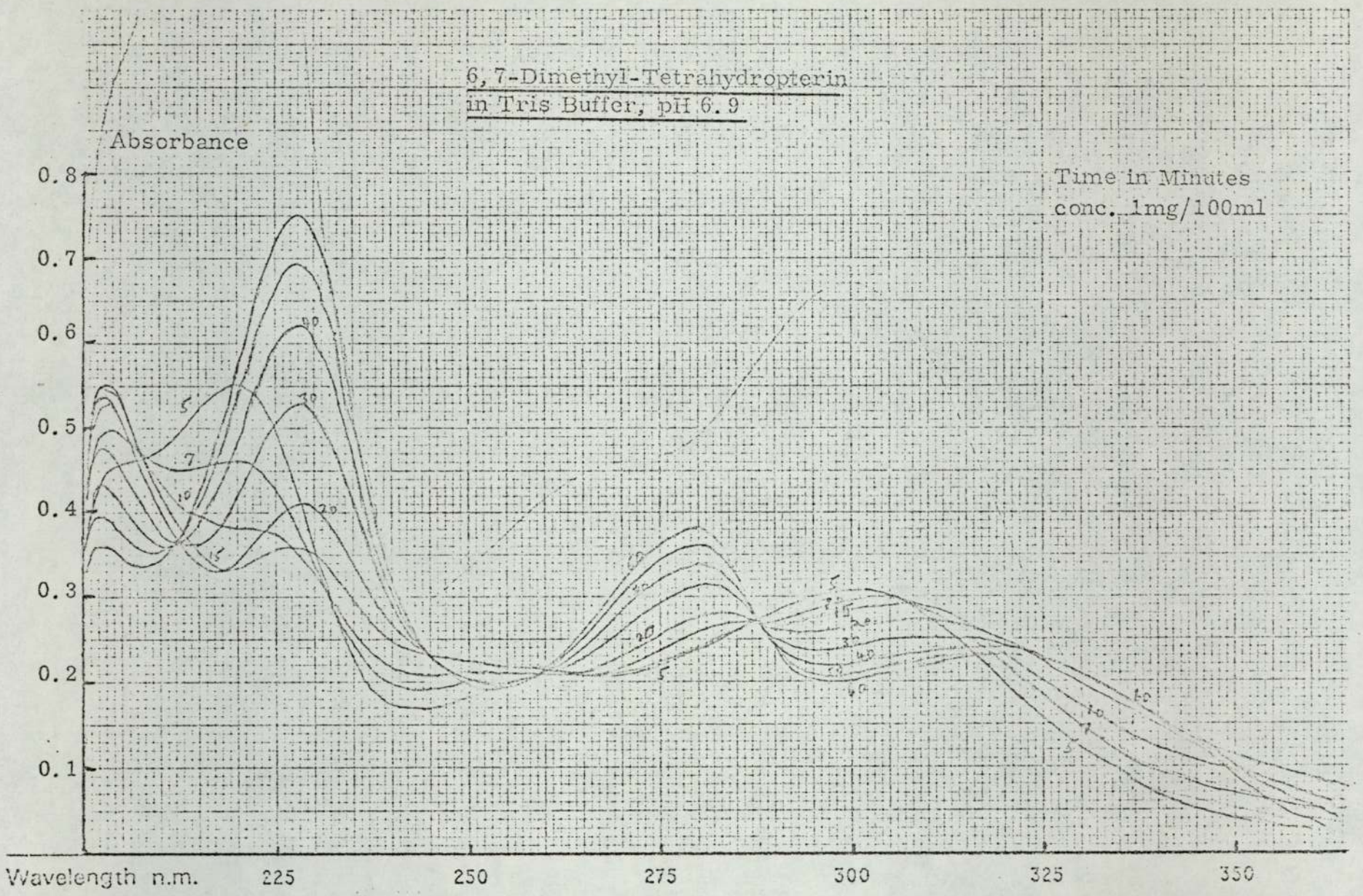
Tetrahydrobiopterin in 0.1N-NaOH pH 13.

Time in Minutes

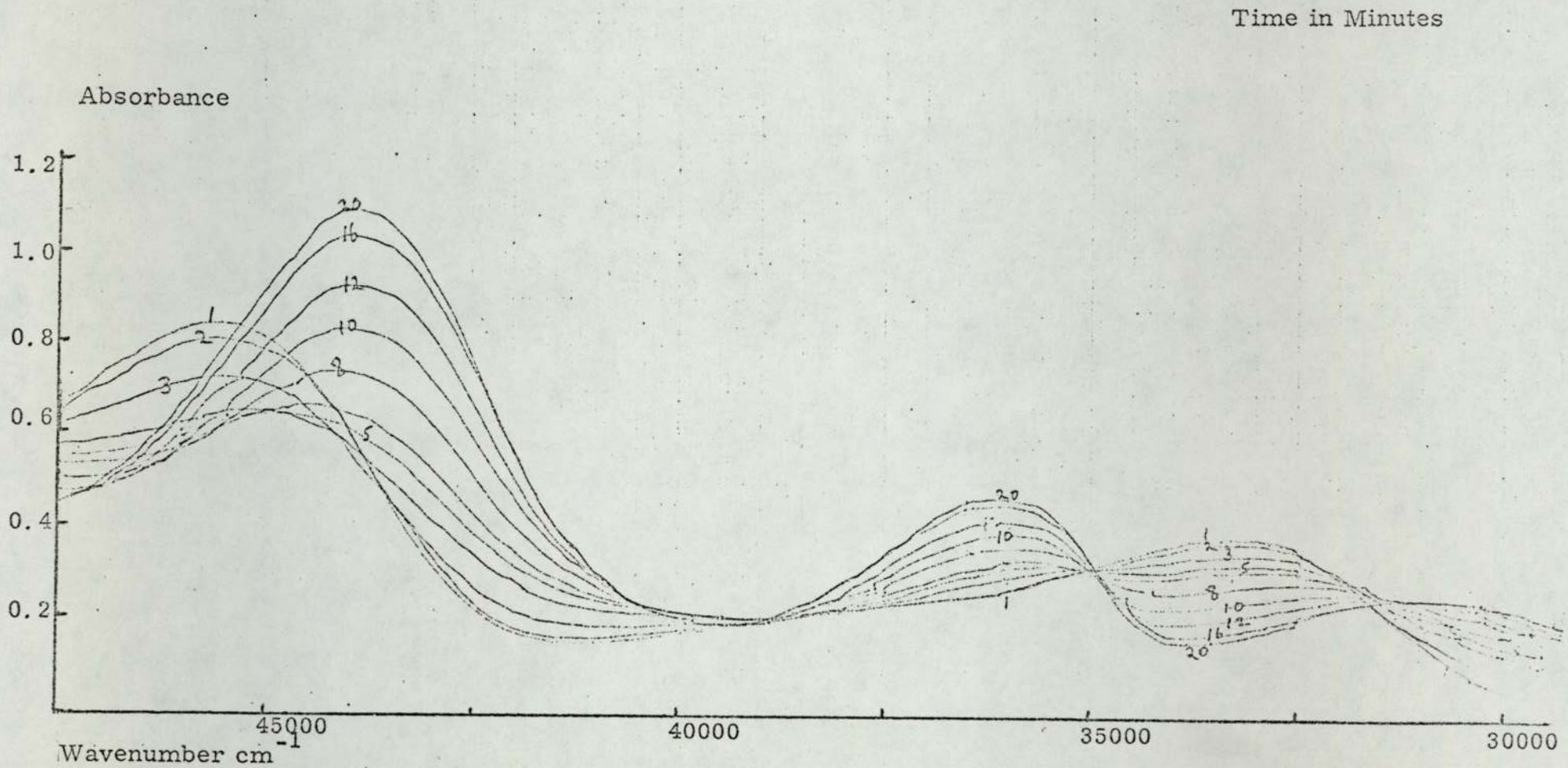
concn. 2.4mg/100ml




Absorbance
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
6, 7-Dimethyl-Tetrahydropterin
in 0.1 Molar Phosphate Buffer pH 7.
Time in Minutes
concn. 1 mg/100ml
Wavelength n.m.
225
250
275
300
325
350

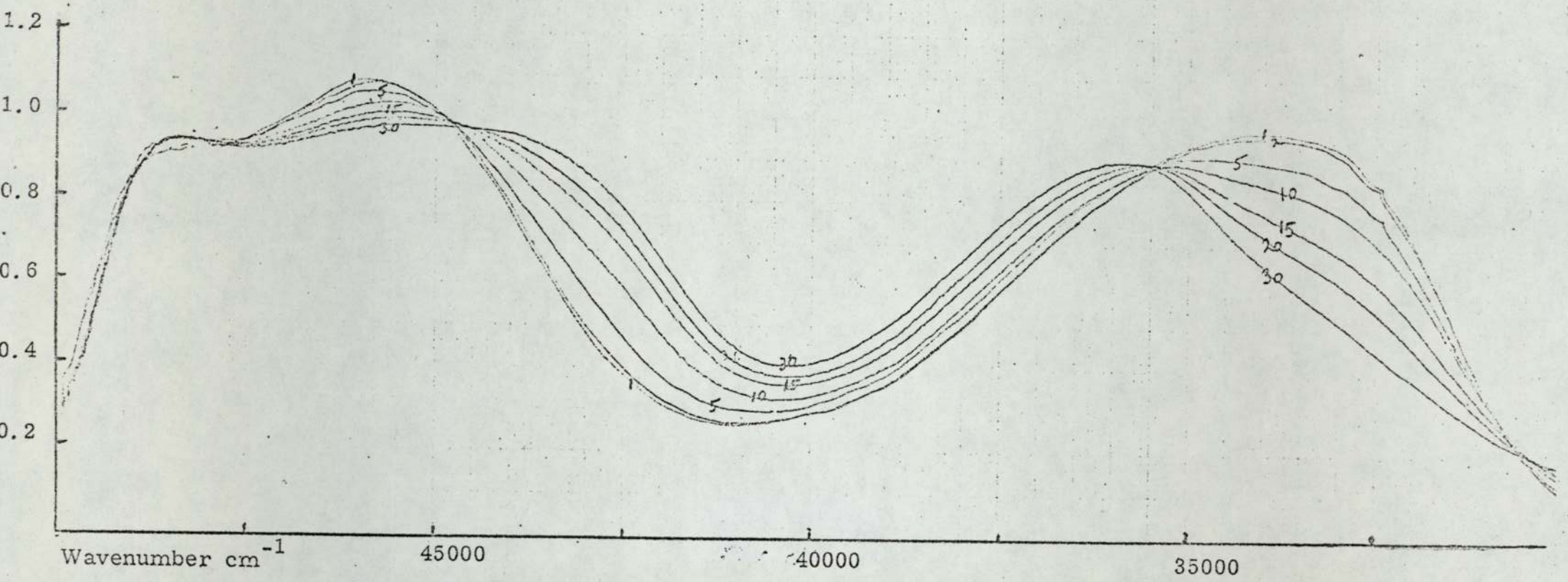

6, 7-Dimethyl-Tetrahydropterin
in Tris Buffer, pH 6.9
Absorbance
Time in Minutes
conc. 1mg/100ml
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Wavelength n.m.
225
250
275
300
325
350

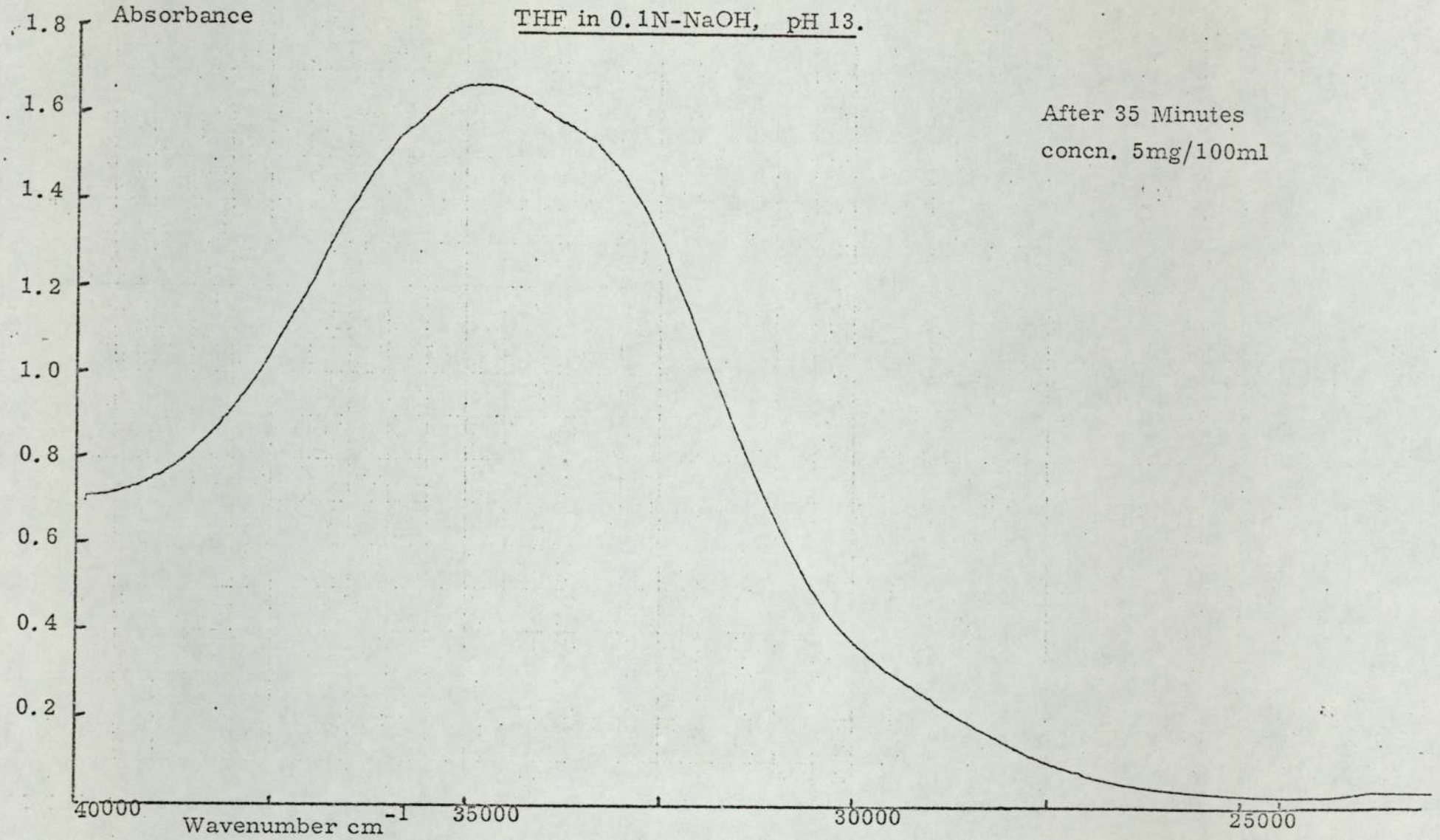
6-Methyl-Tetrahydropterin in 0.1 Molar Phosphate Buffer, pH 7.



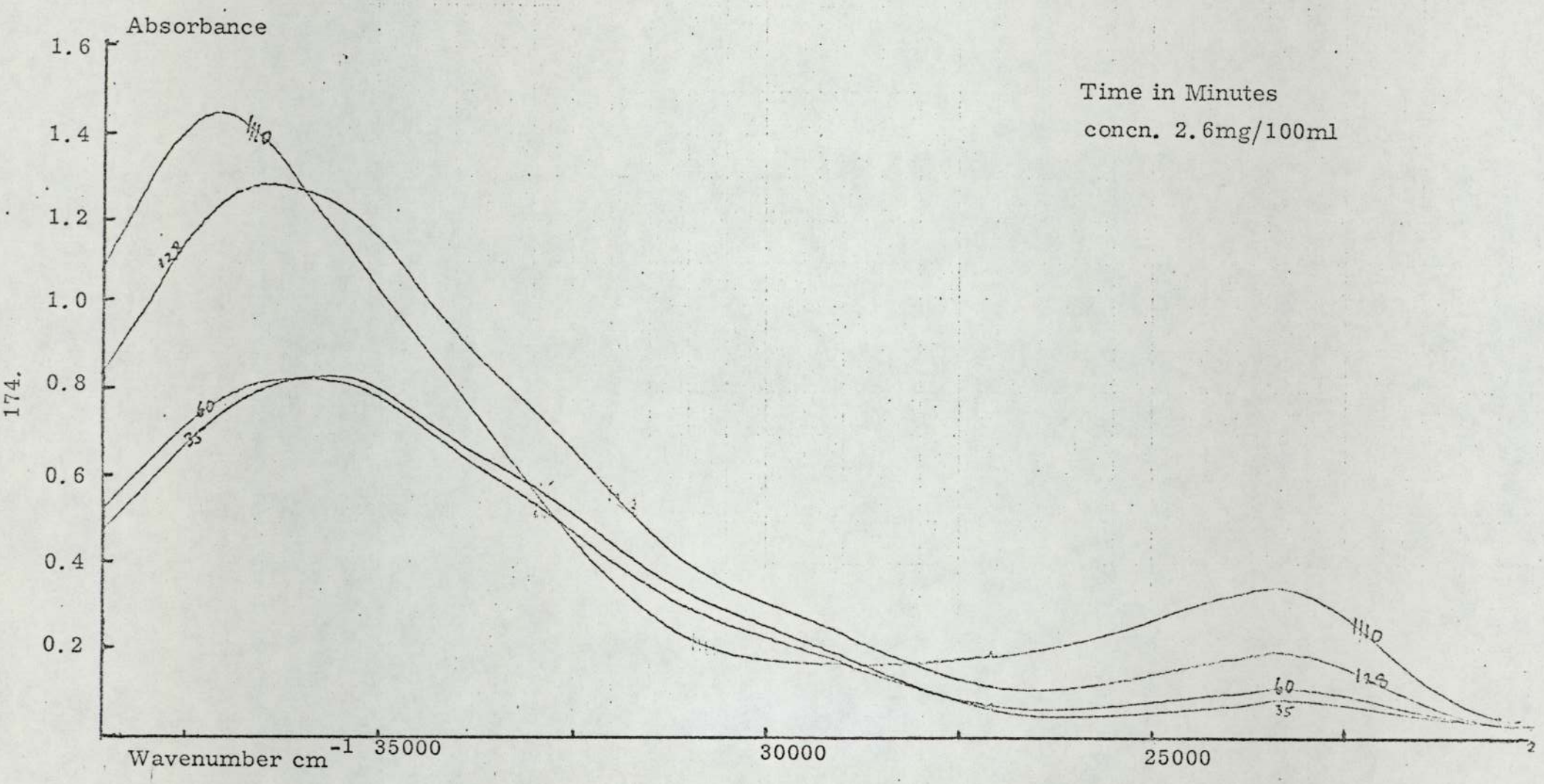
THF in 0.1 Molar Phosphate Buffer, pH 7

Time in Minutes
concn. 2.7mg/100ml

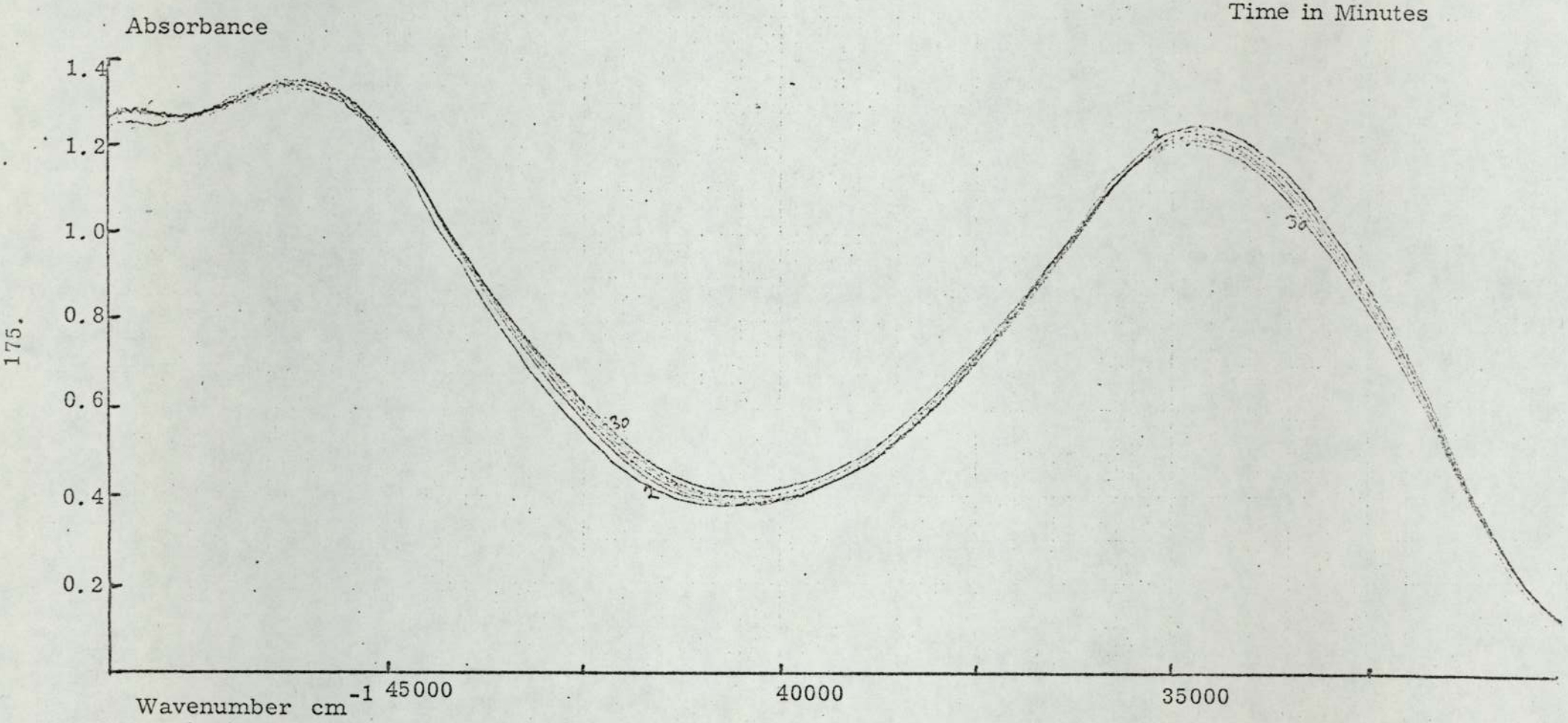



Absorbance
THF in 0.1N-NaOH, pH 13.
After 35 Minutes
concn. 5mg/100ml
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
40000
35000
30000
25000
Wavenumber cm-1

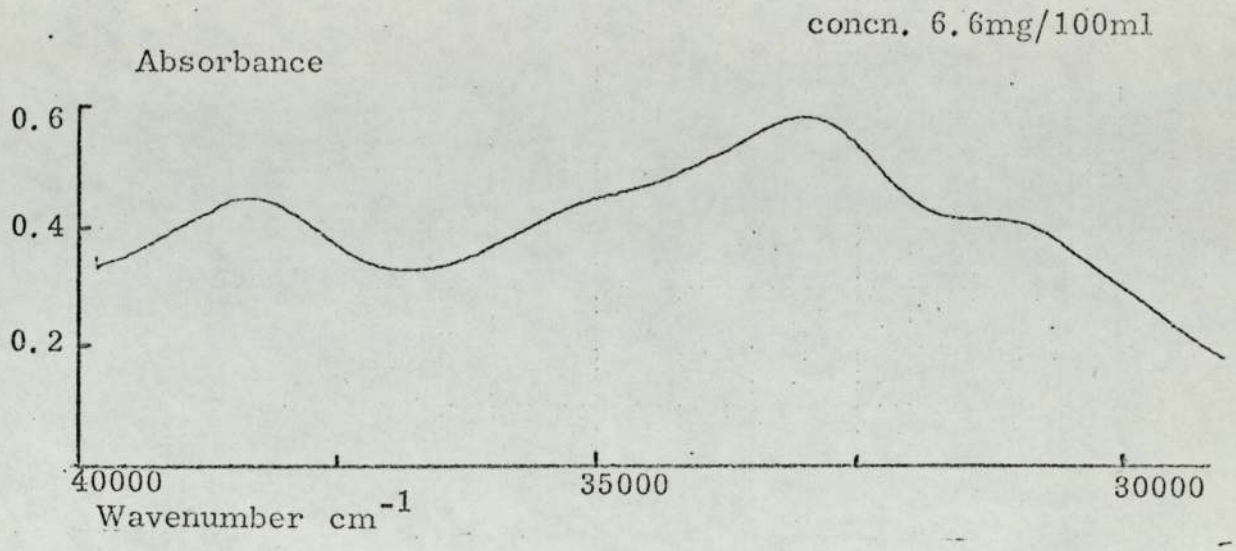
DHF in 0.1 Molar Phosphate Buffer, pH 7



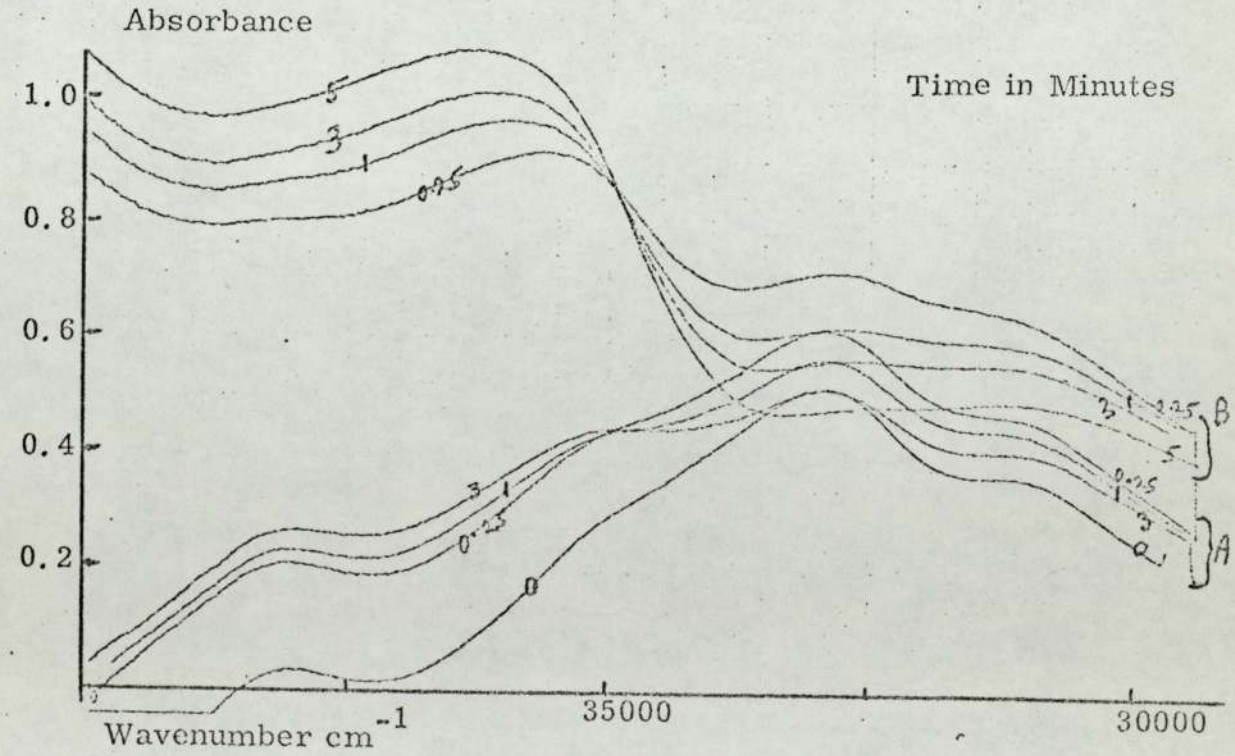
5-Methyl-THF in 0.1 Molar Phosphate Buffer, pH 7



$K_3Fe(CN)_6$ in 0.1 Molar Phosphate Buffer, pH 7



Addition of Tetrahydrobiopterin to a solution of $K_3Fe(CN)_6$ in 0.1 Molar Phosphate Buffer, pH 7.

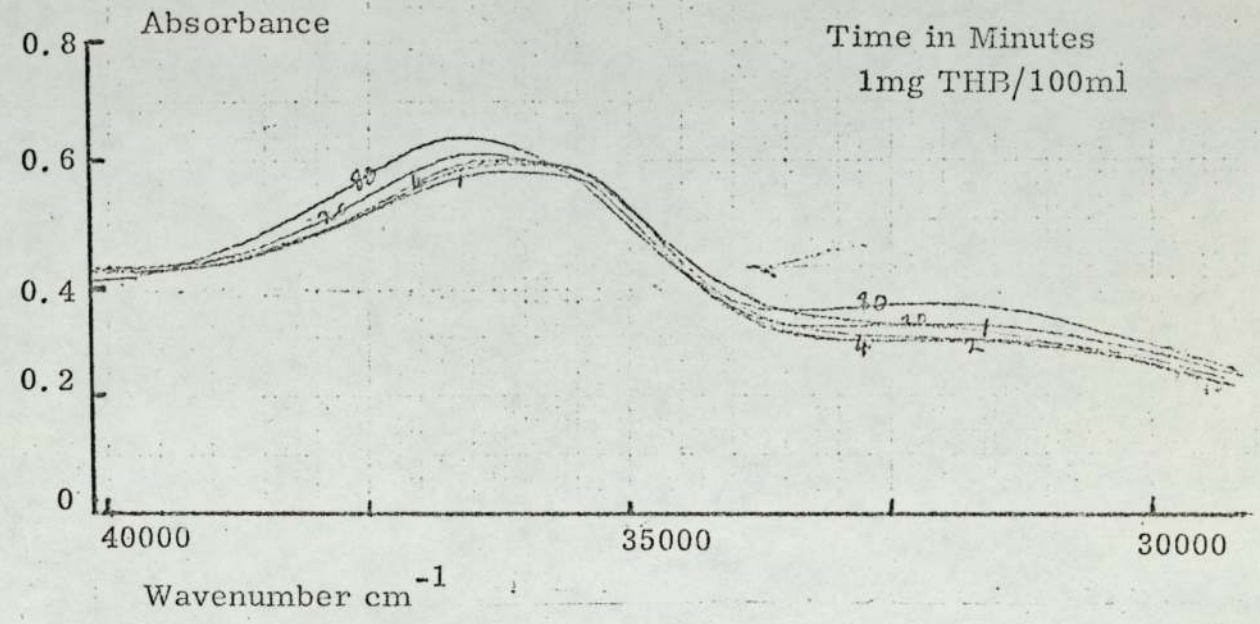


A First addition

B Further addition

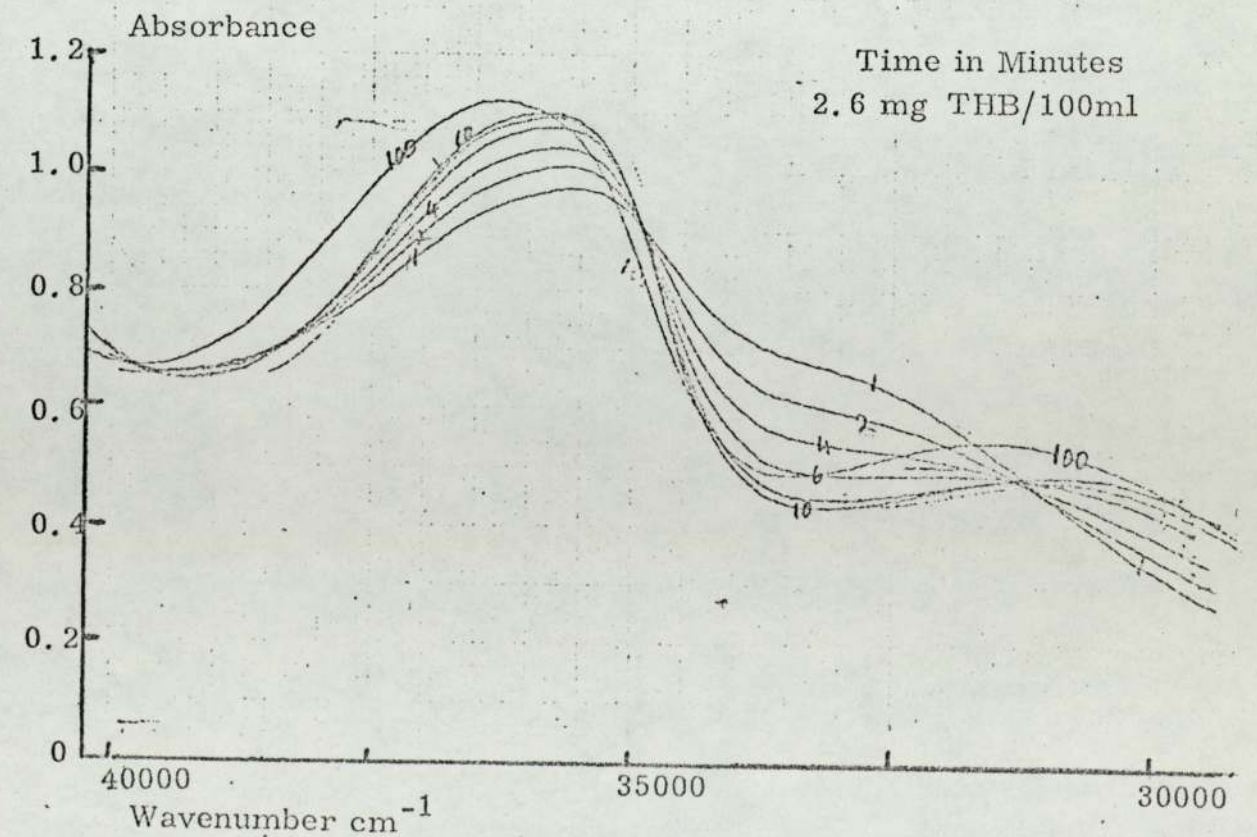
Tetrahydrobiopterin plus 2,6-Dichlorophenolindophenol

1 : 1 Molar Ratio in Phosphate Buffer pH 7



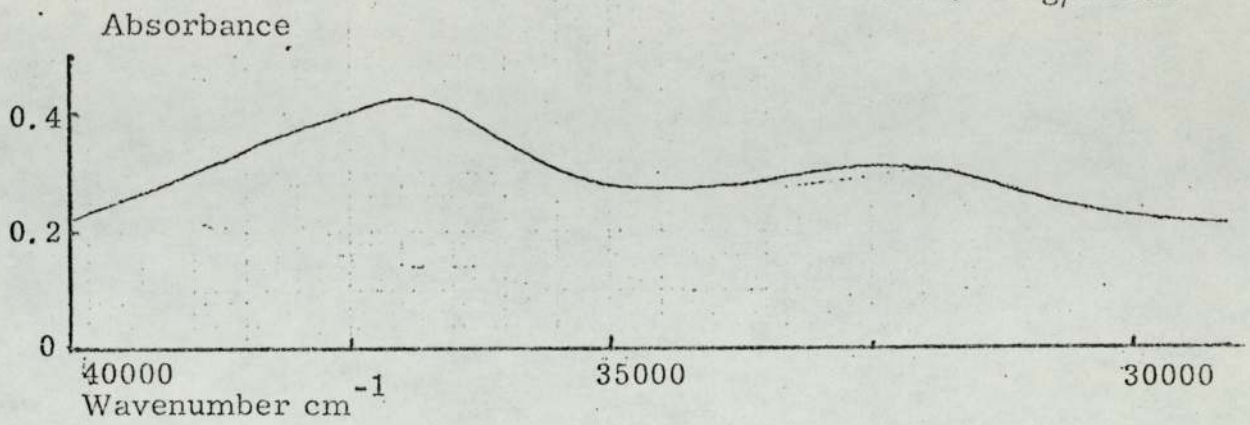
Tetrahydrobiopterin plus 2,6-Dichlorophenolindophenol

2 : 1 Molar Ratio in Phosphate Buffer pH 7

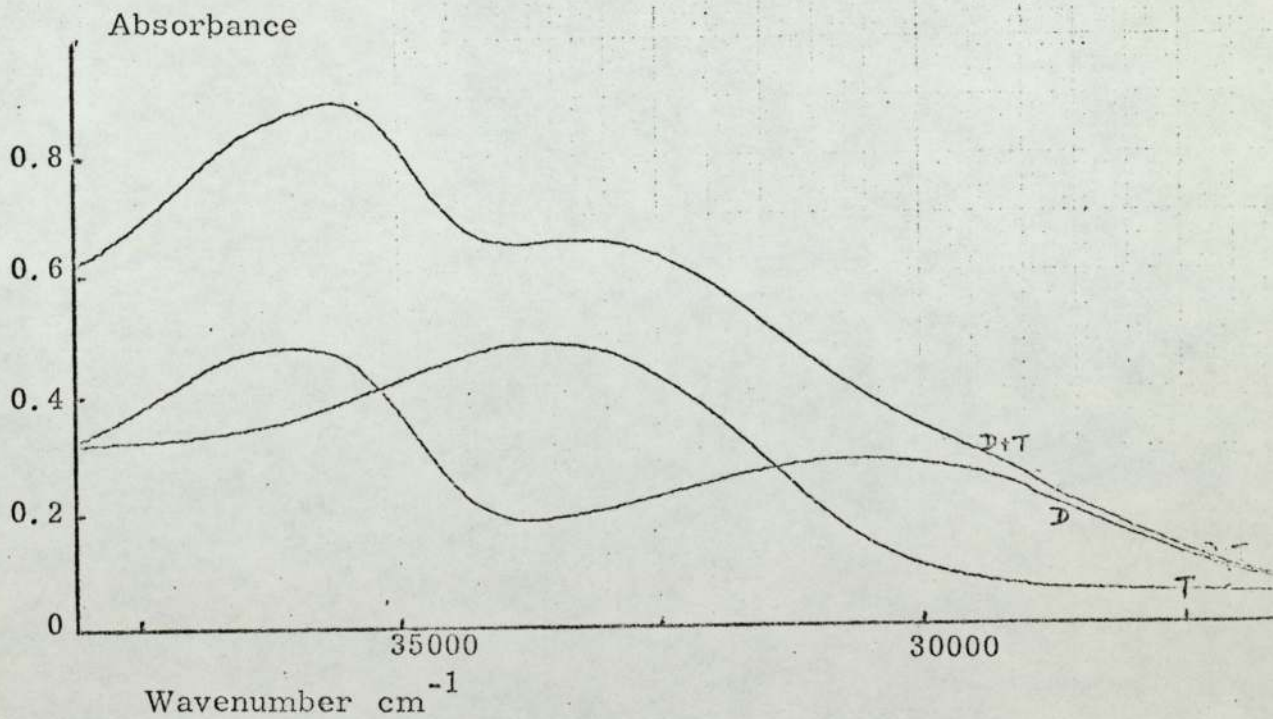


2, 6-Dichlorophenolindophenol in Phosphate Buffer, pH 7

concn. 1mg/100ml



Tetrahydro and Dihydrobiopterin
1 : 1 Molar Ratio in Phosphate Buffer, pH 7

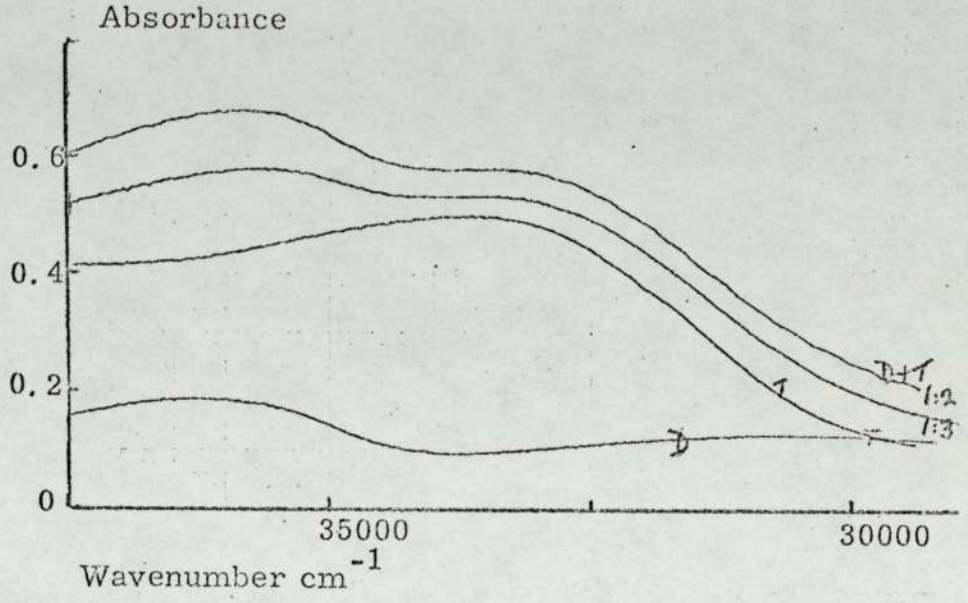


T is Tetrahydrobiopterin concn. 1.5 mg/100 ml.

D is Dihydrobiopterin concn. 1.5 mg/100 ml.

D + T is the syperimposed spectrum

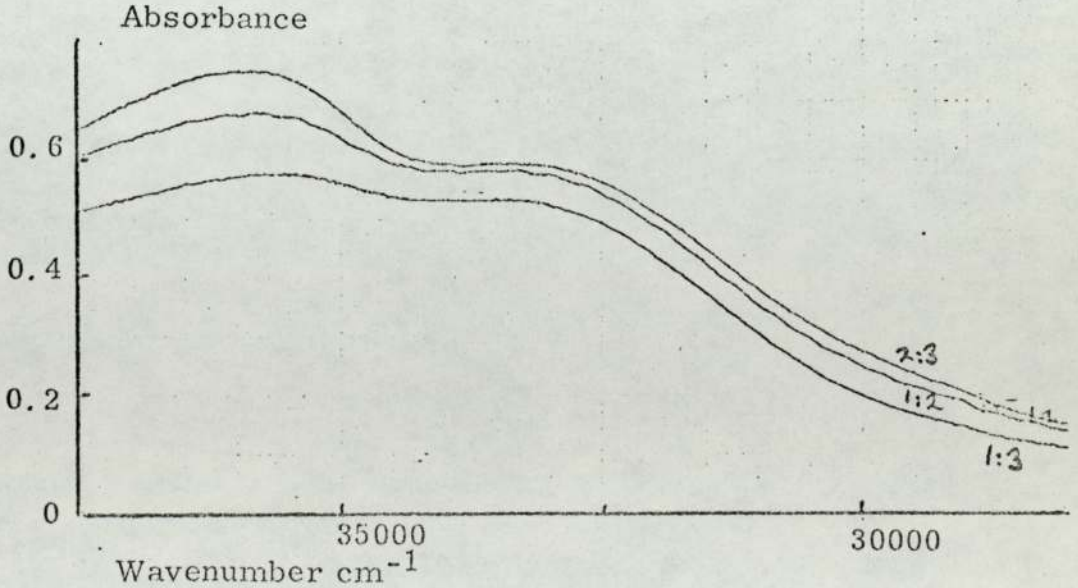
Dihydro and Tetrahydrobiopterin in Phosphate Buffer, pH 7.



T is Tetrahydrobiopterin concn. 1.5 mg/100ml.
D is Dihydrobiopterin concn. ca 0.5 mg/100ml.
Superimposed Spectra in Molar Ratios of Dihydro:Tetrahydro -biopterin

---

Dihydro and Tetrahydrobiopterin in Phosphate Buffer, pH 7.



Superimposed Spectra in Molar Ratios of Dihydro: Tetrahydro -biopterin