Oxidation Mechanisms of Tetrahydrofolic Acid, Tetrahydrobiopterin and Related Compounds.

by

Anthony John Pearson

A Thesis presented for the Degree of

Doctor of Philosophy

in

The University of Aston in Birmingham.

July, 1974.

SUMMARY.

The autoxidation of tetrahydrofolic acid and tetrahydrobiopterin, 6-substituted derivatives of 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine (tetrahydropterin), has been investigated by product analysis and kinetic methods using gas manometric and spectrophotometric techniques. The overall reaction is first order in tetrahydropterin and shows a combination of zero- and first order dependence on oxygen, the relative contributions of which are affected by the exclusion of visible light. There is a linear dependence of rate on percentage ionisation of the 3,4-amide group and the reaction is retarded by protonation of N(5), suggesting that oxygen attack occurs at C(4a). Use of appropriately deuteriated compounds leads to no kinetic isotope effects, showing that 6- and 7- CH bond cleavages are not rate-determining. Kinetic data suggest a free radical chain reaction in which the chain carrier is the hydroperoxyl radical (HO_2.) and that intermediate hydroperoxides are not involved. Quinonoid dihydropterins are obligatory intermediates, their rearrangement leading to the observed products.

Oxygen-18 labelling experiments show that the autoxidative ring contraction of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione does not provide evidence for the intermediacy of 8a-hydroperoxides as suggested in the literature.

The mechanism is rationalised on the basis of simple Huckel molecular orbital calculations.

Solutions of autoxidising tetrahydrobiopterin in the presence of ferrous ion are shown to induce non-specific hydroxylation of phenylalanine, and a radiochemical product analysis method is described. Effects of catalase indicate the partial involvement of hydrogen peroxide, generated during tetrahydrobiopterin autoxidation. Use of superoxide dismutase indicates that the hydroperoxyl radical is not active in the system, but N.I.H. shift determinations suggest a free radical species. The complexity of the reaction is recognised, and the involvement of iron-oxygen complexes and hydroxyl radicals (OH.) is considered.

ACKNOWLEDGEMENTS.

The following people are gratefully acknowledged.

Dr. J. A. Blair for his supervision, encouragement and helpful discussion.

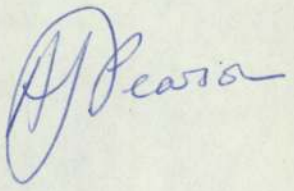
Mr. E. Hartland for n.m.r. spectra, Mr. M. Houghton for mass spectra and Mrs. V. Clenton for i.r. spectra.

Dr. R. F. Long (Roche Products Ltd., Welwyn Garden City) for gifts of tetrahydrobiopterin and 6,7-dimethyl tetrahydropterin, Dr. H. I. X. Mager (Delft University of Technology, the Netherlands) for 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione, and Dr. A. M. Stokes (University of Oxford) for superoxide dismutase.

Mrs. P. M. Johnson, who typed the manuscript.

Financial support was provided by means of University of Aston and S.R.C. studenships.

The work described was carried out between 1971 and 1974 at the University of Aston in Birmingham. It has been done independently and has been submitted for no other degree.



A. J. Pearson.

CONTENTS.

continued ...

continued ...

continued ...

APPENDICES

FIGURES.

continued ....

FIGURES (continued)

TABLES

continued .....

TABLES (continued) Page

continued ....

TABLES (continued) Page

SCHEMES.

NOTE: Schemes showing mechanisms derived from the present work only are listed. Other schemes occur in appropriate sections.

CHAPTER 1.

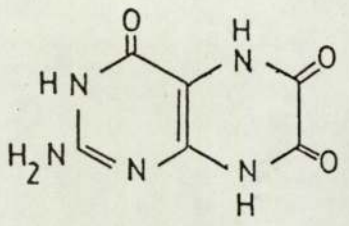
Introduction.

Structure, Nomenclature, and Abbreviations.

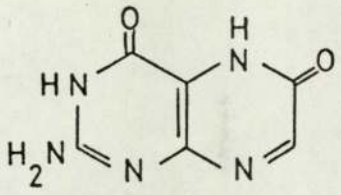
The pteridines are a class of poly-aza naphthalenes originally isolated from butterfly wings at the end of the nineteenth century by Sir Frederick Gowland Hopkins. The ring system constituting these compounds is extremely widespread in nature, and is common to a wide variety of biologically important molecules. The structures of leucopterin, 1(i), and xanthopterin, 1(ii), the first pteridines to be isolated, proved very difficult to determine and it was not until 1940 that they were elucidated by Purrman[1,2].

The compounds considered in the following pages are derivatives of pyrimido [4,5-b] -pyrazine, usually called "pteridine" (a term introduced by Wieland[3]). The structure and commonly used numbering of the ring system is shown in 1 (iii). The numbering used by some continental workers, 1 (iv), is less common and rather cumbersome to apply, it being an attempt to relate pteridine to purine, 1 (v).

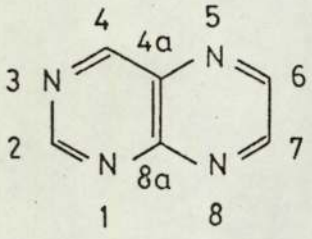
Perhaps the most abundant pteridines are those known collectively as pterins, a name arising from the first sources (Gk. pteron wing), and these are all derivatives of 2-amino-4-hydroxypteridine, 1 (vi). The continued use of the term pterin is justified, since it serves as a useful abbreviation in naming many of the naturally occurring pteridines. It will be observed that the structure shown in 1 (vi) appears to be a



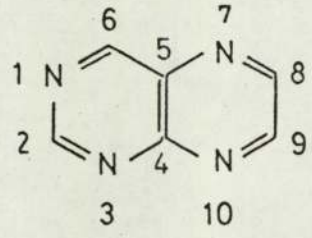
1(i) Leucopterin



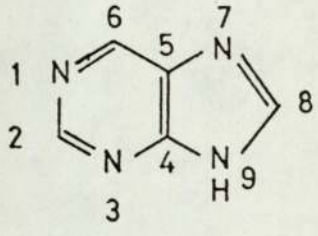
1(ii) Xanthopterin



1(iii) Pteridine numbering

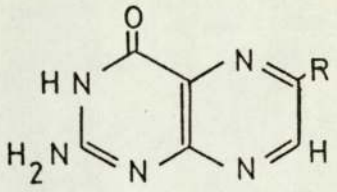


1(iv) Less common numbering

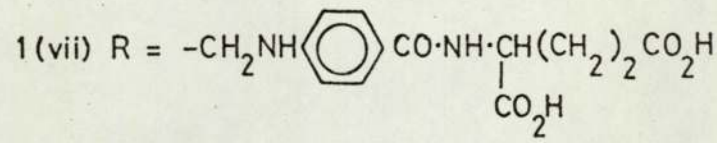


1(v) Purine numbering

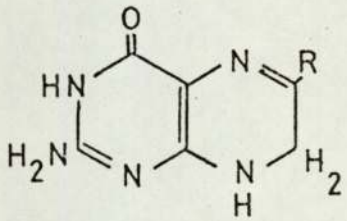
misrepresentation of 4-hydroxypteridine, since it is written rather as a 4-oxo tautomer. However, both spectroscopic and theoretical evidence[4-14] indicate that the tautomeric equilibrium favours the latter, obvious exceptions being O-methylated derivatives. For the same reasons, 6- and 7- hydroxy pterins are written as 4,6- and 4,7- dioxopteridines, respectively. Folic acid, an essential compound in mammalian biochemistry, has the structure shown in 1 (vii). Here we have an N-methyl-p-aminobenzoyl-glutamic acid residue attached to the pteridine ring, and this may be named N-[4-{[(2-amino-4-hydroxy-6-pteridinyl)methyl]amino}benzoyl] glutamic acid. The name 'pteroylglutamic acid' was proposed by the group who originally elucidated the structure[15], though the even simpler name 'folic acid', proposed by Mitchell et al.[16], is now accepted as standard practice, and all related structures are referred to as folic acid derivatives. The reduced compounds, 7,8-dihydrofolic acid,1 (viii), and 5,6,7,8-tetrahydrofolic acid, 1 (ix), are the ones participating in biochemical processes, and although the standard practice[17] is to abbreviate them to DHF and THF, respectively, this might cause confusion between tetrahydrofolic acid and tetrahydrofuran (also referred to as THF), so in the present thesis the abbreviations DHFA and THFA will be employed.



1(vi) R = H

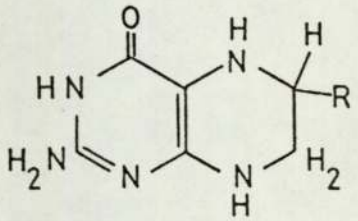
1(vii) R = 

1(x) R = $-CH(OH)\cdot CH(OH)\cdot CH_3$



1(viii) R as in (vii)

1(xi) R as in (x)



1(ix) R as in (vii)

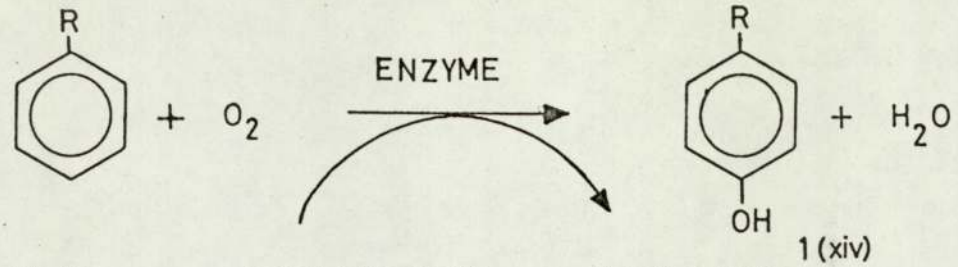
1(xii) R as in (x)

Natural Occurrence and Biological Importance of Pterins.

The occurrence of pterins as pigments in the eyes and wings of insects, and the skin of reptiles, and also their possible role in melanin synthesis in fish and amphibia have been extensively reviewed by Blakley[18], and will not be dealt with any further here, since these aspects have little direct bearing on the contents of the present study. However, the importance of the compounds as metabolic cofactors is relevant to the work undertaken, and these aspects will now be briefly discussed.

In the mid-1950's Patterson *et al.*[19] isolated from human urine a new pteridine required for the growth of the micro-organism Crithidia Fasciculata. A year later a description of its separation and purification was described by the same group[20] and the compound, biopterin, was shown by spectroscopic methods, elemental analysis, and oxidative degradation to be 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine, 1 (x). This was subsequently confirmed by synthesis[21].

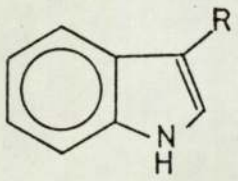
Following this discovery, Kaufman and his colleagues[22-25] demonstrated that a compound with properties of the pteridines was an essential cofactor in the enzymatic hydroxylation of phenylalanine, 1 (xiii), to tyrosine in rat liver slices. It was demonstrated that 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridines carrying methyl substituents at C(6) or C(6) and C(7) could replace the natural cofactor, whereas the 2-hydroxy-4-amino



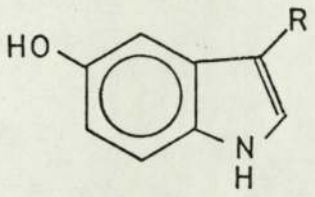
1(xiii) tetrahydropterin dihydropterin 1(xiv)

R = $-CH_2CH(NH_2)CO_2H$

SCHEME 1.1 Enzymatic hydroxylation of phenylalanine

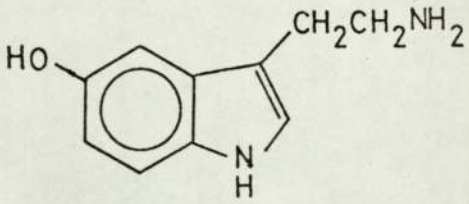


1(xv)



1(xvi)

R as in 1(xiii)



1(xvii)

analogue was inactive[26,27]. The need for a supply of molecular oxygen was also noted, and when the reaction was carried out under ^{18}O-labelled oxygen gas, it was found that ca. 60% incorporation of label into the hydroxyl group of tyrosine occurred, demonstrating that this substituent derives from molecular oxygen[28]. Further work led to the isolation of 7,8-dihydrobiopterin, 1 (xi), from the liver enzyme system[29], though it was also found that this compound was not active in the hydroxylating system in the absence of dihydrofolate reductase, an enzyme which catalyses the reduction of 7,8-dihydropterins to tetrahydro- compounds. Thus, it was concluded that the natural cofactor is tetrahydrobiopterin[30], 1 (xii). The latter appears to be the primary electron donor in the hydroxylating system, the terminal electron donor being NADPH[22] (which probably serves a role in reduction of the primary oxidation product of the cofactor back to starting material). The hydroxylation is shown in Scheme 1.1, and its importance cannot be over-emphasised, since it is a key reaction in the biosynthesis of adrenalin.

It was found during these studies that if the reaction was carried out in the absence of a sheep liver enzyme and NADPH a much lower yield of tyrosine was obtained for a given consumption of tetrahydropterin, and a further addition of cofactor was required to effect further hydroxylation[26]. NADPH was found to increase

the yield significantly in the absence of sheep liver preparations, as were ascorbate and 2-mercaptoethanol, but if the enzyme were allowed to run down prior to the addition of these compounds no stimulation was observed. The product of tetrahydropterin oxidation in the depleted system was judged, from its ultraviolet spectrum, to be a 7,8-dihydropterin[26,27], and it is this which accumulates in the absence of NADPH. Consequently, Kaufman concluded that an intermediate dihydropterin was formed subsequent to tetrahydropterin utilisation by the enzyme, and that this may then be reduced back to cofactor, or it may undergo rearrangement to the inactive 7,8-dihydropterin. The latter cannot be reduced by NADPH, ascorbate or thiols.

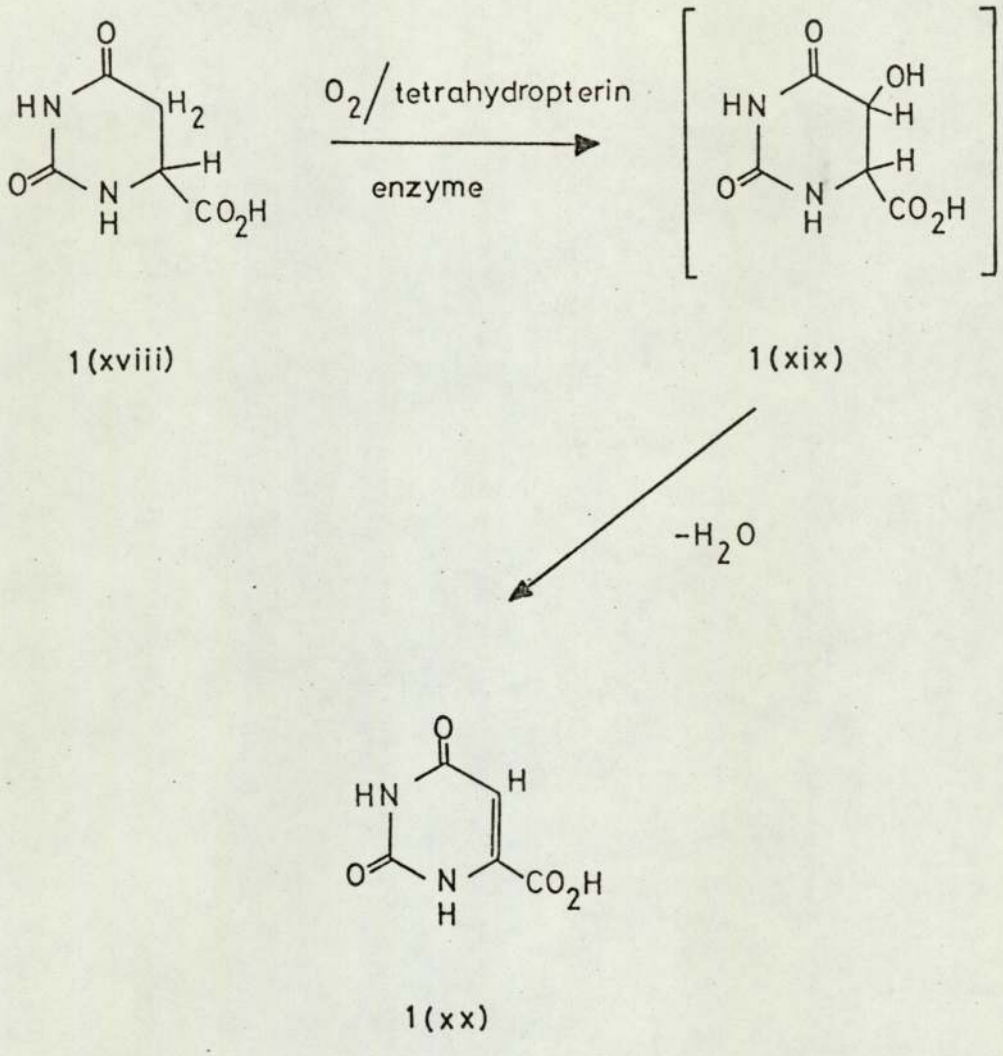
Much work has been done which indicates that this intermediate is a quinonoid dihydropterin, and this is discussed later.

Further enzymatic hydroxylation reactions requiring tetrahydropterins as cofactor have been described in the literature. For instance, Renson *et al.*[31] claim that the phenylalanine hydroxylase system described above also effects the conversion of tryptophan, 1 (xv), to 5-hydroxytryptophan, 1 (xvi), a reaction of potentially great importance owing to its intermediacy in the biosynthesis of serotonin, 1 (xvii). More recently, an enzyme responsible for this hydroxylation has been isolated from the distal one-fourth of the rat small intestine, and shown to require ferrous ion, tetrahydropterin, and oxygen for full activity[32].

Tetrahydropterins have also been shown to be active cofactors in the Mandelic acid-4-hydroxylase system[33], the hydroxylation of p-coumaric acid by Spinach-Beet Phenolase[34], and in a dihydroorotate hydroxylase from Crithidia Fasciculata[35]. The latter reaction is particularly interesting, since the authors propose the intermediacy of a hydroxyorotate, 1 (xix), during the dehydrogenation of dihydroorotate, 1 (xviii), as shown in Scheme 1.2. This implies hydroxylation at a saturated carbon atom, whilst all the reactions discussed previously are aromatic hydroxylation reactions. However, the hydroxyorotate was not isolated from the reaction mixture, and its existence requires further proof. Nevertheless, this is an important observation, since dihydroorotate and orotate are key intermediates in the biosynthesis of pyrimidine nucleotides[36].

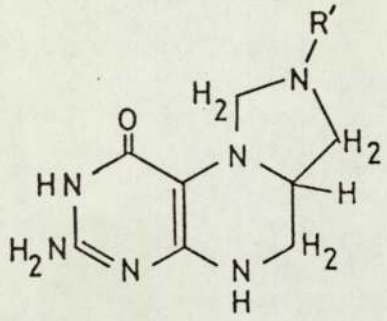
Despite much intensive research, the exact mechanism of the activation of molecular oxygen by the monooxygenases is still unknown.

Although folic acid is obtained from animal and plant tissues, probably in the form of conjugates having two or more glutamic acid residues in the side chain linked by peptide bonds, it does not itself act as cofactor in enzymic transformations. However, it is enzymatically reduced first to DHFA and then to THFA, and the latter is found to be implicated in many biochemical processes involving transfer of one carbon atom units at the oxidation levels of formate, formaldehyde, and methyl. As an example

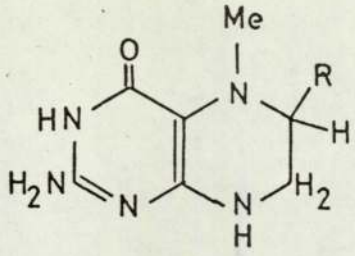


1(xviii) 1(xix) 1(xx)

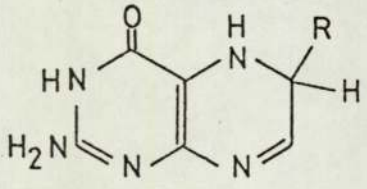
SCHEME 1.2 Proposed conversion of dihydroorotate to orotate[35]



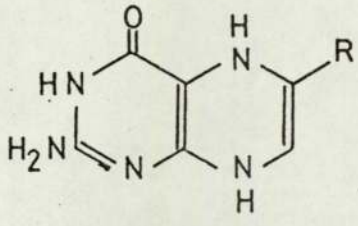
1(xxi)



1(xxii)

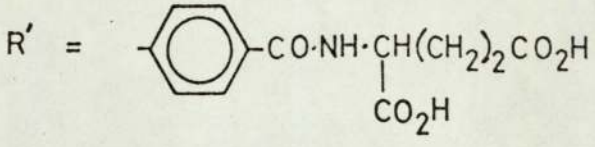


1(xxiii)



1(xxiv)

R as in 1(vii)



of the general importance of folates it may be mentioned that 5,10-methylene THFA, 1 (xxi), is involved in the enzymatic synthesis of thymidine and its phosphorylated derivatives, and 5-methyl THFA, 1 (xxii), is essential for the conversion of homocysteine to methionine, these being but two of the wide range of such reactions. The first is an extremely important step in the biosynthesis of nucleic acids, and the second in amino acid, and therefore protein, synthesis. However, these will be discussed no further here, and the reader is referred to the excellent book by Blakley[18], and the review by Stockstad and Koch[37].

Methods of Reduction of Pterins.

Since most of the starting materials for the oxidation studies described later are obtained by reduction of the corresponding fully oxidised compounds (e.g. THFA from folic acid), it is perhaps pertinent at this point to mention the various methods described in the literature.

The method of choice for the reduction of folic acid to THFA is by catalytic hydrogenation, over platinum or palladium, in neutral aqueous media or glacial acetic acid, a method first described by O'Dell *et al*.[38] and refined somewhat by Hatefi *et al*.[39]. Two moles of hydrogen are consumed and THFA is formed, although a further slow uptake of gas is observed resulting in the formation of unknown products. It is noteworthy that DHFA is not

detected in the reaction mixture when glacial acetic acid is used, so this provides a fairly clean method of preparation of THFA, which is obtained as the diacetate after lyophilisation, the catalyst being previously removed by filtration in an inert atmosphere. The use of high purity folic acid is recommended[39], and in order to avoid the formation of colloidal platinum which is difficult to remove, the Adams' catalyst (PtO_2) should be reduced prior to addition of folic acid. The most convenient solvent is in fact glacial acetic acid, owing to the extremely high solubility of THFA relative to folic acid and partially reduced compounds, and therefore the low risk of contamination by the latter compounds. THFA diacetate obtained as such may be purified by chromatography on DEAE-cellulose, provided 2-mercaptoethanol is incorporated into the solvent as an antioxidant[40,41]. In 0.1M sodium hydroxide, catalytic reduction of folate is reported to yield mainly DHFA[38], but hydrogen is readily consumed in excess of one mole[42,43], so that pure DHFA cannot be obtained in this way.

Less useful methods for the preparation of THFA include catalytic hydrogenation in 98% formic acid (though formylation of the product occurs to give a mixture of THFA and formyl-THFA[44]) and the high temperature reduction[45] of folic acid with sodium dithionite at ca.pH 6, a process which leads to reaction mixtures which are rather heavily contaminated with sulphur dioxide and inorganic materials, and therefore requiring extensive purification.

The THFA prepared by these methods is generally assumed to be 5,6,7,8-tetrahydrofolic acid, and the more facile reduction of the pyrazine ring compared to pyrimidines supports this, as do the properties of THFA. (N.m.r. is presented in Appendix A.)

Scrimgeour and Vitols[46] have described a method of preparation of THFA by the reduction of folate or DHFA with sodium borohydride. Although this method is particularly useful for producing tritiated THFA, in the light of the findings described later concerning the oxidative lability of reduced pterins at high pH, the method may result in appreciable formation of degradation products and therefore an impure material.

Several reports have appeared in the literature describing the reduction of folate by dithionite at ambient temperatures to yield DHFA[47-52], and although O'Dell *et al.*[38] had earlier suggested the structure 7,8-dihydrofolate, 1 (viii), for this compound, a great deal of controversy regarding this was apparent at the time. All the possibilities, 5,6-dihydro-,1 (xxiii), 5,8-dihydro-,1 (xxiv), as well as the 7,8-dihydro- structures have been suggested and it was not until the n.m.r. spectrum was determined and tritium labelling studies were done[51] that it was concluded that the latter structure is correct.

It has also been reported[53] that pteridines may be reduced by exposure to ultraviolet light in the presence of a hydrogen donor such as allyl-thiourea, and this was originally believed to have some bearing on the visual

processes in insects, in which the eyes are often found to contain a number of pteridines.

The Chemical Oxidation of Reduced Pterin Derivatives.

Reasons for Study.

It is generally accepted that, since folic acid itself is not biosynthesised in mammals, both the reduced derivatives and folic acid itself are tremendously important dietary constituents. Consequently, any study of the lability of these compounds to oxidation and other chemical degradations is useful, especially at the present time when foodstuffs are stored for relatively long periods by use of freezing techniques, etc. Questions of major importance are whether the products of oxidation retain the folate structure (i.e. is the molecule fragmented to useless products) and how do a variety of conditions affect the rate of decomposition and the products obtained.

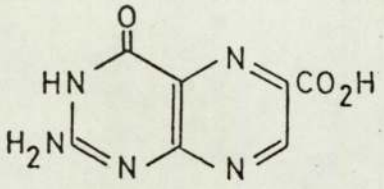
With regard to other reduced pteridines, oxidation studies are important in connection with the role of tetrahydropterins as cofactors for monooxygenase reactions, in which molecular oxygen is activated. The reduced flavins, which contain the pteridine ring system, are involved in a variety of other biological electron transfer processes.

The above aspects have prompted numerous investigations of tetrahydropterin oxidation, though these have tended to revolve around product analysis

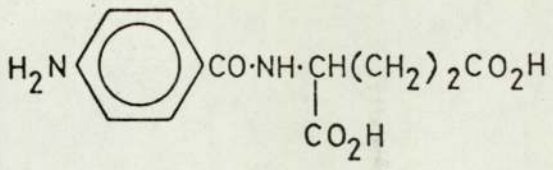
and non-quantitative assessment of reactivity, with only a few kinetic studies of oxidation by reagents other than oxygen. The results and implications of such experiments will now be considered.

Oxidation Products.

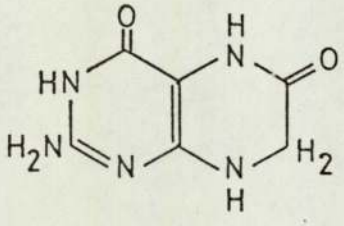
The facile oxidation of pteridines at all oxidation levels by a variety of reagents has been known for some time. Wittle *et al*.[54] described the oxidative cleavage of the folic acid side-chain by treatment with permanganate or chloric acid. The products are pterin-6-carboxylic acid, 1 (xxv), and p-aminobenzoylglutamic acid, 1 (xxvi), the latter undergoing simultaneous chlorination of the benzene ring when chloric acid is the oxidant. Later, Dion and Loo[55] showed that the folic acid analogue, 1 (xxvii), undergoes similar cleavage of the 9,10- bond on treatment with permanganate, as shown in Scheme 1.3. With regard to stability of reduced compounds, it was recognised by O'Dell *et al*[38] that THFA undergoes rapid autoxidation by one mole of oxygen, whilst Zakrzewski and Nichol[56] showed that solutions of THFA may be oxidised under air to give at least five products, separable by paper chromatography. More recently, it has been shown that THFA is oxidised at low pH to pterin (via 7,8-dihydropterin) and dihydroxanthopterin, 1(xxviii), whilst at higher pH DHFA appears to be the initial product[57, 58]. The latter is reported[57] to autoxidise



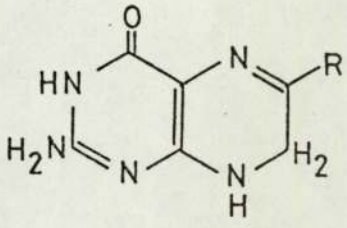
1(xxv)



1(xxvi)

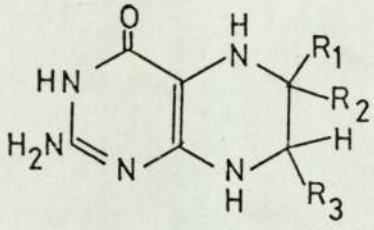


1(xxviii)



1(xxix) R = -CHO

1(xxxii) R = $-CH_3$

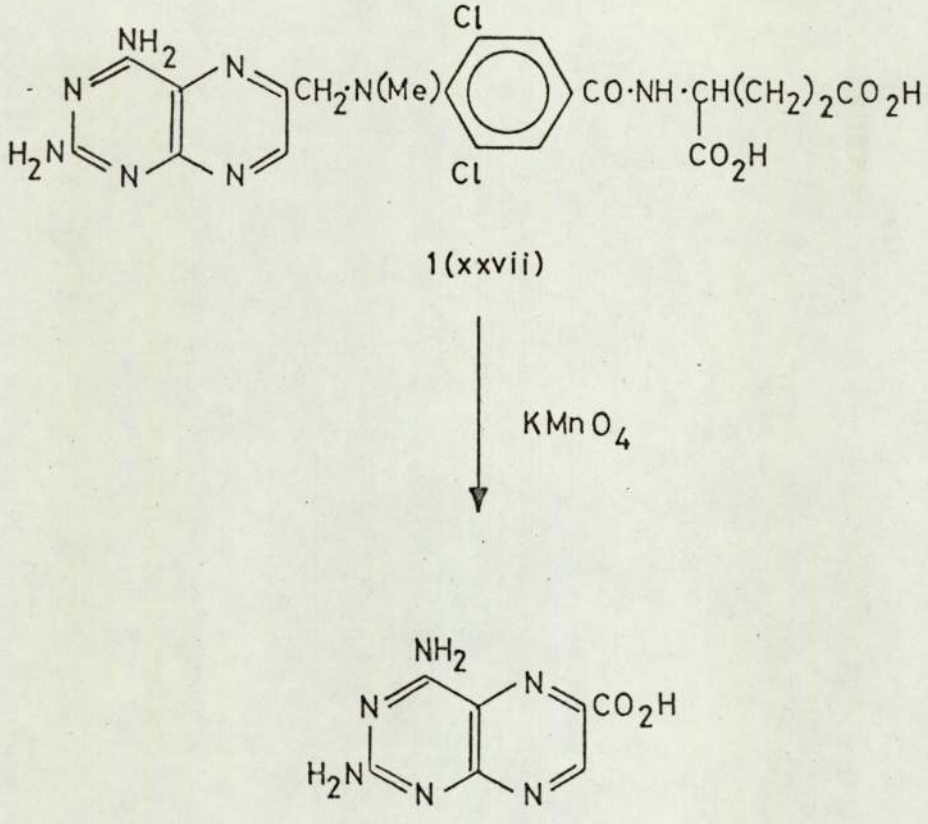


1(xxx) R_1 = $-CH(OH)CH(OH)CH_2OH$

$R_2 = R_3 = H$

1(xxxi) $R_1 = -CH_2 \cdot NH_2$ $R_2 = -CH_3$ $R_3 = H$

1(xxxii) $R_1 = -CH_2 \cdot NH_2$ $R_2 = R_3 = -CH_3$



SCHEME 1.3 Oxidative cleavage of folate analogues[55]

to folate, dihydroxanthopterin, formaldehyde, p-aminobenzoylglutamic acid, and 7,8-dihydropterin-6-carboxaldehyde, 1 (xxix), the yields of which show a marked dependence on pH and temperature. The formation of the latter compound during DHFA autoxidation had also been recognised earlier by Whitely et al.[59], who identified it by comparison of the ultraviolet spectrum and thin layer chromatographic behaviour with those of authentic material. Autoxidation (as distinct from $KMnO_4$ oxidation) is also noted to result in cleavage of folic acid itself at high pH, to give pterin-6-carboxylic acid[60].

With regard to simpler pteridines, it has been recognised that whereas the introduction of OH, SH or NH_2 groups at positions 2- and 4- in the ring result in decreased solubility and an increased stability of the compounds towards hydrolysis[61], the same substituents in the corresponding dihydro- or tetrahydro- compounds lead to molecules showing a greater lability towards oxidising agents[62]. The hydrolytic stability of the substituted compounds is due to the filling of depleted molecular orbitals (by substituent lone pair electrons) and partial restoration of aromatic character which is rather lacking in pteridine itself, whilst the change in oxidative stability of the reduced compounds is due to increased electron availability. The latter is discussed more fully in the later chapter on molecular orbital theory, and is empirically illustrated by the fact that 5,6,7,8-tetrahydropteridine itself can be synthesised, isolated

and purified by recrystallisation and/or chromatography without the use of antioxidants[63], though it is noted that the product becomes rapidly brown on exposure to air and even in coal gas (and, therefore, not necessarily due to oxidation). On the other hand, tetrahydropterin solutions are reported to be rapidly oxidised by air to a complex mixture of products[64], whilst the tetrahydro- derivatives of 6-methyl pterin, 8-(2-hydroxyethyl)-6,7-dimethyl pterin,[65] 2,4-dihydroxypteridine,[62] 2,4-diamino pteridine[66] and 6-aminopterin[67] undergo easy oxidation to the dihydro- and parent compounds. The presence of products due to loss of the C(6) substituent seems to be characteristic of compounds in which this moiety contains the system $\geqslant C-\ddot{X}$ bonded via the carbon atom to the pterin ring. X may be any atom carrying a lone pair of electrons, such as nitrogen or oxygen. Furthermore, this cleavage appears to be more facile when X is nitrogen than when it is oxygen, as demonstrated by the ready formation of xanthopterin and pterin in autoxidation of THFA[57, 58] at ambient temperatures, compared to the cleaner conversion of tetrahydrobiopterin to dihydrobiopterin[56, 68] under similar conditions. It is known, however, that at 100°C tetrahydrobiopterin does indeed autoxidise to dihydroxanthopterin (36%), biopterin (4%), and pterin (36%).[69] The same publication showed that the side chain of tetrahydroneopterin, 1 (xxx), was lost as glyceraldehyde. Later, a mechanism for this reaction, involving a quinonoid dihydropterin intermediate, was suggested in the absence of any experimental justification[70]. Similarly, 6-aminomethyl-6-methyl-

tetrahydropterin, 1 (xxxi), shows oxidative loss of the aminomethyl group to give 6-methyl-7,8-dihydropterin, 1 (xxxii), formaldehyde and ammonia[71], though in later experiments[72] 6-aminomethyl-6,7-dimethyltetrahydropterin, 1 (xxxiii), showed a small loss of the 7-methyl group, and the authors suggested a minor oxidation pathway involving opening of the pyrazine ring followed by re-cyclisation. Under acid conditions, oxidation of 5-methyl-6,7-diphenyl-5, 6-dihydropterin results in loss of the methyl group and formation of 6,7-diphenylpterin[73].

The lability of reduced pterins presents problems with regard to isolation, storage, and use in many experiments, particularly when dealing with biological systems. It is usually recommended that DHFA be stored as a frozen suspension in 1 mM hydrochloric acid containing mercaptoethanol as antioxidant[74], or as a suspension in 10% potassium ascorbate, pH 6.0, at 4°C [75]. Oxidation of THFA in solution is retarded by 2,3- dimercaptopropanol and mercaptoethanol, an effect which does not appear to be due to complexing of transition metals, since strong chelating agents such as EDTA show a much smaller inhibition[76]. Ascorbate is reported to be an even better antioxidant for THFA[77], and is used to protect it during the synthesis of 5-formyl THFA[44], and in the isolation of THFA derivatives from biological materials[78]. Usually, a large excess of ascorbate is used, and in such cases the high degree of protection afforded may be mostly due to removal of oxygen from the solutions by ascorbate auto-

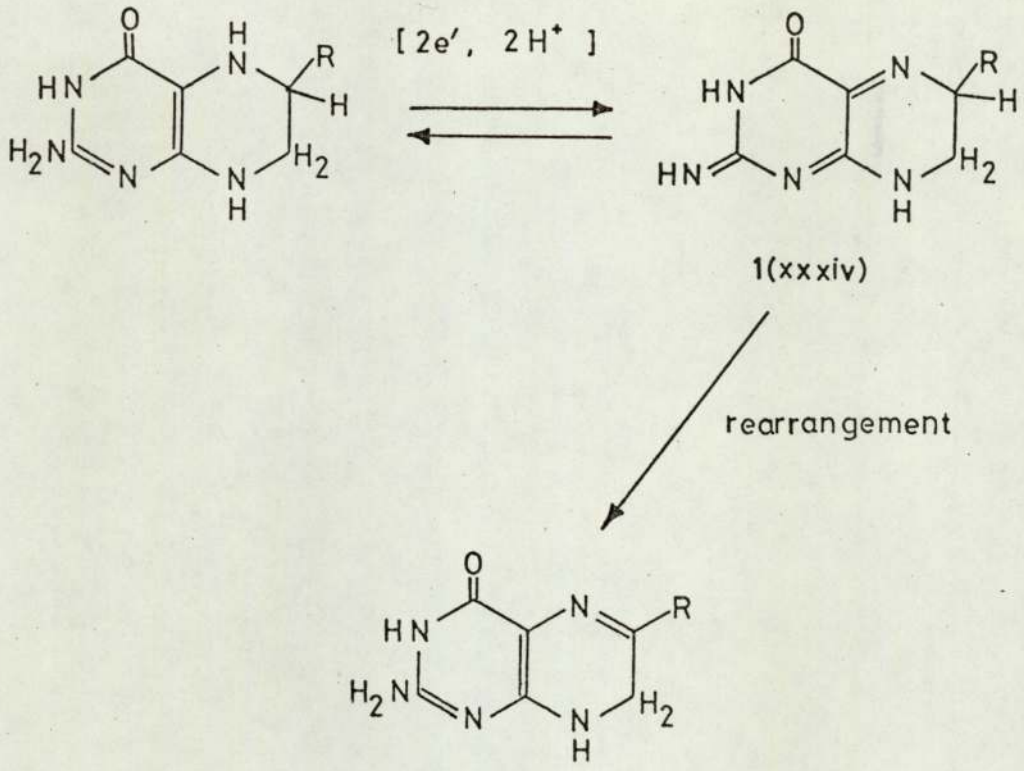
xidation, although it is possible that reducing agents such as these may act partly via the reduction of the unstable dihydropterin intermediate mentioned above.

Oxidation of Tetrahydropterins - Mechanistic Interpretations.

It was mentioned earlier that, during his studies on the role of tetrahydropterins as cofactors in enzymatic hydroxylations, Kaufman suggested the primary oxidation product was a dihydropterin which could be reduced back to the tetrahydro- level, or might rearrange irreversibly to give the isolatable 7,8-dihydropterin. This author then carried out studies indicating the intermediate to have a quinonoid dihydropterin structure, along the following lines. 6,7-Dimethyltetrahydropterin was instantaneously oxidised with a stoichiometric quantity of the dye, 2,6-dichlorophenolindophenol, a good oxidising agent, and the dye removed by rapid ether extraction[79]. The resulting solution gave a single absorption maximum at 302-304 nm., and showed a rapid change to the spectrum of 6,7-dimethyl-7,8-dihydropterin. It was assumed that the compound giving this absorption was a dihydropterin on the basis of the 2-electron stoichiometry of the reaction, and it was a dihydropterin other than 7,8-dihydropterin because it could be reduced back to the tetrahydro- level with NADPH, a conversion which does not occur with 7,8-dihydro- compounds. Evidence that the intermediate was not a 5,8-dihydropterin, such as 1 (xxiv), consisted of demonstrating that, during

the oxidation-reduction cycle between starting material and intermediate in the presence of NADPH, a racemisation at C(6) does not occur when an optically pure tetrahydropterin is used, as shown by the constancy of cofactor activity in the phenylalanine hydroxylase system[79]. That the intermediate was not a 5,6-dihydropterin, such as 1 (xxiii), was shown using NADPH-4-^{3}H as reducing agent in the presence of sheep liver enzyme, when no non-exchangeable label appeared in the tetrahydropterin. The same result was obtained using tritiated water and unlabelled NADPH[65]. Both 5,6- and 5,8- dihydropterins should incorporate non-exchangeable tritium during the reduction process. Furthermore, very little tritium loss is observed from 6,7-dimethyltetrahydropterin-7-^{3}H, when subjected to the above reversible process, again mitigating against the formation of 5,6- or 5,8- dihydro- compounds. From these studies, Kaufman proposed that the primary oxidation product has a quinonoid structure, 1 (xxxiv), and the primary site of oxidation is N(5). The mechanism accepted by many workers in this field is shown in Scheme 1.4.

Most of the more recent evidence in the literature indicates the presence of such a quinonoid intermediate. For example, during polarographic studies of the anaerobic oxidation of tetrahydropterins with Cu^{2+} and Fe^{3+}, Vonderschmitt and Scrimgeour[80] observed that 2 moles of oxidant produced 7,8-dihydropterin, but that this oxidation occurs in two distinct steps, the first of which was proposed to be formation of the quinonoid intermediate.



SCHEME 1.4 Intermediacy of p-quinonoid dihydropterin in oxidation of tetrahydropterins

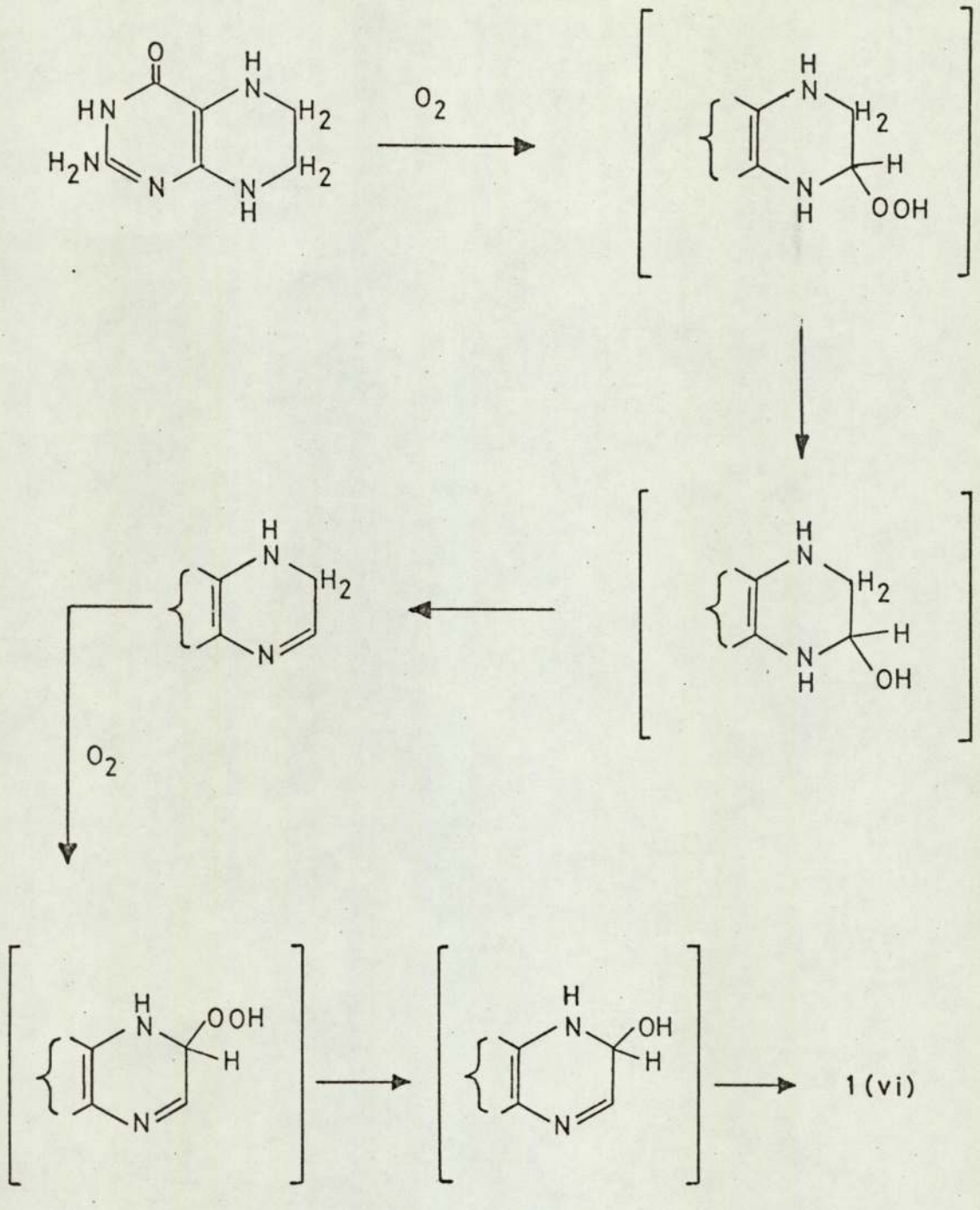
They also reported that the rearrangement of the latter to the 7,8-dihydropterin is relatively slow and that the course of the reaction followed by polarography is first order in quinonoid compound. Further polarographic work by Archer and Scrimgeour[81] on the reversible oxidation to quinonoid dihydropterins indicates the presence of two overlapping one-electron steps, with the intermediacy of a semiquinone free radical. Kinetic studies by these authors[82] showed that an extremely rapid reduction of ferricyanide by tetrahydropterins occurred under anaerobic conditions, and this was followed by the slower rearrangement of an intermediate to the stable 7,8-dihydropterin. The kinetics of the latter process were determined by following the formation of the 7,8-dihydropterin spectrophotometrically, and rearrangement was found to be first order in intermediate, first order in buffer concentration and dependent on the pKa values for the buffer used. A large primary isotope effect was obtained when 6-deuterio tetrahydropterins were used, and the activation energy for the rearrangement was estimated at 10.1 Kcal mol^{-1}. These facts provide good evidence that the observed intermediate is indeed a quinonoid dihydro compound, since its rearrangement is both proton- and base- catalysed and involves heterolysis of the 6-CH bond. Later studies by these workers[83] examined the rate of disappearance of the oxidant (Fe^{III}) by anaerobic stopped-flow methods, thus determining actual rates of oxidation of the tetrahydropterins to quinonoid compound, finding a 300-fold decrease in rate on protonation of N(5)

at pH's 7 to 1 and a further small increase in rate on raising the pH from 9 to 10.4. Similar changes were observed when air was used as oxidant, though inclusion of oxygen in the Fe^{III} reaction has no effect on the kinetics, seeming to preclude oxygenated intermediates.

Jaenicke and Kretzschmar[84] have also reported polarographic studies on the oxidation of THFA in aqueous media, and conclude that free radicals are involved and the reaction proceeds via a quinonoid intermediate, which can rearrange to DHFA or undergo proton-catalysed loss of side chain, although no detailed mechanism was set out. These authors report that the autoxidation is first order with respect to THFA.

Electron spin resonance has been used[85] to show that the red colourations produced when tetrahydropterin and THFA are treated with hydrogen peroxide in trifluoro-acetic acid/methanol are due to cationic radicals which can be trapped at liquid nitrogen temperatures. Such red colourations are very familiar to workers experienced in handling these compounds who have had the misadventure to allow air into acetic acid solutions of their hydrogenation products. Further observations of the e.s.r. spectra of these and the N(5)-methylated radicals[86, 87] have resulted in the resolution of hyperfine structure and determination of coupling constants, showing that there is a high unpaired electron density at N(5).

Although most workers accept the involvement of free radicals, the presence of quinonoid intermediates has



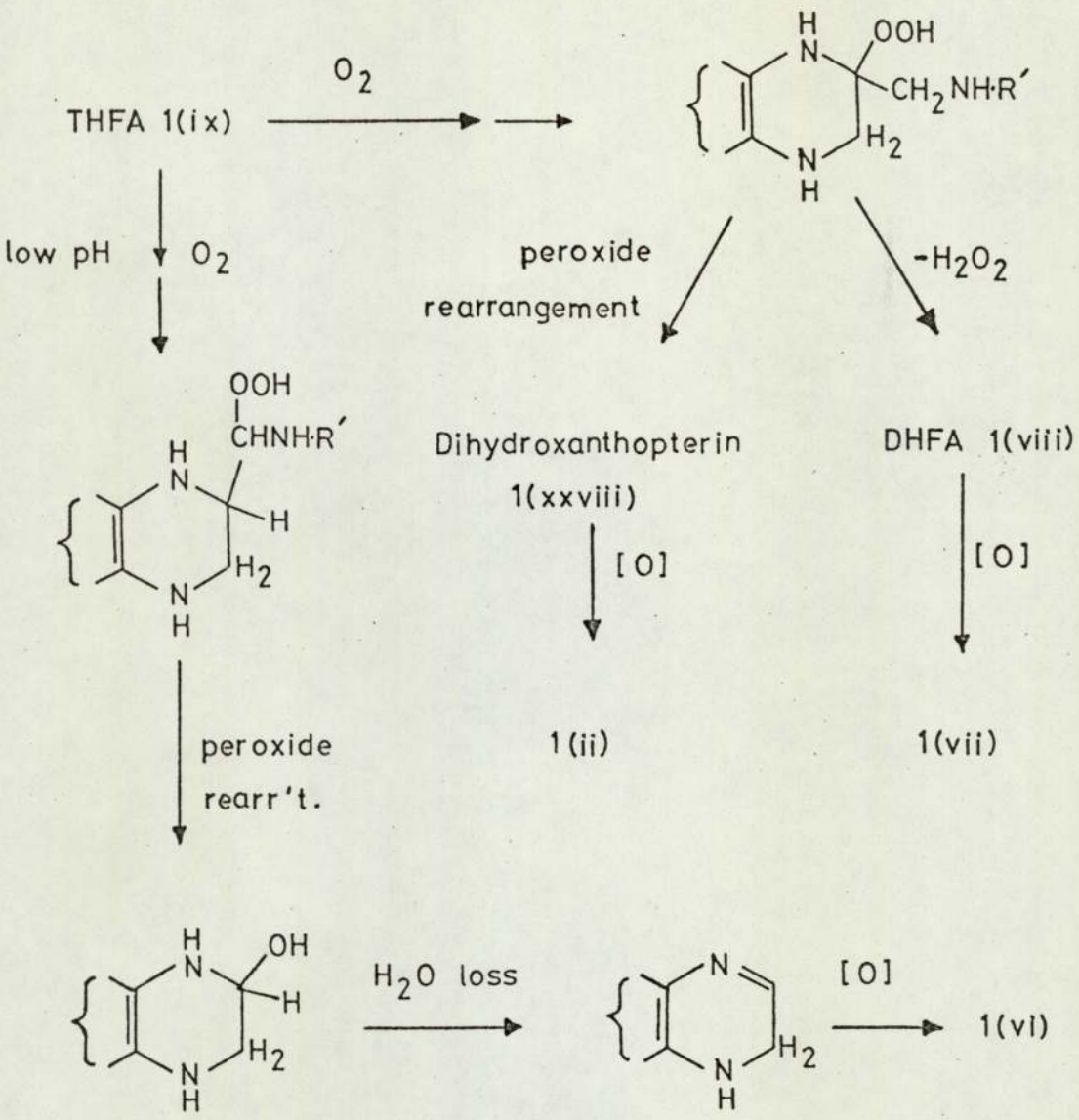
SCHEME 1.5 Hawkins' autoxidation mechanism[89]

remained a subject of some controversy. Results on the autoxidation of tetrahydropterin published by Viscontini and Weilenmann[88] have been interpreted by Hawkins[89] as evidence for the intermediacy of hydroperoxides (Scheme 1.5), whilst Huennekens[90] appears to believe that oxidation of THFA occurs initially at the 5,6- position, notably at C(6). Based on the pH- dependence of xanthopterin: pterin ratios found in the final products after three days autoxidation of THFA, Stocks-Wilson[58] discussed the possible involvement of hydroperoxides formed by oxygen attack at C(6) or C(9), the xanthopterin or pterin arising by peroxide rearrangement as shown in Scheme 1.6. The main objection to this hypothesis is that hydroperoxide rearrangement is usually acid-catalysed, whereas xanthopterin yields decrease as pH is lowered (Table 1.1). No satisfactory explanation of this discrepancy was presented.

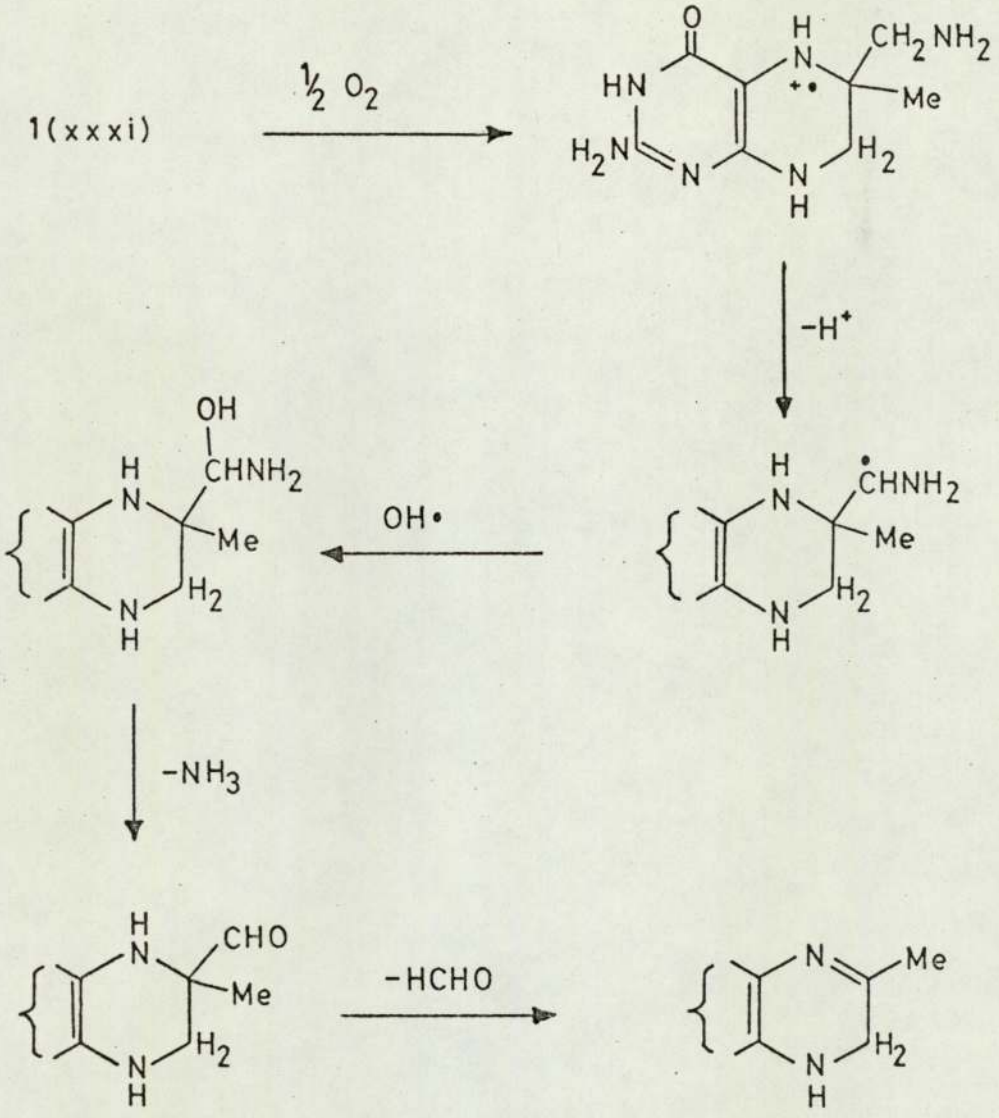
Despite the above objections, Viscontini's group have interpreted most of their findings[71, 91, 92, 93] in terms of either a quinonoid intermediate for simple compounds, or hydroxylated derivatives for compounds where quinonoid intermediates are considered unlikely, as shown in Schemes 1.4, 1.7, and 1.8. The proposed mechanism shown in Scheme 1.7 is not particularly satisfactory. One would not expect OH. radicals to be present in autoxidation reactions to any large extent, and also the addition of OH. radicals to the side chain as shown represents radical recombination, a process usually accepted as being minor, and therefore unlikely to be responsible for products on

TABLE 1.1. pH - Dependence of pterin: xanthopterin ratio in oxidation products of tetrahydrofolic acid at ambient temperatures.[58]

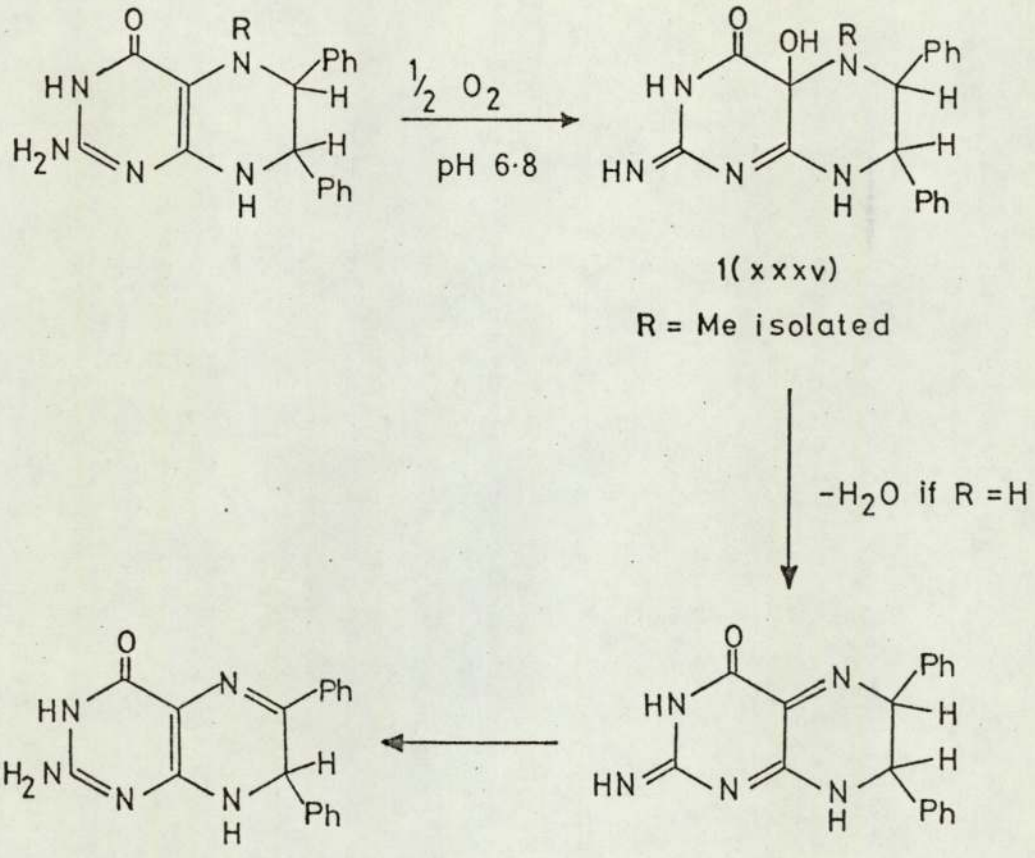
Buffer and pH	Pterin : Xanthopterin ratio
Dilute Acetic acid pH 3	12.64 : 1.00
0.1 M Ammonium acetate pH 7	1.00 : 1.90
NaOH - $NaHCO_3$ pH 10	1.00 : 17.38



SCHEME 1.6 Outline of Stocks-Wilson mechanism of THFA autoxidation.[58]



SCHEME 1.7 Oxidative side-chain loss according to Viscontini



SCHEME 1.8 Viscontini & Okada[93] — detailed autoxidation mechanism

any large scale. This has been offered as a mechanism of side chain loss during autoxidation of THFA and tetrahydrobiopterin[94]. Scheme 1.8 was proposed[93] as a general mechanism of tetrahydropterin autoxidation based on the observation that the 4a-hydroxylated compound 1 (xxxv) can be isolated when 5-alkylated tetrahydropterins are used. This seems feasible, although it was not demonstrated whether the hydroxyl substituent was derived solely from molecular oxygen, or whether the isolated compound was merely a pseudo base of the corresponding quinonoid intermediate, which would have a quaternary ($=\overset{\oplus}{N}<$) nitrogen function.

Mager and Berends[95-100] have approached the problem from a slightly different angle. They accept the involvement of semiquinone radicals and quinonoid dihydropterin intermediates, and propose that the formation of the latter is preceded by addition of molecular oxygen to the angular carbon 8a, resulting in an alkylperoxy radical which, by electron transfer, is converted to an alkyl hydroperoxide. This may then oxidise a second tetrahydropteridine molecule to give an 8a-hydroxy compound, which then loses water to form the quinonoid intermediate. (Scheme 1.9). The evidence for the presence of hydroperoxides is:

a) the stimulated uptake of oxygen in the presence of mercaptoethanol and other reducing compounds, and

b) studies on the relationship between the stoichiometry of the reaction (determined manometrically) and the quantity of hydrogen peroxide present (determined by

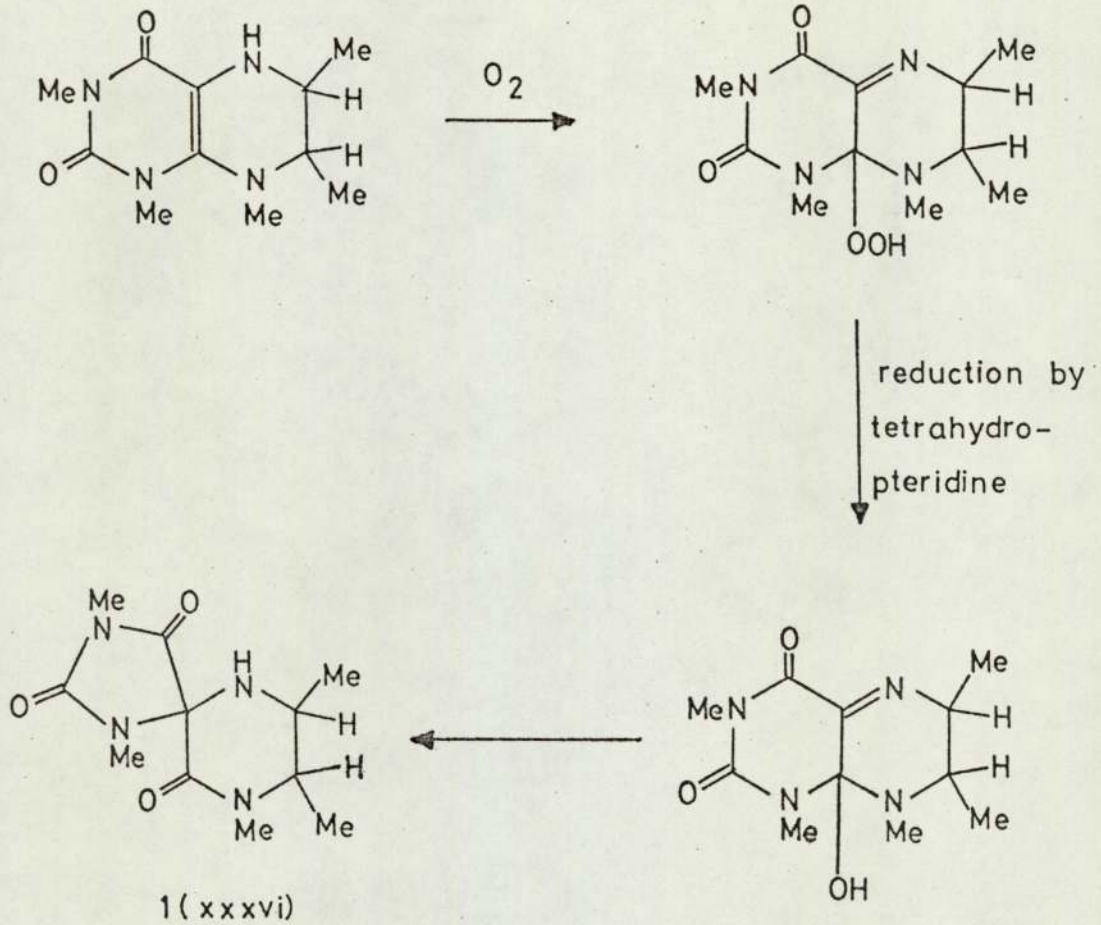
oxygen evolution after addition of catalase), both being due to peroxide forming and peroxide consuming reactions.

It must be pointed out, however, that all of these effects can be equally well explained by replacing organic peroxides with hydrogen peroxide in the theory. Their evidence for the structure of the hydroperoxides is based on the observations that N-alkylated model compounds undergo autoxidative ring - contraction to the well-characterised spiro hydantoin, 1 (xxxvi) [96,97], the proposed mechanism being as shown in Scheme 1.9. This will be discussed in Chapter 5.

The possible importance of such hydroperoxide intermediates has been discussed by Mager's group,[98,99,100] who have suggested that they may be responsible for the activation and transfer of oxygen in the mono oxygenase reactions, such as the tetrahydropterin-dependent hydroxylation of phenylalanine.

Kaufman has mentioned that these proposals of hydroperoxide intermediates remain to be established[101].

Further to the above discussion it is known that when tetrahydropterins are allowed to autoxidise in the presence of ferrous iron and an aromatic substrate such as phenylalanine, the latter is hydroxylated non-specifically[98-100, 102], and the mechanism of this system is unknown. An understanding of the mechanism of autoxidation of tetrahydropterins is a pre-requisite to any study of this hydroxylating system.



1 (xxxvi)

SCHEME 1.9 Outline of Mager & Berends autoxidative ring contraction mechanism

The Aims of the Study.

Although, as mentioned above, autoxidation of tetrahydropterins has been investigated by product analysis, the reaction has not been subjected to rigorous kinetic study, although this has been done with more powerful oxidants such as ferricyanide[82, 83].

The aims of the series of experiments described in Chapters 2 - 6 were therefore:

a) to qualitatively examine the products of THFA autoxidation at various pH values and at discrete times in the oxidation pathway and thereby establish the reaction sequence more fully than has hitherto been described;

b) to determine the products of tetrahydrobiopterin autoxidation at ambient temperatures and similarly write down the reaction pathway;

c) to determine the kinetics of autoxidation as rigorously as possible and thereby determine the factors affecting the stability of THFA and tetrahydrobiopterin as typical naturally-occurring tetrahydropterins. The ultimate aim is to develop a practical mechanism for the autoxidation reactions which is consistent with both the reaction pathway data and kinetics, and which establishes whether or not hydroperoxides may be considered as distinct intermediates; if so, to determine the most probable structure;

d) to obtain kinetic information about the autoxidation of DHFA;

e) Using molecular orbital theory, to build a sound theoretical framework for the derived mechanism, taking into account possible structures for any transition states involved, and to rationalise any kinetic differences shown by various reduced pterins.

At this premature stage, a mechanism will not be proposed as such, but rather it is hoped to take the reader through a series of logical steps, arriving at a reasonable mechanistic interpretation of all the data accumulated.

Chapter 8 takes a brief glimpse at the non-enzymatic tetrahydropterin-mediated hydroxylations of phenylalanine, and an attempt will be made to understand this complex system by comparison with the well-known Fenton reagent.

CHAPTER 2.

Products of Autoxidation of Tetrahydrofolic Acid and Tetrahydrobiopterin. Derivation of reaction pathway.

In Chapter One it was noted that the products of oxidation of THFA with ferricyanide, and molecular oxygen, have been characterised. The purpose of the experiments described here was to determine the approximate times of appearance of the products, with special reference to the mechanism of autoxidation suggested by Stocks-Wilson[58]. Thus, the problem is: are dihydropterin and dihydroxanthopterin both formed directly from THFA, as suggested by this mechanism, or is either one of them preceded by the formation of some other detectable compound? In this context it may be pointed out that Chippel and Scrimgeour[57] suggest that dihydroxanthopterin production only occurs subsequent to the formation of DHFA during the ferricyanide oxidation of THFA.

Also, a rather closer examination has been made of the oxidation products of tetrahydrobiopterin and 6,7-dimethyltetrahydropterin, with special reference to observations of C(6) substituent loss under certain conditions.

The detection and characterisation of hydrogen peroxide in the autoxidation products of tetrahydrobiopterin is described, together with attempts to detect organic peroxides at low temperatures. Although solutions of oxidised material have been reported[58, 97, 98] to give positive results to chemical tests for peroxides, no absolute discrimination between hydrogen peroxide and organic derivatives has been given.

The preparations, or sources, of all starting materials are given in Appendix A.

(1) Product Detection and Reaction Pathway during Autoxidation of Tetrahydrofolic acid.

Method.

Solutions of THFA (25 mg) in the appropriate buffer (25 cm^3; dilute acetic acid for pH 3 oxidations; 0.1M - sodium phosphate buffer, pH 7; sodium hydroxide adjusted to pH 11; 0.1M-sodium hydroxide pH 13) were shaken under air to achieve oxidation, samples withdrawn at the times indicated in the results, and eluted together with appropriate authentic materials (DHFA, pterin, xanthopterin and dihydroxanthopterin) using cellulose MN 300 layer (0.1 mm) in the solvent systems shown in Table 2.1. The developed chromatograms were viewed under ultraviolet (366 nm) and the observed fluorescent products compared with the standards. (R_f values in Table 2.1.)

TABLE 2.1. Standard R_f values for oxidation products of Tetrahydrofolic acid.

Compound and appearance under UV light.	R_f value in solvent indicated (1)	(2)	(3)
THFA (absorbing)	0.78	0.35	0.43
DHFA (blue fluorescence)	0.22	0.12	0.43
Pterin (blue fluorescence)	0.52	0.38	0.46
Dihydroxanthopterin (green fluorescence)	0.37	0.23	0.40
Xanthopterin (green fluorescence)	0.49	0.25	0.42

Solvent systems: -

(1) 0.1M - sodium phosphate buffer.

(2) n-Propanol - 1% aqueous ammonia 2:1 v/v

(3) Organic phase from n-Butanol - acetic acid - water 4:1:5v/v after 24 h. equilibration period.

Results and Discussion.

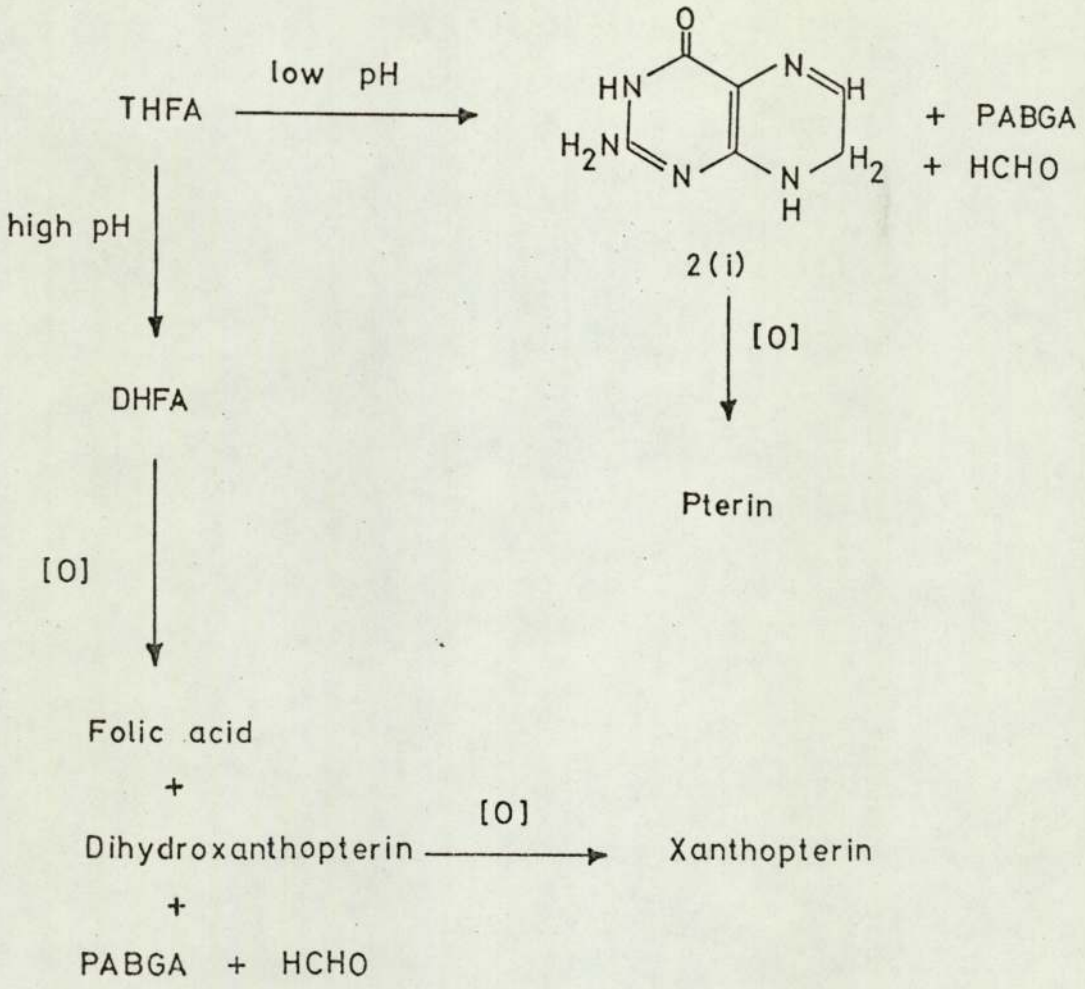
From the results of product appearance in Table 2.2, it is clear that during oxidation at pH 3 pterin arises directly from oxidative cleavage of THFA. The intermediacy of 7,8-dihydropterin, 2(i), during this conversion is obligatory owing to the stepwise nature of the oxidation process. However, its presence can only be inferred from the slight differences between the R_f values of the observed product in the earlier stages of reaction and those of pterin itself. Attempts were made to synthesise dihydropterin as a t.l.c. standard, using dithionite reduction of pterin, but a clean product could not be obtained, leading to very poor chromatographic separation and inaccurate R_f values for the dihydro-compound. Similar difficulties have been encountered by Watt[103] who obtained only UV spectral evidence for pterin reduction to dihydropterin using zinc in hydrochloric acid.

At pH 7, pterin is similarly observed in the initial products, together with DHFA, but no xanthopterin or dihydroxanthopterin. Further reaction gives a mixture in which pterin is still present, DHFA has disappeared, and no xanthopterin could be observed. However, a yellow compound having R_f 0.35 (1) was found in this mixture, and this is probably 7,8-dihydropterin-6-carboxaldehyde, which is a known product of DHFA autoxidation[57, 59, 104]. Examination of the UV spectrum of this oxidation mixture showed a peak at 423 nm, characteristic of this compound, but since it represented only a very minor product, its detection was pursued no further.

TABLE 2.2. T.l.c. investigation of reaction pathway during tetrahydrofolic acid autoxidation.

Oxidation medium and pH.	Sampling time	Observable products, appearance and R_f values. (solvent in parentheses).
Dilute acetic acid pH 3	1.5 h.	Pterin or dihydropterin, blue fluorescence, 0.55 (1), 0.42 (2), 0.45 (3).
	39 h.	Ditto. 0.52 (1), 0.40 (2), 0.46 (3).
Phosphate buffer pH 7	40 min.	Dihydrofolic acid major product, blue fluorescence, 0.20 (1), 0.13 (2), 0.42 (3). Pterin or dihydropterin, blue fluorescence 0.58 (1), 0.42 (2), 0.48 (3).
	24 h.	7,8-dihydropterin-6-carboxaldehyde, yellow, 0.35 (1). Pterin, blue fluorescence, 0.53 (1), 0.43 (2), 0.46 (3).
NaOH; pH 11	40 min.	Dihydrofolic acid major product, blue fluorescence, 0.20(1) Dihydroxanthopterin minor, green fluorescence 0.37 (1). Pterin or Dihydropterin minor, blue fluorescence, 0.58 (1), 0.42 (2), 0.50 (3).
	24 h.	Xanthopterin major product, green fluorescence, 0.50 (1), 0.24 (2), 0.42 (3).
NaOH; pH 13	35 min.	Dihydrofolic acid, blue fluorescence, 0.21(1), 0.12 (2), 0.43 (3).
	24 h.	Xanthopterin, green fluorescence, 0.49 (1), 0.25(2) 0.42(3)

Solvent systems as in Table 2.1.



PABGA = p-aminobenzoylglutamic acid

SCHEME 2.1 THFA autoxidation pathway

At higher pH values, it can be seen that formation of xanthopterin is definitely preceded by production of DHFA, and is a known oxidation product of the latter, probably via dihydroxanthopterin which was just detectable in the earlier stages of oxidation at pH 11. The fact that this conversion can also be effected by oxidation with ferricyanide under anaerobic conditions[57] also suggests that the 6-oxo group in xanthopterin arises from the water used as solvent.

Therefore, the reaction pathway appears to conflict with the mechanism of Stocks-Wilson, but the pterin:xanthopterin ratios recorded by this worker also reflect to some extent the relative rates of formation of dihydropterin and DHFA, and give valuable information about the pH-dependent rearrangement of any intermediates. The overall reaction pathway may be written as in Scheme 2.1.

(2) Products and Reaction Pathway during autoxidation of Tetrahydrobiopterin.

Although UV spectrophotometric evidence has been recorded[70] for the oxidation sequence tetrahydrobiopterin ⟶ 7,8-dihydrobiopterin ⟶ biopterin in phosphate buffer at pH 7, and significant loss of side chain was observed[69] at 100°C, no other evidence has been presented for the occurrence of the former and no investigation of side chain loss under other conditions has been carried out.

Here, a brief investigation has been conducted in order (a) to examine the products formed at different pH values and different temperatures, and (b) to obtain further evidence for the formation of 7,8-dihydrobiopterin under conditions where no side chain loss was detectable.

(a) Effect of reaction conditions on oxidative side chain loss.

Method:

Tetrahydrobiopterin was dissolved in distilled water (pH 5) or 0.1M-sodium hydroxide (pH 13) to give a concentration of approximately 1 mg. cm^{-3}. The solutions were then autoxidised as follows:

(i) The distilled water solution was allowed to stand overnight without shaking in the refrigerator dew box (ca. 1°C).

(ii) The 0.1M-sodium hydroxide-solution was shaken under air at ambient temperature overnight.

(iii) Both solutions were placed in a water bath at 40°C and a steady stream of oxygen gas was bubbled through for six hours.

Samples of the solutions were then applied to 0.1 mm MN 300 cellulose layer t.l.c. plates, together with appropriate authentic materials, and eluted with the solvent systems used in the preceding section and others indicated in Table 2.3. (results).

TABLE 2.3. Effect of reaction conditions on products of tetrahydrobiopterin autoxidation as judged by t.l.c.

Appearance and R_f values of authentic materials. Solvent system in parentheses.	Distilled water 1°C overnight.	pH 13, 23° air overnight.	pH 13, 23° O_2/1h.	Distilled water 40° O_2/6h. *	pH 13, 40° O_2/6h.
Compound characterised as 7,8-dihydrobiopterin. Blue fluorescence	Intense blue			Intense blue	Faint blue
0.60 (1)	0.60 (1)			0.60 (1)	0.60 (1)
0.46 (2)	0.46 (2)			0.45 (2)	0.45 (2)
0.35 (4)	0.35 (4)			0.34 (4)	
Xanthopterin green-blue fluorescence.		Weak green-blue	Weak green-blue	Weak green-blue	Intense green-blue
0.42 (1)		0.42 (1)	0.42 (1)	0.39 (1)	0.42 (1)
0.23 (2)		0.23 (2)	0.23 (2)	0.26 (2)	0.23 (2)
0.41 (4)		0.41 (4)	0.41 (4)	0.42 (4)	0.42 (4)
Dihydroxanthopterin green fluorescence			Weak green		
0.24 (1)			0.24 (1)		
0.20 (2)					
0.30 (4)					
Biopterin blue fluorescence	Very weak blue	Intense blue	Intense blue	Intense blue	Intense blue
ca.0.5 (1)	0.52 (1)	0.54 (1)	0.52 (1)	0.48 (1)	0.48 (1)
0.50 (2)		0.50 (2)	0.52 (2)	0.45 (2)	0.50 (2)
0.43 (4)	0.45 (4)	0.44 (4)	0.44 (4)	0.42 (4)	0.42 (4)

Solvent systems as in Table 2.2., and:

(4) n-butanol-acetic acid-water 20:3:7

* Several other minor products (not characterised) were also present.

Results.

The results of t.l.c. analysis are shown in Table 2.3. Only a qualitative estimation of the relative amounts of xanthopterin and other products was available, as judged by the relative fluorescence intensities of each spot when viewed under UV light (366 nm). The results of the pH 13 study are easier to interpret, owing to the outstanding green-blue fluorescence of xanthopterin compared to other pterins. It was judged that at 23°C (room temperature) this compound represented only a minor reaction pathway, whilst its yield was greatly increased on increasing the temperature. Oxidations in distilled water at room temperature gave no xanthopterin, as expected from the THFA results, although some xanthopterin and biopterin were formed at 40°. Minor products were not identified, it being sufficient to judge the degree of side chain loss by the formation of xanthopterin at pH 13. Even under these conditions, the major product at room temperature appeared to be biopterin, and this is in agreement with the UV spectra of the reaction mixture, after dilution and adjustment to the required pH, which gave:

pH 13, λ_{max} 251, 360 nm.

pH 1, λ_{max} 245, 318 nm.

Literature (Blakley[18] p. 69) pH 13 λ_{max} 251, 361 nm.

pH 1 λ_{max} 247, 320 nm.

The oxidation at low temperature in distilled water showed only one major product having R_f values

identical to those of the compound isolated and characterised as 7,8-dihydrobiopterin (next section).

(b) Isolation and characterisation of 7,8-dihydrobiopterin.

Method.

The oxidation of tetrahydrobiopterin in distilled water appears to be a fairly clean reaction, with very little side chain loss. A solution of tetrahydrobiopterin (50 mg) in water (50 cm^3) was stirred under oxygen at 25° until 0.8 mol. equiv. of oxygen had been absorbed (measured manometrically - see Chapter 3). This partially ensures that any hydrogen peroxide formed during autoxidation (see later) does not lead to over-oxidation of the resulting compound. The product was freeze-dried, and since it appeared reasonably homogeneous on t.l.c. (R_f values in Table 2.3) with only minor contamination by biopterin, no further purification was attempted, owing to the small amounts available. Samples of the solid were examined by u.v. and n.m.r.

Results.

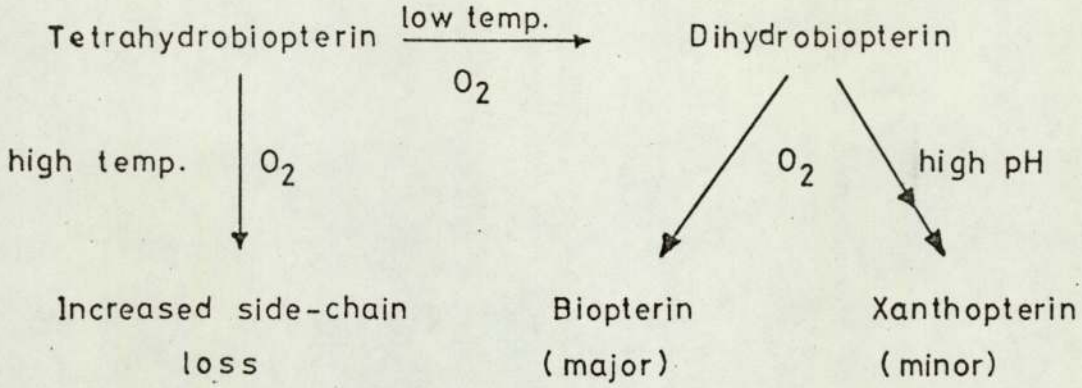
The spectral properties of the compound were consistent with 7,8-dihydrobiopterin:
u.v. λ_{max} (pH 1) 267 and 350 nm, λ_{max} (pH 7) 240, 280 and 330 nm, λ_{max} (pH 13) 280 and 330 nm (cf. literature[18])
n.m.r. (Trifluoroacetic acid) (τ) 1.5 br (amino-groups), 4.6 (2H,s,7-H_2, characteristic of 7,8-dihydropterins[18,105])

5.3 and 5.7 (1H each, poorly resolved, CH groups of side chain),and 8.55 (3H, d, J 8 Hz, methyl group of side chain). The n.m.r. spectrum also showed a small singlet (<1H) at τ 0.83, indicating contamination with biopterin, the oxidation product of dihydrobiopterin. It is well known that attempts to obtain solid dihydrobiopterin always result in some contamination with biopterin.[106]

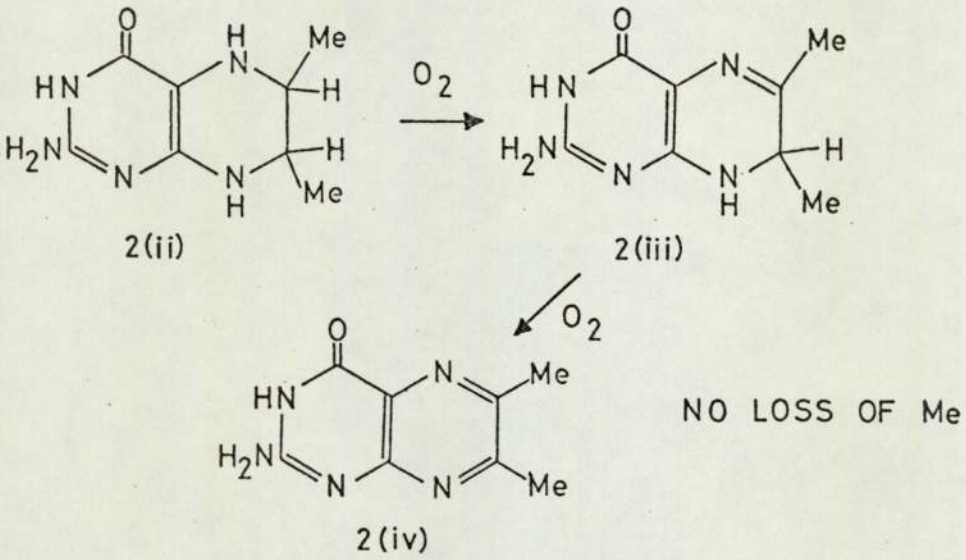
Discussion.

The results described above allow us to write a similar reaction pathway for tetrahydrobiopterin autoxidation as was presented for THFA. This is summarised in Scheme 2.2.

The loss of side chain is common to both reactions, though it seems to occur more readily with THFA than with tetrahydrobiopterin, as indicated qualitatively from the fact that xanthopterin appears to represent only a minor product from the latter at pH 13 and ambient temperatures, whilst THFA shows an approximately 50% cleavage to xanthopterin under similar conditions[58]. That this cleavage is dependent on the presence of a heteroatom (with a lone pair of electrons) in the β-position of the side chain is indicated by the results of the next section.



SCHEME 2.2 Schematic autoxidation pathway: Tetrahydrobiopterin



SCHEME 2.3 6,7-dimethyl tetrahydropterin autoxidation pathway

(4) The Autoxidation of 6,7-dimethyltetrahydropterin - the absence of side chain loss.

In order to ascertain whether the autoxidative loss of C(6) substituent required the presence of a heteroatom in the β-position as suggested, it was decided to examine the products from oxidation of the 6,7-dimethyl derivative, 2 (ii). Owing to the easily recognisable fluorescence of xanthopterin on t.l.c., which is the sole product of cleavage at pH 13, oxidations were carried out in 0.1M sodium hydroxide. Thus, any presence of xanthopterin would indicate side chain loss.

Method.

Solutions of the tetrahydropterin in 0.1M aqueous sodium hydroxide (ca. 1 mg in a volume of 0.5 cm^3) were:

(a) Shaken under air at ambient temperatures overnight;

(b) allowed to stand under oxygen in a stoppered tube at 40^o overnight.

The reaction solutions were examined by t.l.c. in the usual way, using 0.1 mm MN300 cellulose and 0.25 mm MN-Silica Gel G. layers.

Results and discussion.

The results of the t.l.c. examination are shown in Table 2.4. Although the major component of the reaction mixture could not be positively identified,

TABLE 2.4. Oxidation products of 6,7-dimethyltetrahydropterin at pH 13 - search for xanthopterin

Fluorescence and R_f values of standards and products.			
6,7-dimethyl pterin Literature [109]	Xanthopterin standard	pH 13, air, 23° products	pH 13, O_2, 40° products
		Weak blue 0.27 (1) 0.43 (4)	Weak blue 0.27 (1) 0.43 (4)
	Green-blue 0.42 (1) 0.23 (2) 0.41 (4)	xanthopterin absent	xanthopterin absent.
		Intense blue	Intense blue
		0.45 (1)	0.45 (1)
		0.59 (2)	0.59 (2)
		0.54 (4)	0.54 (4)
0.65 ± 0.10 (5)*		0.74 (5)*	0.74 (5)*
0.46 ± 0.02 (6)*		0.4 (6)*	0.4 (6)*
0.88 ± 0.05 (7)*		0.80 (7)*	0.80 (7)*
0.81 ± 0.03 (8)*		0.79 (8)*	0.79 (8)*
		Weak blue	Weak blue
		0.82 (1)	0.82 (1)
		0.75 (4)	0.75 (4)

* Using 0.25mm silica gel layer; others using MN 300 cellulose.

Solvent systems (1), (2), (4) as before. (5) Dimethylformamide/water 19:1

(6) 5% Citric Acid. (7) 5% aq. ammonia (8) 5% aq. ammonia/propan-2-ol 1:1

owing to the unavailability of the authentic material, the R_f values for t.l.c. on silica gel compare reasonably well with the literature values shown in the table for 6,7-dimethylpterin, though this technique is not expected to be unambiguous. The observation is also in agreement with Kaufman[65], so no synthesis of authentic material was carried out, it being considered unnecessary in view of the fact that loss of side chain was being sought by looking for xanthopterin in the products. The absence of this compound, even at elevated temperatures, confirms retention of C(6) substituent, and supports the contention that a heteroatom in the β-position of this substituent is essential for its loss. The oxidation of this compound is shown in Scheme 2.3.

U V spectral examination of the reactant solution gave:

λ_{max} pH 13 250, 350 nm λ_{min} 295 nm

literature: 6,7-dimethylpterin[107] λ_{max} pH 13 245, 355 nm.

λ_{min} 290 nm.

7,8-dihydro-6,7-dimethylpterin[26] λ_{max} pH 13 281, 320 nm.

(5) Peroxides - Organic or Inorganic?

Since most "normal" autoxidation reactions do proceed with the formation of organic hydroperoxides, attempts were made to detect these qualitatively, using the usual chemical test reagents, and to establish their identity.

Methods.

(a) Isolation of solid peroxidic material.

Tetrahydrobiopterin is soluble in a mixture of ethanol and aqueous 0.1M-sodium hydroxide (4:1) and the mixture remains liquid at temperatures of -78°C (dry ice - acetone bath). Solutions of tetrahydrobiopterin in this medium (2 mg. cm^{-3}) were oxidised under air or oxygen with constant stirring at -78° for 2 - 4 hours to allow accumulation of peroxides. Half of the reaction mixture was neutralised slowly by dropwise addition of a previously determined volume of 0.1M hydrochloric acid (Analytical Reagent grade) care being taken to keep the temperature below - 70°. The two solutions were then connected to a rotary vacuum pump, via a liquid nitrogen trap, and the solvent slowly removed whilst keeping the temperature at -78° to -65°. The solid material collected in both cases was tested for peroxides with freshly prepared ferrous thiocyanate solution (ferrous ammonium sulphate and ammonium thiocyanate dissolved in 0.1M sulphuric acid), the most sensitive test reagent.

(b) T.l.c. characterisation of peroxides.

Since the process of solvent removal takes considerable time at the temperatures employed, this might result in decomposition of peroxidic material. Therefore, samples of the alkaline solution from (a) were examined chromatographically. The solution was rapidly spotted on to chilled silica gel (0.25 mm) t.l.c. plates (0^{o}) alongside standards of hydrogen peroxide dissolved in the NaOH-ethanol solvent used. (Silica gel plates previously eluted with methanol-concentrated hydrochloric acid (10:1) to remove any iron contaminant, which may destroy peroxides, and re-activated at 110^{o} for 35 min.[108]). The chromatograms were eluted at 0^{o} in the refrigerator in the solvent systems shown in Table 2.5, and developed by spraying with a 1% aqueous solution of N,N-dimethyl-p-phenylenediamine and warming, when pink spots appeared where peroxides were present.

Results and discussion.

The dried material obtained prior to neutralisation in (a) gave an intense red colouration when treated with ferrous thiocyanate, thus indicating the presence of a peroxide. However, the neutralised material did not give a positive test result. Thus, it seems that the peroxide present was sodium peroxide, which liberated hydrogen peroxide on neutralisation. The latter would be lost on removal of solvent, whilst non-volatile organic peroxides would be expected to have remained.

The results of t.l.c. are recorded in Table 2.5, and show that hydrogen peroxide is positively identified in the reaction mixture. No other peroxide was found on the chromatograms, so again it appears that organic hydroperoxides are not produced.

TABLE 2.5

Detection of hydrogen peroxide in low-temperature oxidation of tetrahydrobiopterin.

Solvent System	R_f values of peroxides detected.	
	H_2O_2 standard	Oxidised tetrahydro-biopterin.
Water-ethanol-chloroform 20:17:2	0.82	0.82
Propanol-1% aq. ammonia 2:1	0.56	0.55
Carbon tetrachloride-acetone 2:1	0.27	0.27

In conclusion, it may be said that although the product analysis gives information about the nature of C(6) substituents likely to be lost during autoxidation of tetrahydropterins, these observations tell us very little

about the actual intermediates involved. However, the fact that when this substituent is methyl no xanthopterin is observed in the products seems to militate against the proposal of C(6) hydroperoxide intermediates suggested by Stocks-Wilson[58]. Such intermediates would be expected to rearrange (to give xanthopterin) when a methyl substituent is present, as in the case of other reactions involving methyl group shifts (e.g. Wagner-Meerwein and Wolff rearrangements). Furthermore, it may be argued that the failure to detect organic hydroperoxides at low temperatures may be due to the extreme lability of any such compounds, which might easily decompose to give hydrogen peroxide and an oxidised pterin compound (e.g. the dihydropterin intermediate discussed in Chapter One).

Therefore, it is necessary to consider in some detail the kinetics of the autoxidation process, and to look for possible kinetic methods of determining the nature of individual steps in the reaction sequence. This is approached in Chapters 3 and 4, which give information revealing important aspects of the autoxidation process.

CHAPTER 3.

The basic kinetics of tetrahydropterin autoxidation using tetrahydrofolic acid and tetrahydrobiopterin as model compounds.

At the outset of a kinetic study of the type undertaken here, it is necessary to consider the methods which may be available for determining reaction rates, and their particular merits or deficiencies. It is well known that at neutral pH's, autoxidation of tetrahydropterins is accompanied by a considerable change in the ultraviolet absorption spectrum. However, apparent rates of reaction judged by this phenomenon are found[58] to be slower in 0.1M sodium hydroxide than in phosphate buffer, pH 7, whereas the results of the present study show just the opposite. The reason for this apparent slowness of reaction at pH 13 is that under these conditions the u.v. spectra of starting compound and products are very similar, so that very little change is observed. Therefore, in this study, the potential reproducibility of spectrophotometric methods was forfeited in favour of a slightly more inaccurate manometric technique for measuring the quantity of oxygen consumed during reaction, since no spurious results due to changes in conditions can occur. Changes in oxygen solubility under the varying solution conditions employed are very slight[110] and unlikely to introduce large errors into the reaction rates computed from data obtained in this way.

Methods.

The apparatus consisted of a 250 cm^3 glass reaction flask connected to a conventional Warburg water manometer, and having an inlet for oxygen which could be closed by

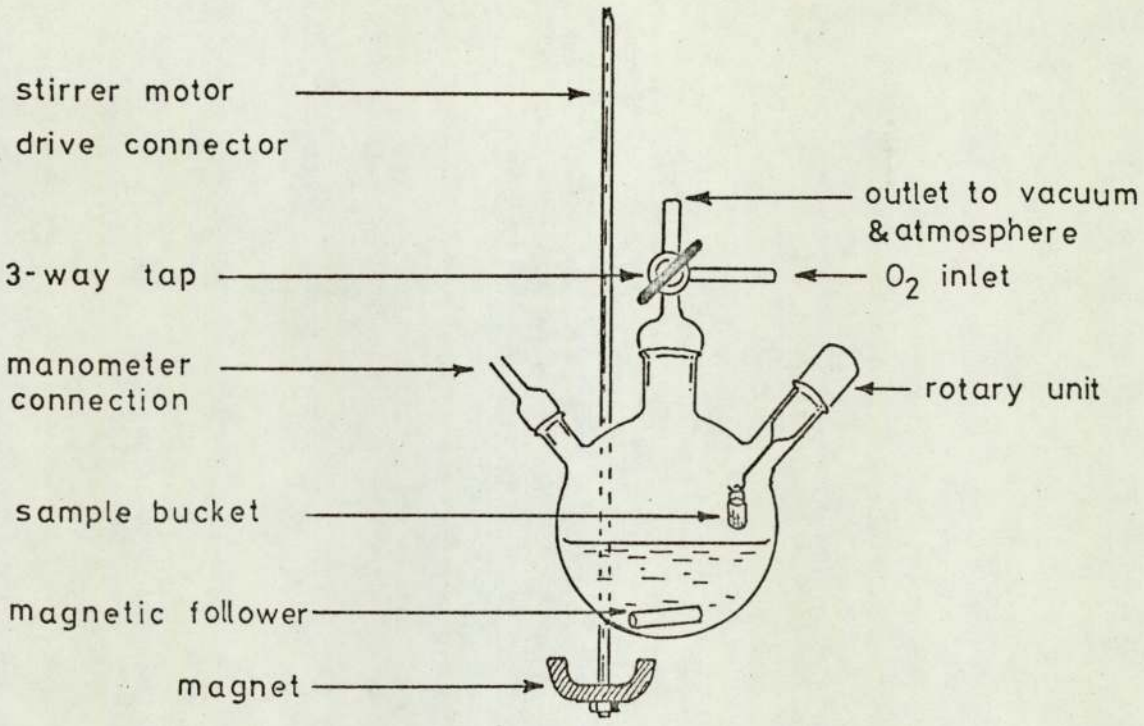


FIG. 3.1 Reaction vessel for manometric studies

means of a three-way glass tap (Fig. 3.1.) The flask, containing 100 cm^3 of aqueous solvent made up using glass-distilled water, was immersed in a thermostated water bath (temperature variation not greater than $\pm$ 0.01°) and was purged with a stream of oxygen gas for 20 min. with rapid magnetic stirring. In assembling the apparatus, all ground-glass joints were made gas-tight with a thin smear of Vaseline prior to connection. The weighed tetrahydropterins were introduced into a side-neck in an open glass ampoule and suspended above the solvent, the gas supply shut off, and thermal equilibration awaited. Thus, the sample was kept dry and under these conditions did not oxidise. The ampoule was then allowed to fall into the rapidly stirred solvent and the pressure change after adjustment to constant volume was noted at intervals of 1 min. or less depending on the rate obtained. Correction for variation in atmospheric pressure and bath temperature was made using a similar reaction vessel, containing water, as thermobarometer. Calibration of the apparatus was achieved by determining the weight of water required to completely fill it, hence calculation of the volume of gas contained in the system and conversion of pressure change to moles of oxygen could be made (details in Appendix B). Reproducibility of reaction stoichiometries using different reaction vessels showed calibration to carry an error of ca. $\pm$ 5%. The oxygen uptake curves were analysed by the initial rates method (after any induction period had ceased), and although this carries inherent inaccuracies

due to the rather subjective drawing of tangents involved, it overcomes difficulties due to oxidation of the initial products (e.g. 7,8 dihydro-compounds) and interference from secondary reactions with the hydrogen peroxide generated (Chapter Two). In most runs, tangents to the initial part of the curve were easy to estimate owing to an almost linear uptake of oxygen for 2-5 mins., and runs done under identical conditions showed accuracy to within $\pm$ 10%.

Variation of oxygen partial pressures was achieved by mixing known volumes of oxygen and nitrogen and introducing the mixture into a vacuum de-gassed reactor. Dark rates were determined using a reaction vessel coated with black paint and enclosed in aluminium foil.

The pH-dependence was studied by incorporating into the solvent the quantity of 0.1M sodium hydroxide or 0.1M hydrochloric acid required to give the desired solution pH, which was then measured immediately after oxidation. Examination of solution pH before and after a pilot run showed a change of less than $\pm$ 0.1 pH units during oxidation.

Spurious results due to contamination of the reaction vessel were eliminated as far as possible by frequent overnight soaking in chromic acid followed by thorough washing with cold glass-distilled water.

RESULTS.

Oxygen uptake curves and stoichiometry of reaction.

Fig. 3.2 shows some typical plots of oxygen

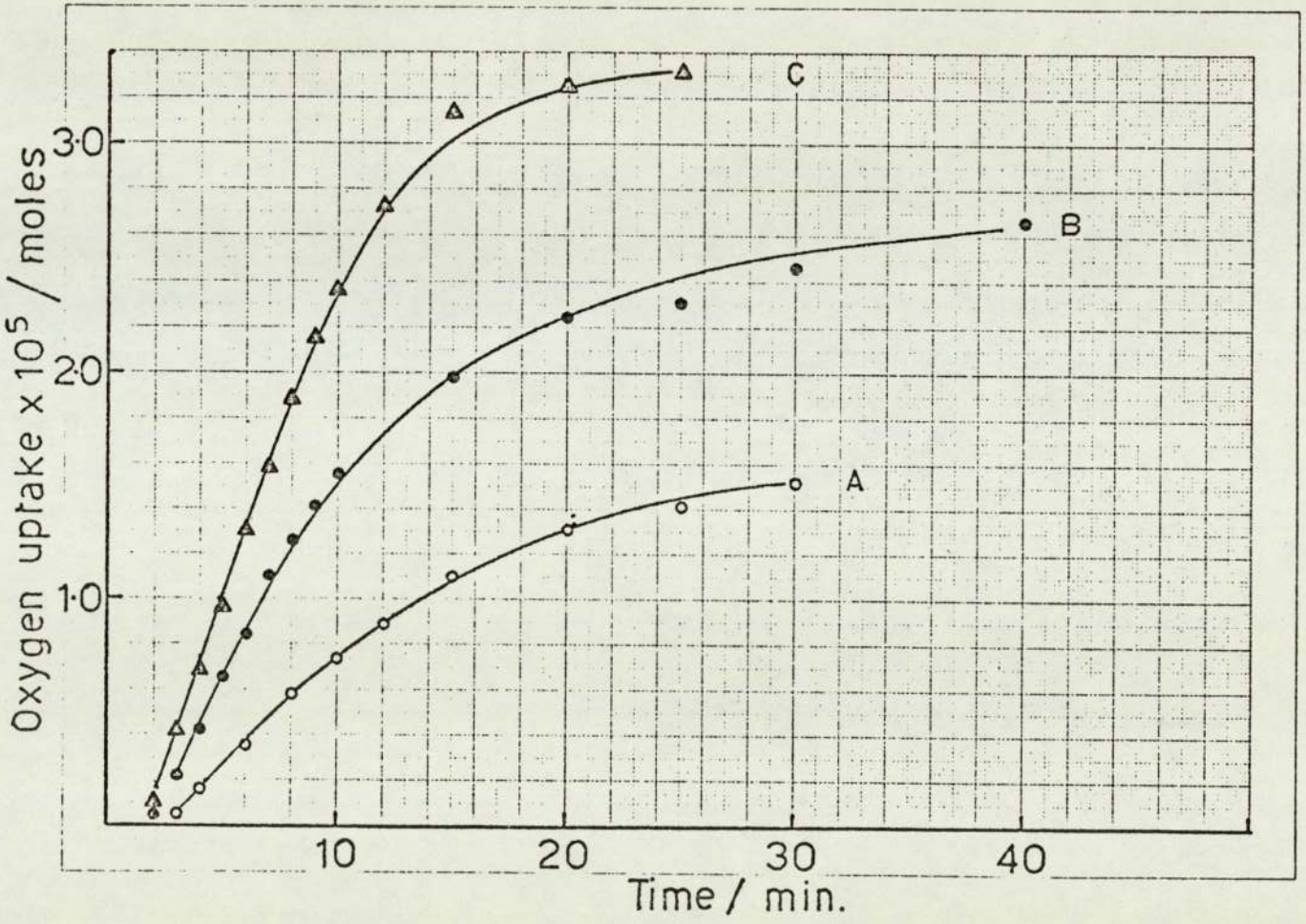
FIG. 3.2.

Typical oxygen-uptake curves for autoxidation of tetrahydrobiopterin in 0.1M-sodium phosphate buffer under oxygen gas at 25°C.

A) 0.156 mM - tetrahydro-biopterin.

B) 0.312 mM

C) 0.468 mM



absorption against time under an atmosphere of oxygen gas from which initial rates were determined. It can be seen that there is a levelling out of the curve after about 20-30 minutes oxidation, although oxygen uptake is observed to continue at a much slower rate. This is indicative of two steps to the reaction; a fairly rapid oxidation of the tetrahydropterin to 7,8-dihydropterin (and corresponding products from side chain loss) followed by a slower conversion of the dihydro-compounds to fully oxidised materials. Approximate stoichiometries of the first oxidation step can be obtained by determining the quantity of oxygen consumed after about 40 mins. reaction, and at low concentrations of the tetrahydropterin it is observed that 1 mole of oxygen is consumed per mole of starting material. However, increase in concentration of tetrahydropterins in the region 0.4 - 1.0 mM causes a decrease in the stoichiometry (Oxygen: tetrahydropterin ratio) which approaches ca. 0.7 (Table 3.1), in agreement with observations by Mager and Berends and Viscontini (Chapter 1).

This is readily explained as being due to secondary oxidation of tetrahydropterin by the hydrogen peroxide generated during the reaction. At low concentrations there is very little hydrogen peroxide formed, but as the concentration becomes higher the secondary reaction becomes more appreciable. Hence, the adoption of the initial rates method for the kinetic studies.

It may be noted that this effect can be explained <u>without</u> recourse to postulating organic hydroperoxide

TABLE 3.1.

Variation of reaction stoichiometry with tetrahydropterin concentration. (pure oxygen gas phase).

Reactant	Concentration (mM)	Stoichiometry (O_2: tetrahydropterin)
THFA	0.2	1.0
THFA	0.4	0.75
THFA	0.6	0.65
THFA	0.8	0.65
Tetrahydrobiopterin	0.156	1.0
Tetrahydrobiopterin	0.312	0.89
Tetrahydrobiopterin	0.468	0.78
Tetrahydrobiopterin	0.625	0.75

intermediates which cause the secondary oxidations.

Also from Fig. 3.2. it can be seen that there is a short induction period prior to commencement of oxygen absorption. That this is not a lag phase due to poor stirring, affecting diffusion of gas into solution, was demonstrated by the observation that a similar effect is obtained when the reaction is followed spectrophotometrically (Chapter 4).

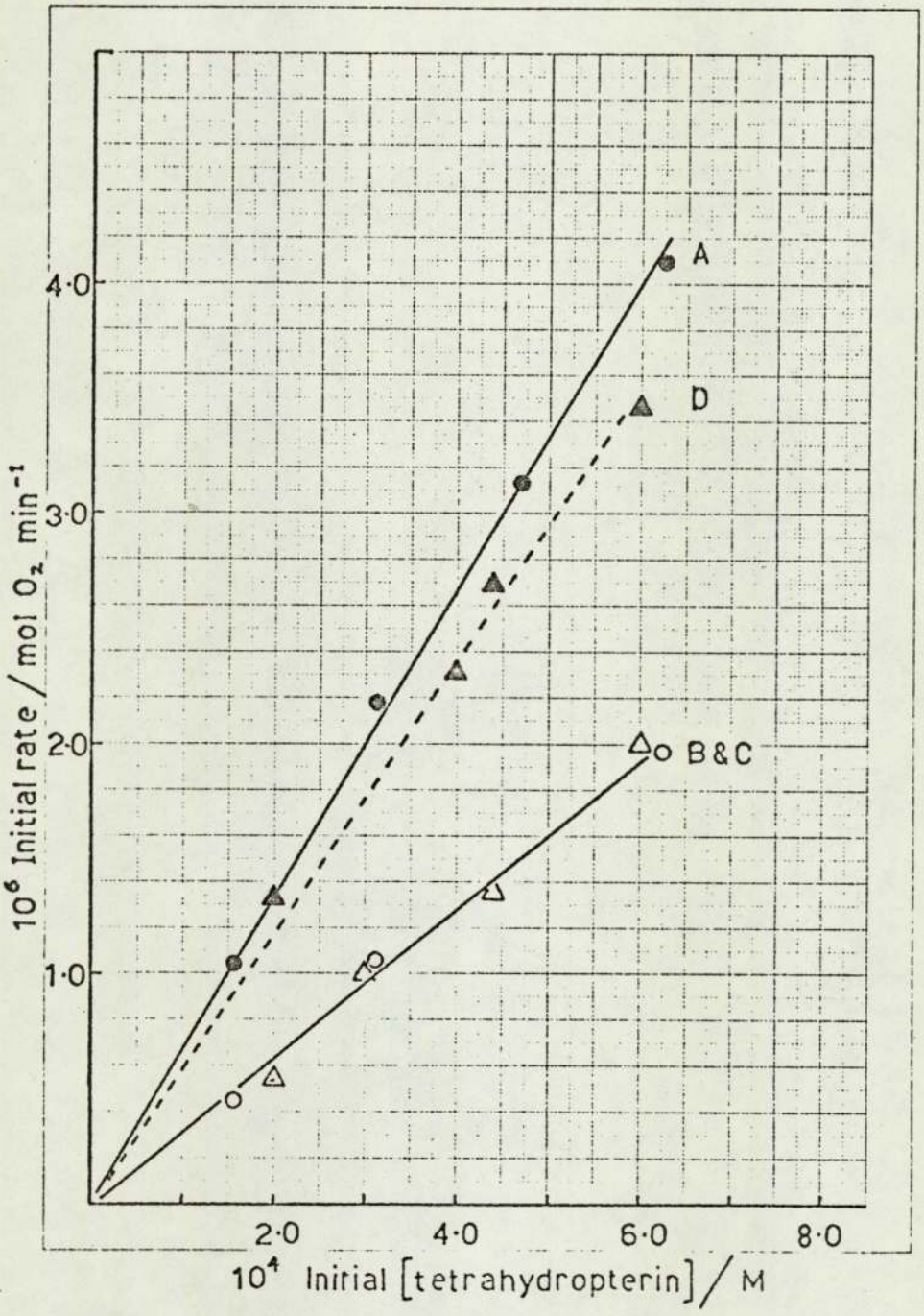
The Kinetic Order of Reaction with respect to Tetrahydropterin Concentration.

The initial rates data over a range of concentration of tetrahydropterins show that there is a linear relationship between initial rate and initial concentration with both air and oxygen as the gas phase and in the absence of light, thus showing that the reaction is first order with respect to tetrahydropterin (Fig. 3.3). This observation has since been confirmed by Jaenicke and Kretzschmar[84]. Such a result is not surprising, and tells us very little about the reaction mechanism in the absence of any other kinetic observations.

Numerical values for all rate data obtained are given in Appendix C for reference.

FIG. 3.3

Linear dependence of initial rates on initial tetrahydropterin concentration for autoxidations at 25°C in 0.1M - sodium phosphate buffer.



A) Tetrahydrobiopterin/oxygen gas light rates

B) (circles) Tetrahydrobiopterin/air light rates

C) (triangles) Tetrahydrofolic acid/air light rates.

D) Tetrahydrofolic acid/oxygen gas dark rates.

The Effect of trace amounts of transition metal ions, chain-breaking inhibitors and other additives or modifications.

Owing to the possibility of heavy metal contamination of any buffers used during isolation and storage of naturally occurring reduced pteridines, it was decided to examine the catalytic effects of some of these on autoxidation of THFA and tetrahydrobiopterin. The results are shown in Table 3.2. Three transition metals were studied, and of these ferrous and ferric ions had no effect at impurity concentration levels, whereas addition of cupric ion gave rise to a marked acceleration and chromate showed slight catalysis. The effect of changing concentration of the cations was not investigated since the results are subject to ambiguous interpretation (see later). Incorporation of EDTA into the solution completely eliminated the catalytic effect of cupric ion. The apparently differing catalysis of various buffers has been suggested by Blakley[76] as being due to different levels of heavy metal impurities. That this is probably true can be seen by examination of the results of Table 3.2 (a) for sodium phosphate and ammonium acetate buffers (both at pH 7). For instance, when the concentration of the latter is increased from 0.1M- to 2.5M- an approximately 2-fold increase in rate is observed, and the catalysis is not due to increasing acetate anion concentration, since incorporation of EDTA reduces the rate to the normal value. In 0.1M-sodium phosphate, very little apparent inhibition

TABLE 3.2.

Inhibitor and catalyst effects on autoxidation rates - expressed as initial rate relative to oxidations in 0.1M-phosphate buffer, under oxygen gas.

3.2 (a) Tetrahydrobiopterin oxidations. 25°C.

Buffer Solution	Gas phase	Additive or other modification	Relative rate
0.1M-Phosphate; pH 7	Oxygen	None	1.00
0.1M-Phosphate; pH 7	Oxygen	0.02M EDTA	0.98
0.1M-Phosphate; pH 7	Oxygen	2mM-8-hydroxy-quinoline-5-sulphonic acid	0.56
0.1M-Phosphate; pH 7	Oxygen	2 mM - Phenol	0.53
0.1M-Phosphate; pH 7	Oxygen	2% v/v Ethanol	0.62
0.1M-Phosphate; pH 7	Oxygen	2% v/v Methanol	0.69
0.1M-Phosphate; pH 7	Oxygen	Dark rate	0.92
0.1M-Sodium hydroxide	Oxygen	None	3.44
0.1M-Sodium hydroxide	Oxygen	0.24 mM - EDTA	3.67
2.5M - Ammonium acetate	Air	None	1.03
2.5M - Ammonium acetate	Air	0.24 mM - EDTA	0.37
0.1M - Ammonium acetate	Air	None	0.53
20 mM - EDTA/water	Air	0.1 mM Cu(II) (chelated)	0.37
20 mM - EDTA/water	Air	0.5mM Cu(II) (chelated)	0.36
20 mM - EDTA/water	Air	0.3mM hydrogen peroxide	0.38
0.1M-Phosphate; pH 7	Air	0.02 M - EDTA	0.45

continued ....

TABLE 3.2. (continued)

3.2 (b) Tetrahydrofolic acid oxidations. 25°C.

Buffer solution	Gas phase	Additives or other modifications.	Relative Rate
0.1M-Phosphate; pH 7	Air	None	1.00
0.1M-Phosphate; pH 7	Air	0.1 mM-8-hydroxy-quinoline-5-sulphonic acid.	0.45
0.1M-Phosphate; pH 7	Air	0.3 mM-Phenol	0.55
0.1M-Phosphate; pH 7	Air	0.1 mM Cu (II)	5.00
0.1M-Phosphate; pH 7	Air	0.1 mM Cu(II); 0.24 mM - EDTA	0.91
0.1M-Phosphate; pH 7	Air	0.1 mM Fe(III)	1.0
0.1M-Phosphate; pH 7	Air	Powdered glass (8 g.p.l.)	1.0
0.1M-Phosphate; pH 7	Air	0.15 mM Chromic acid (as CrO_3)	1.82
NaOH; pH 7	Oxygen	None	2.52
NaOH; pH 7	Oxygen	10^{-5}M-Fe(II)	2.82

TABLE 3.3.

Atomic absorption analysis of buffer solutions for copper and iron content.

Buffer	Copper (M)	Iron (M)
0.1M - Sodium phosphate	8.8×10^{-7}	4.28×10^{-6}
0.1M - Sodium hydroxide	6.0×10^{-7}	1.61×10^{-6}
2.5M - Ammonium acetate	14.2×10^{-7}	1.07×10^{-6}

is produced by EDTA, and the rates for these two buffer systems including the chelating agent are very similar. Using atomic absorption spectroscopy, appreciable concentrations of copper were found in 2.5M-ammonium acetate solution, whilst concentrations in 0.1M-phosphate are rather lower. The concentrations of iron show the reverse trend, indicating that catalysis is probably specific to copper (Table 3.3). The reaction is not catalysed by Cu(II) - EDTA complexes, since change in concentration of this produces no noticeable change in rate. Therefore, great care must be exercised over choice of reagent for buffer solutions when working with reduced pteridines.

Hydrogen peroxide at low concentrations had no effect on initial rate, so that autocatalysis by this is unlikely.

It may also be noted from Table 3.2 that the incorporation of finely powdered soda-glass into the autoxidation reaction has no catalytic effect. This particular aspect was investigated owing to reports[111] that liquid phase autoxidation of tetralin shows a surface catalysis (e.g. by colloidal silica, alumina, powdered glass) which is not due to heavy metal content of the substances used, and which is related to the surface area employed.

Table 3.2. also shows the effects of small concentrations of various known inhibitors of autoxidation reactions. It is seen that phenol and 8-hydroxyquinoline-5-sulphonic acid both reduce the rate by a factor of two.

TABLE 3.4.

Further inhibition studies of tetrahydrobiopterin autoxidation; ascorbate and ethanol effects.

Solution phase	Gas phase	Specific rate* (rate ÷ concentration).
0.1M - Phosphate; pH 7	Air	0.037 min^{-1}
0.1M-Phosphate/0.01% sodium ascorbate; pH 7	Air	0.016 min^{-1}
Distilled water ** pH ca 4.5	Air	55.6×10^{-4} min^{-1}
Ethanol/Water, 8:1 **	Air	3.44×10^{-4} min^{-1}

* determined spectrophotometrically at ambient temperature.

** following increase in absorbance at 330 nm

That the latter is not acting solely to remove Cu(II) by chelation is seen by comparing this with the effect of EDTA. At low concentrations, ascorbate shows a similar inhibition (Table 3.4) although this had to be measured by spectrophotometric determination of the specific initial rates for tetrahydrobiopterin oxidation at room temperature (method as described in Chapter Four), owing to the ready oxygen uptake by ascorbate itself. Aliphatic alcohols, notably ethanol, also produce inhibition effects at low concentration (Table 3.2). At much higher concentrations (8:1 Ethanol/water) a very large reduction in rate was observed spectrophotometrically (Table 3.4; O_2 uptake not measured owing to (a) slowness of reaction and (b) large vapour pressure of ethanol giving inaccurate pressure readings) and this cannot be due to oxygen solubility effects because the gas is in fact more soluble in ethanol than in water (volume % absorbed O_2 is 2.98 in water and 5.66 in 80% aqueous ethanol[110, 112]).

Exclusion of light does not markedly affect rates measured under pure oxygen (but see later) indicating the involvement of ground state oxygen. (Table 3.2 (a)).

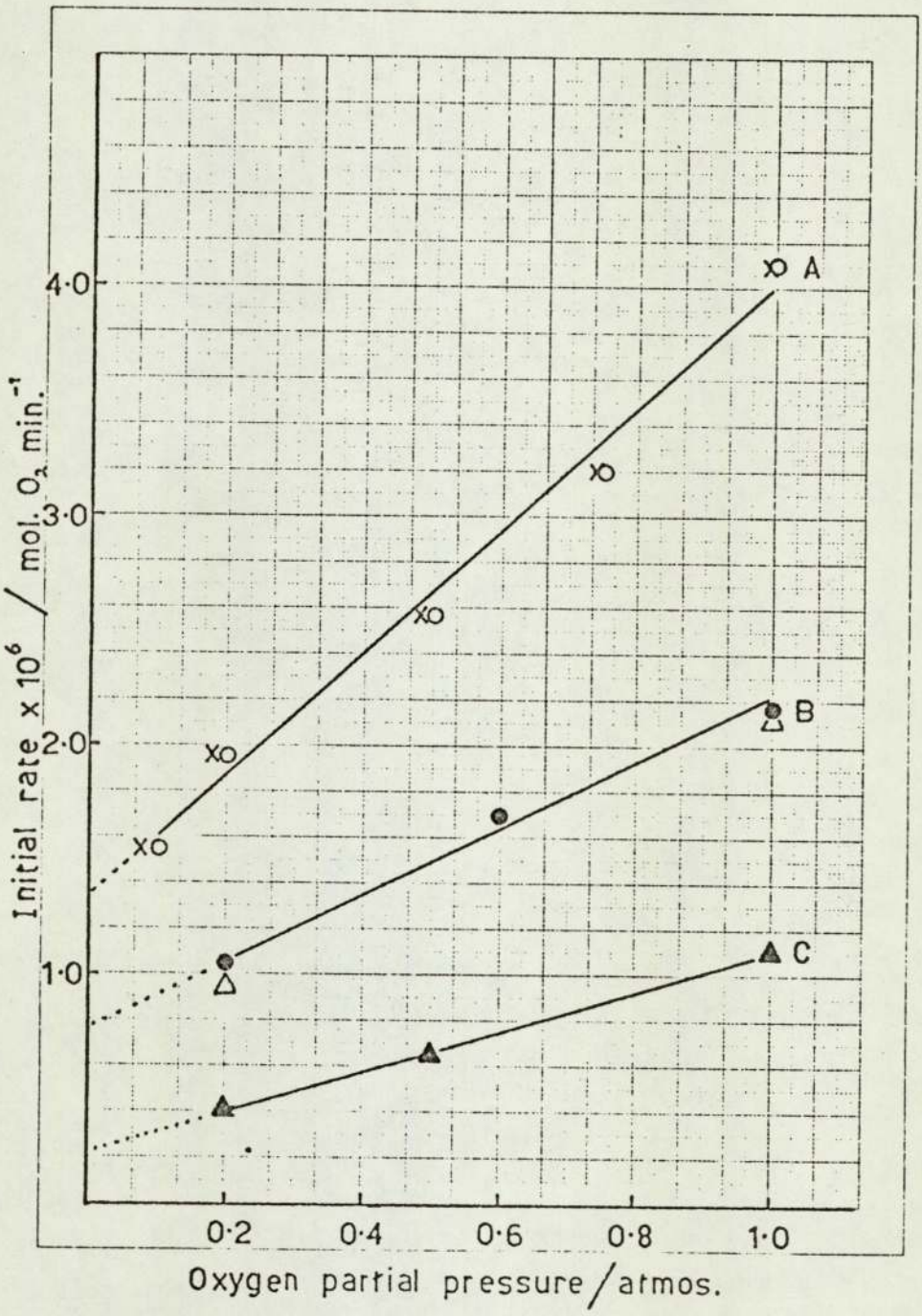
The dependence of reaction rate on partial pressure of oxygen in the gas phase.

In contrast to the simple first order relationship between initial rate and initial tetrahydropterin concentration, the kinetic dependence on oxygen concentration is rather more complex. Most of the investigations were carried out using tetrahydrobiopterin, it being assumed that THFA behaves similarly. The results will be presented first, and a full discussion given later.

Figures 3.4 and 3.5 show the oxygen pressure dependence of both light and dark rates for tetrahydrobiopterin, whilst Fig. 3.6 shows the dependence for THFA. It can be seen that correction of oxygen partial pressure for water vapour pressure has little effect. Although some plots of log (rate) vs. log (oxygen pressure) for the light rates give a reasonable straight line of slope approximately 0.5 (Fig. 3.7), it is observed that the reaction in fact does not have a kinetic order of 0.5 with respect to oxygen (note the curvature in Fig. 3.7 A). This is shown by the poor correlation in a plot of rate vs. square-root of oxygen pressure, as compared to the rather better linearity of a graph of rate vs. oxygen pressure (compare Figs. 3.4 and 3.5 with Fig. 3.8). It appears, therefore, that for both light and dark rates there is a combination of zero- and first-order dependence on oxygen concentration, and the kinetics obey the

FIG. 3.4

Dependence of tetrahydrobiopterin light rates on gas phase oxygen pressure in 0.1M - sodium phosphate buffer at 25°C.



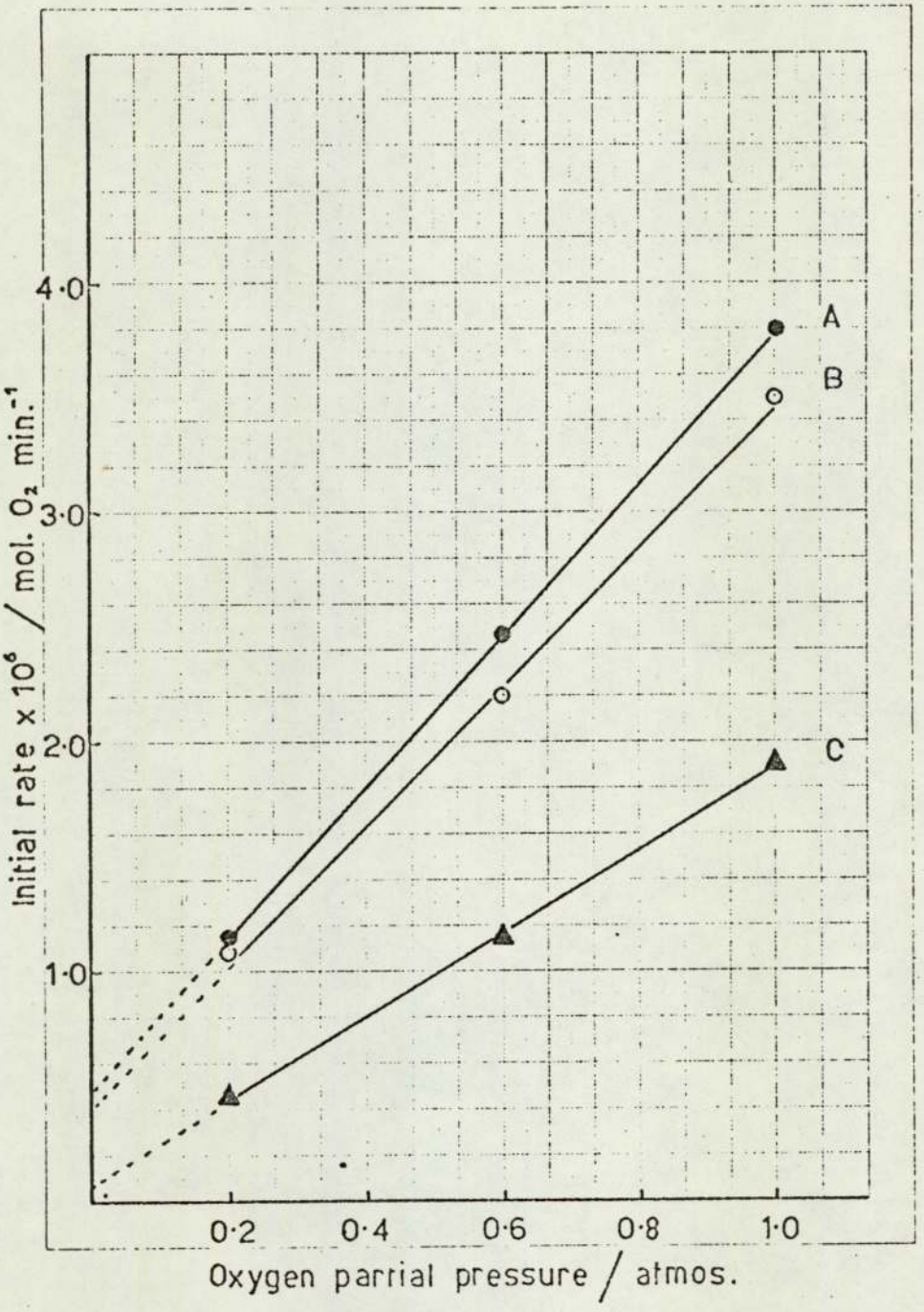
A) 0.625 mM-tetrahydrobiopterin. Crosses show pressures corrected for water vapour pressure.

B) 0.312 mM (dark circles). Open triangles show corresponding rates in presence of EDTA (0.02M).

C) 0.156 mM.

FIG. 3.5

Dependence of Tetrahydrobiopterin dark rates on gas phase oxygen pressure at 25°C.



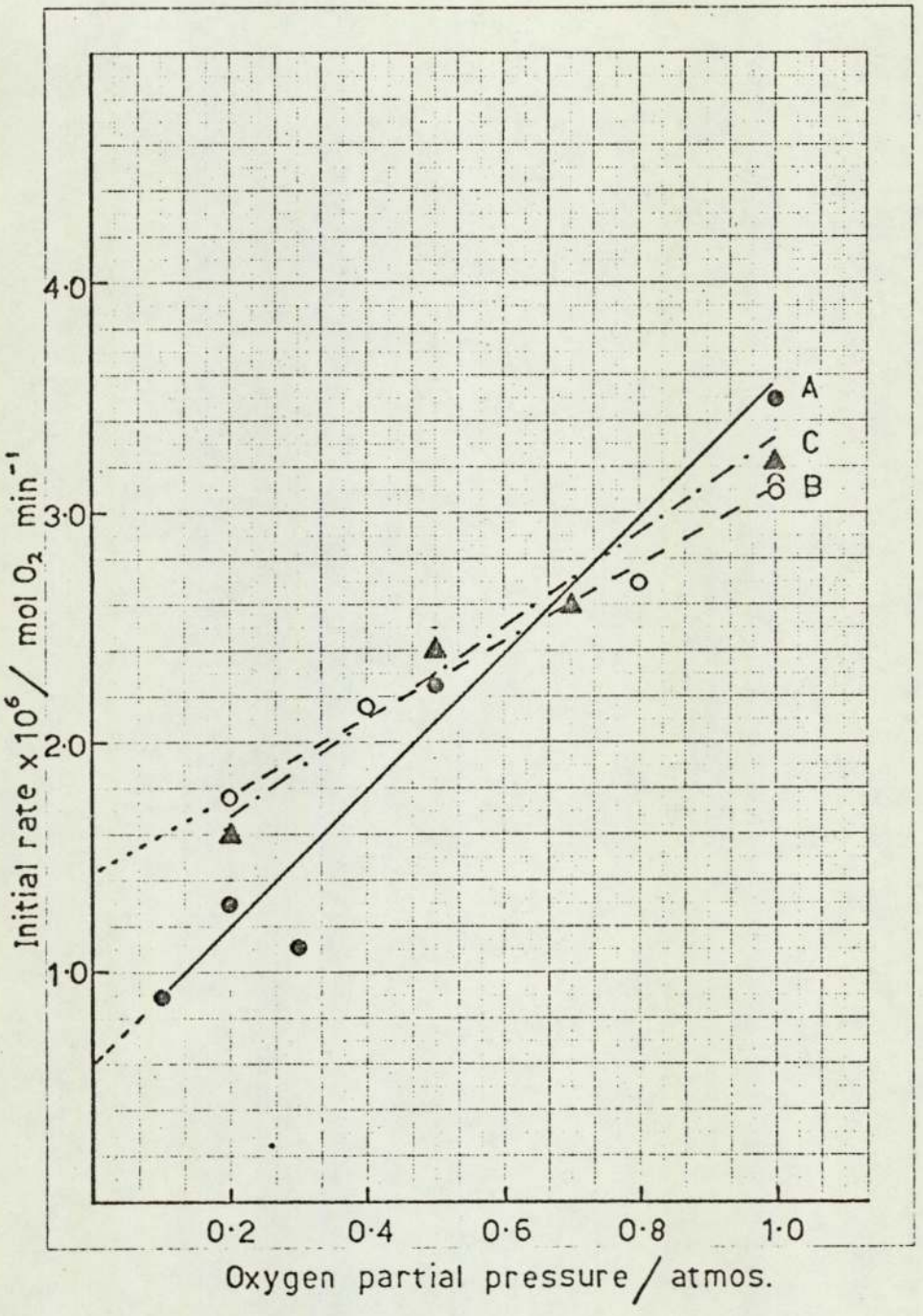
A) 0.625 mM in 0.1M - sodium phosphate buffer.

B) 0.625 mM in 0.01M - sodium phosphate.

C) 0.312 mM in 0.1M - sodium phosphate.

FIG. 3.6

Oxygen dependence of Tetrahydrofolic acid autoxidation rates at 25°C in daylight.



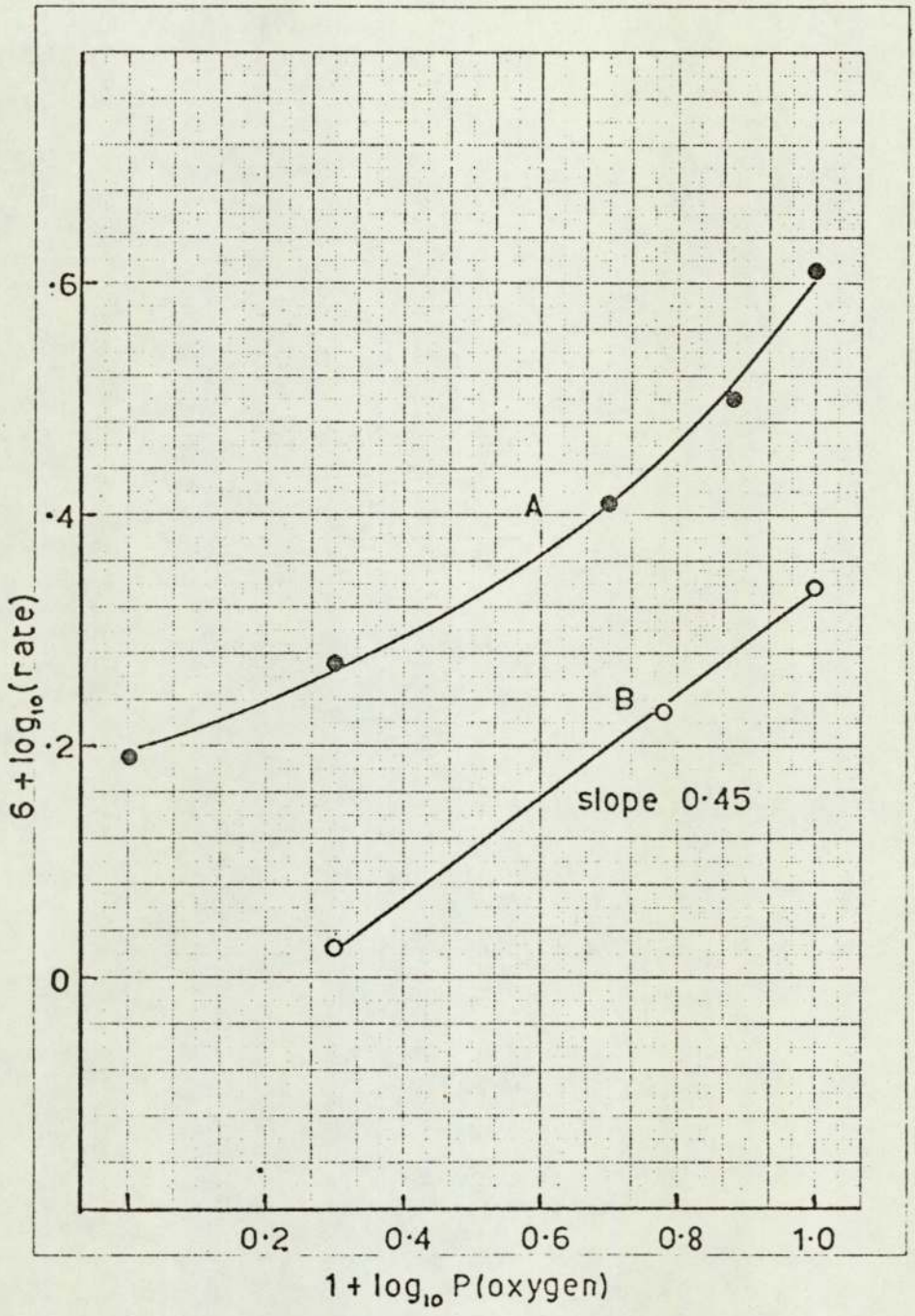
A) Closed circles. 0.7mM-THFA in 50 ml. of 0.1M-sodium phosphate buffer, using small soda glass reaction vessel (150 cm^3).

B) Open circles. 0.4mM-THFA in 100 ml. of 0.1M-sodium phosphate buffer using large Pyrex glass vessel.

C) Triangles 0.23 mM-THFA in 100 ml. of 0.1M-sodium hydroxide using large Pyrex glass vessel.

FIG. 3.7

Log-log plots of rate dependence on oxygen pressure in 0.1M-sodium phosphate buffer at 25°C. shows possibility of half-order kinetics.

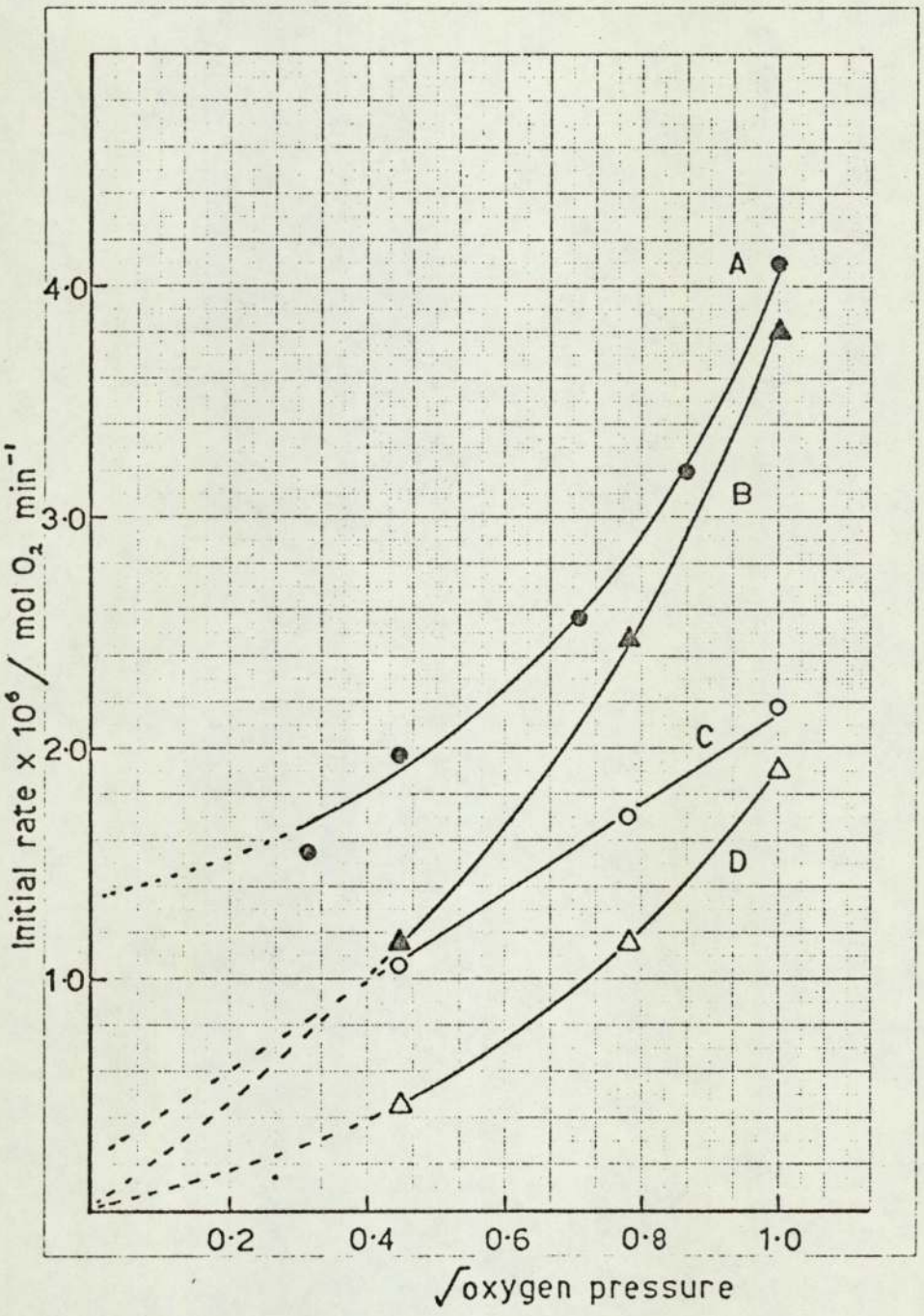


A) 0.625 mM tetrahydrobiopterin light rates.

B) 0.312 mM tetrahydrobiopterin light rates.

FIG. 3.8

Attempted fit of data to half-order dependence on oxygen for oxidations of tetrahydrobiopterin in 0.1M-sodium phosphate buffer at 25°C.



A) 0.625 mM light rates.

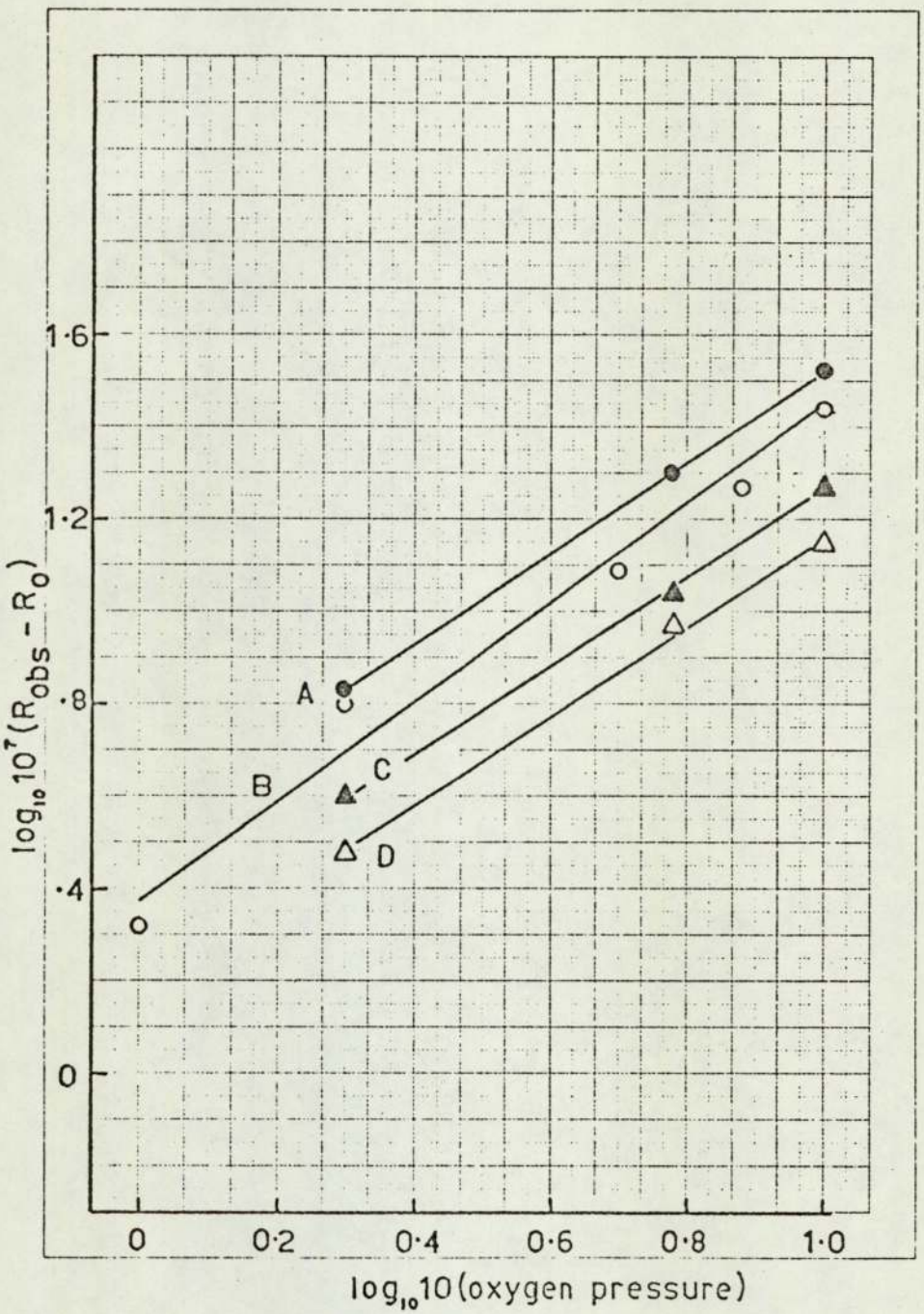
B) 0.625 mM dark rates.

C) 0.312 mM light rates.

D) 0.312 mM dark rates.

FIG. 3.9

Log-log plots showing validity of combined zero- and first-order dependence on oxygen. Using tetrahydrobiopterin in 0.1M-sodium phosphate buffer at 25°C. (see text).



A) 0.625 mM; dark rates; slope 0.98
B) 0.625 mM; light rates; slope 1.08
C) 0.312 mM; dark rates; slope 0.95
D) 0.312 mM; light rates; slope 0.97

relationship (1):

$$\text{Rate} = (H_4\text{Pter.}) \left\{ k_a + k_b \, (\text{oxygen}) \right\} \qquad (1)$$

Where H_4Pter represents tetrahydropterin.

That this is so is supported by the observation that a plot of log $(R_{OBS} - R_0)$ vs. log (oxygen pressure), where R_{OBS} = observed rate and R_0 = rate at zero oxygen pressure obtained by extrapolation, gives a good straight line of unit slope in all cases (Fig.3.9). Furthermore, the kinetic order with respect to tetrahydropterin is not dependent on oxygen pressure since the rate shows linear dependence on tetrahydropterin concentration under both air and oxygen (Fig. 3.3). The validity of the empirical rate equation (1) is thereby confirmed, and the observations are not an artefact caused by inefficient stirring resulting in slow diffusion of oxygen into solution.

A striking observation is that the relative values of k_a and k_b in (1) appear to be affected by the exclusion of daylight from the reaction vessel. Earlier reports[113,114] that light rates for THFA and dark rates for tetrahydrobiopterin showed similar oxygen pressure dependences may well have been due to the use of substantially different reaction vessels for the determinations. The former were obtained using a soda-glass vessel of small dimensions (total volume ca. 150 cm^3), whilst the latter used a 250 cm^3 Pyrex glass vessel. Later determinations in the latter vessel gave revised THFA light rates paralleling those of tetrahydrobiopterin (Fig. 3.5). These effects,

TABLE 3.5

Effect of reaction conditions on k_a/k_b ratio (equation 1) at 25°C derived from graphs of rate vs. oxygen pressure for tetrahydrobiopterin.

Reaction conditions (0.31 mM tetrahydrobiopterin)	Intercept x 10^6/mol.O_2.min^{-1}	Slope x 10^6/mol.O_2.min^{-1} atmos.$^{-1}$	Intercept - Slope (k_a/k_b)/atmos.
Daylight, 0.1M-Phosphate; pH 7	0.75	1.40	0.54
Daylight, 0.1M-Phosphate; pH 7 0.02 M - EDTA	0.70	1.40	0.50
Daylight, 0.1M-Phosphate; pH 7; 2 mM - 8 - hydroxyquinoline - 5 - sulphonic acid	0.35	0.75	0.47
Direct irradiation - Hanova photochemical reactor lamp; 0.1M-Phosphate, pH 7. 0.02M - EDTA	0.7	0.9	0.78
Dark rates; 0.1M-Phosphate; pH 7	0.1	1.8	0.056

apparently casting doubt on reproducibility, might possibly be due to differing light-absorption of the glasses, or to the surface area: solution volume ratios permitting the apparent 'photochemical' reaction to occur. The anomaly was investigated no further, since the data obtained using identical vessels shows without doubt the rather unexpected dependence on visible light.

The ratio of $k_a:k_b$ in (1) can be obtained from the ratio of intercept:slope for plots of rate vs. oxygen pressure, and smaller values indicate a better approximation to perfect first order dependence on oxygen. Some values for this quotient under various conditions are given in Table 3.5. Most notably, daylight has a pronounced effect, dark rates approaching first order dependence, whereas in 0.1M-phosphate inclusion of EDTA has no appreciable effect. Since the black-out method used does not lead to total exclusion of light, there remains a small zero-order effect under these conditions, but there seems no doubt that absolute exclusion would yield perfect first-order oxygen dependence. The zero-order effect is not due to transition metal impurities (Table 3.5), though these might well be expected to lead to such effects.

The Overall Apparent Activation Energy.

The initial rates of autoxidation under air were determined over a small range of temperature (25°C to 45°C) for both THFA and tetrahydrobiopterin, and are shown in the form of Arrhenius-type plots in Fig. 3.10. The rates

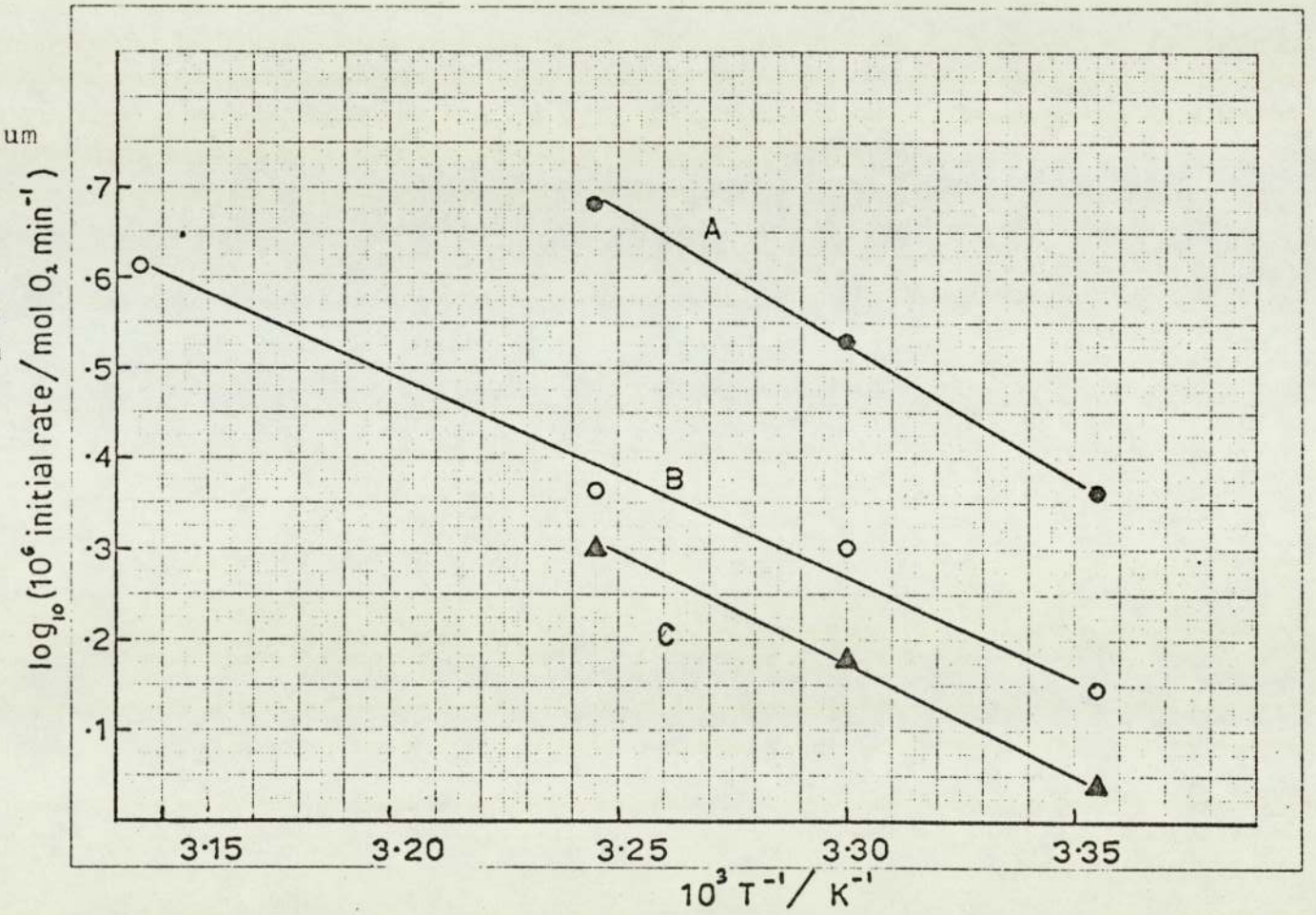
FIG. 3.10

Arrhenius plots for overall oxidation rates.

A) 0.625 mM - tetrahydro-biopterin/air in 0.1M-ammonium acetate.

B) As A plus EDTA (0.02M)

C) 0.7mM-THFA/air in 0.1M-sodium phosphate buffer, using soda glass vessel (early experiments)



are not corrected for the small variation in oxygen solubility with temperature, owing to the fact that under air the oxygen zero-order contribution is comparatively large, making such treatment unnecessary. From the slopes of these plots, it was found that the overall apparent activation energy is around 58 kJ.mol.$^{-1}$. This is a rather low energy barrier for a thermal autoxidation, as compared to, say, styrene which has an activation energy[133] of 23.0 Kcal.mol.$^{-1}$ (96.5 kJ.mol.$^{-1}$).

Discussion of Basic Kinetics in terms of mechanistic interpretation as a free radical chain reaction.

The above observations suggest that the autoxidation is occurring by a free radical chain mechanism.

The induction period noted earlier is characteristic of a chain reaction, since this is the period in which appreciable concentration of chain carrier is formed in the initiation step. Inhibition by low concentrations of phenols was observed, and such effects are normally associated[115-118] with chain-breaking reactions of the phenols with a chain carrier such as alkyl peroxyl (RO_2.) or hydroperoxyl (HO_2.) radicals, to give a stable phenoxy radical which does not initiate further autoxidation chains. Usually the effects reported are quite pronounced (inhibition by, e.g. a factor of ten) compared to the relatively small inhibition noted here. This is probably due to the extremely facile autoxidation of tetrahydropterins

compared to most compounds reported in the literature, making it a more effective competitor for the chain carrier and powerful reductant of, e.g. phenoxy radicals, thereby reducing the scavenging effect of phenols. In this context the comments of Gimblett[119] may be noted, in that very efficient inhibitors use up chain carriers as soon as they are generated by initiation, until all the inhibitor is used, whence the reaction commences at its normal rate. However, with less effective inhibitors, the inhibitor is unable to compete effectively with the initiation process, and now influences the reaction rate through its removal of chain carriers during the propagation steps of the chain reaction. The net effect is that the rate of reaction is not completely suppressed, but the system exhibits a residual rate which may not be reduced further by additional inhibitor. These effects are analogous to the competition observed above.

The acceleration produced by cupric ion is also in agreement with literature observations[120,121] concerning the effect of transition metal ions on free radical chain autoxidations. Usually, metal ion catalysis is proposed to occur by decomposition of peroxides present to give radicals which may act as initiators or chain carriers, e.g.:

$$RO_2H + M^{3+} \longrightarrow RO_2\cdot + M^{2+} + H^+$$

$$\text{or} \quad RO_2H + M^{2+} \longrightarrow RO\cdot + M^{3+} + OH^-$$

However, this does not explain the apparent

specificity for the catalysis of the present reaction by copper, since iron might be expected to act similarly. Also, it was found that EDTA eliminates the catalysis, whereas chelated metal ions are known to cause decomposition of peroxides (e.g. hydrogen peroxide to OH. radicals in Fenton's reagent occurs in the presence of EDTA - see Chapter 8 of this dissertation). Several alternative suggestions of mechanisms for metal ion-catalysis are found in the literature which merit some consideration. Copper (II) catalysis has been observed[123] in the autoxidation of fluorene to fluorenone, and a fairly complex mechanism is proposed to account for the observed kinetics. This consists in the formation of Cu(II) co-ordination complexes with solvent, fluorene anion, and oxygen in which metal ion mediated electron transfer to oxygen occurs. The kinetics of this reaction are first order in base and fluorene, and zero-order in oxygen, so that this is <u>not</u> an analogous reaction to tetrahydropterin autoxidation, since the latter is not appreciably affected by reducing the phosphate buffer concentration by a factor of ten (Fig. 3.5). Similar formation of reductant-metal ion-oxygen complexes has been postulated by Adolf and Hamilton[124] to account for the Fe(III) -catalysed autoxidation of acetoin, but since these authors state that Cu(II) is ineffective it seems likely that a similar mechanism may not be invoked with tetrahydropterins as reductant. Possibly the closest analogy to the present system regarding structure of reductant, is the autoxidation of the enediol ascorbic acid, which is catalysed

by Cu(II) and Fe(III), both aquated and chelated. In some studies by Kahn and Martell[125, 126] a combination of first- and zero-order dependence on oxygen was noted, but this was due to deviations from a perfect first-order relationship as low oxygen pressures were approached, as opposed to the apparent presence of a zero-order reaction at all pressures with the tetrahydropterin reaction. These kinetics were interpreted in terms of a chain reaction involving the formation of ascorbate-metal ion-oxygen complexes and electron transfer within these, similar to the suggestions made earlier by Weissberger *et al*[127,128]. However, the catalytic effect of metal chelates and the differences in kinetic behaviour are sufficient indication that this mechanism is probably not involved in the tetrahydropterin reaction. The most likely explanation of the observed catalysis is that the tetrahydropterin enters the co-ordination sphere of the metal cation and transfers an electron (an *inner sphere* process). The reduced metal may then be re-oxidised by molecular oxygen, depending on the magnitude of its oxidation potential. Also, the metal ion might be reduced by free radicals formed from tetrahydropterin (shown in Chapter Four), so that the apparent catalysis may not be due to chain processes. Such a mechanism, shown in Scheme 3.1, would explain the marked specificity for copper catalysis, since it is known[122] that cuprous ion is rapidly autoxidised whilst ferrous ion appears to be more stable to oxygen.

SCHEME 3.1.

Simple mechanism for Cu(II) catalysis during tetrahydropterin autoxidation.

$$AH_2 + Cu^{2+} \rightleftharpoons (AH_2.Cu)^{2+} \longrightarrow AH. + Cu^{+} + H^{+}$$

$$AH. + Cu^{2+} \rightleftharpoons (AH.\ Cu)^{2+} \longrightarrow A + Cu^{+} + H^{+}$$

$$2\ Cu^{+} + O_2 + 2H^{+} \longrightarrow 2\ Cu^{2+} + H_2O_2$$

where AH_2 represents tetrahydropterin

AH. represents free radical

A represents dihydropterin (e.g. quinonoid intermediate).

Such a scheme also explains the elimination of Cu(II) catalysis by the presence of EDTA, which strongly chelates the cupric ion (log K = 18.7[129]) and the bulk of the ligand prevents even partial co-ordination with tetrahydropterins, thereby totally preventing electron transfer. On this basis, the observed Cu(II) catalysis cannot be utilised as evidence for a free radical chain reaction mechanism.

However, the appreciable rates of oxidation in the dark, compared to light rates, suggests the involvement of ground state oxygen (photochemical reactions involving concerted reactions with singlet oxygen usually require

a quinone sensitiser and use of ultra violet radiation). Such reactions are known to occur[130] by successive one-electron or one-hydrogen abstraction steps and are characteristic of a free-radical chain mechanism. This is because a concerted two-electron transfer to triplet oxygen is a spin-forbidden reaction (it is not consistent with the Pauli principle). Also, the observed complexity in oxygen dependence cannot be rationalised by a non-chain process, as shown in Scheme 3.2.

SCHEME 3.2.

Possible non-chain autoxidation process.

$$AH_2 + O_2 \xrightarrow{k_1} AH. + HO_2.$$

$$AH. + HO_2. \xrightarrow{k_2} A + H_2O_2$$

The rate of oxygen consumption for this process would be given by (2), and would be perfectly first-order

$$\text{Rate} = k_1 (AH_2)(O_2) \qquad (2)$$

in both tetrahydropterin and oxygen. However, with a free-radical chain mechanism, the overall rate law is

determined largely by the types of initiation and termination reactions occurring, and may show complex dependence on oxygen pressure. For instance, Scheme 3.3 shows a type of oxygen-initiated free radical autoxidation chain reaction in which the termination step chosen leads to the rate law (3) on application of steady-state approximation (Appendix D), and this is the type of kinetic law approximated to by the dark reactions discussed above. (It should be pointed out here that in the general schemes considered it is assumed for the sake of brevity that oxygen and the free radical (AH.) combine to give the peroxyl radical (AHOO.) during propagation. This is an arbitrary supposition based on the normal mechanism assumed for these reactions[131], though it could equally well be hydrogen abstraction or electron transfer resulting in the hydroperoxyl radical[132] as chain carrier. The rate expression obtained is the same for all these cases and for the present purposes the former representation will be employed). The choice of termination (AH. + AHOO.) is based on reports[132] that this recombination predominates over the normal alternative (AHOO. + AHOO.) at oxygen pressures below 1000 mm Hg for more stable radicals (hence a valid assumption considering the extent of conjugation in the semiquinone free radical derived from tetrahydropterins - see later). In the presence of daylight, there appear to be secondary effects, possibly in the initiation process, causing a deviation from first order dependence on oxygen. Possibly this is due to impurity effects,

SCHEME 3.3.

Possible oxygen initiated chain reaction.

$$AH_2 + O_2 \xrightarrow{k_1} AH. + HO_2. \qquad \text{initiation}$$

$$AH. + O_2 \xrightarrow{k_2} AHOO.$$

$$AH_2 + AHOO. \xrightarrow{k_3} AH. + AHOOH \qquad \Big\} \text{ propagation}$$

$$AH. + AHOO. \xrightarrow{k_4} \text{non-radical products termination.}$$

Rate law (neglecting slow initiation): (see Appendix D)

$$-\frac{d(O_2)}{dt} = \left\{\frac{k_1 k_2 k_3}{k_4}\right\}^{\frac{1}{2}} (AH_2)(O_2) \qquad (3)$$

SCHEME 3.4.

Possible impurity initiated chain reaction.

$$\text{initiator} \xrightarrow{k_i} 2\ R^1.$$

$$R.^1 + O_2 \longrightarrow R^1O_2.$$

$$R^1O_2. + AH_2 \longrightarrow R^1O_2H + AH.$$

$$AH. + O_2 \xrightarrow{\text{fast}} AHOO.$$

$$AHOO. + AH_2 \xrightarrow{k_p} AHOOH + AH.$$

$$2\ AHOO. \xrightarrow{k_t} \text{inactive products.}$$

Rate law[134]

$$\text{rate} = \left\{\frac{k_i}{k_t}\right\}^{\frac{1}{2}} k_p\ (\text{initiator})^{\frac{1}{2}}\ (AH_2) \qquad (4)$$

though not transition metals, since EDTA was not found to eliminate the zero-order dependence, and it is not due to decomposition of hydrogen peroxide formed since addition of small amounts of hydrogen peroxide does not lead to catalysis. An alternative is some kind of photolysis, such as decomposition of peroxides by light. This is unlikely, since such high energy bond homolyses usually require short wavelength radiation, and the present reaction appears to be susceptible to visible light, a conclusion supported by the fact that glass and water absorb ultraviolet radiation so that the reaction medium would be effectively shielded by the water bath and reaction vessel. Also, direct irradiation with a powerful u.v. source produced only a small enhancement of the intercept in the rate equation (Table 3.5). The assumption of a possible impurity initiation effect, though very attractive since it would lead to zero-order dependence on oxygen as in Scheme 3.4, does not seem operative here. For mechanistic theory it must be remembered that the observed kinetics approach the rate law predicted by the oxygen-initiated reaction of Scheme 3.3 when daylight is excluded. Consequently, it would appear that a better scheme would involve an oxygen initiation which is in some way assisted by the absorption of visible light. Kulevsky *et al.*[135] have in fact proposed that certain hydrocarbon to oxygen electron transfers may proceed via a transitory contact charge-transfer complex. The electron transfer within this is light-dependent and

and for electron rich compounds may well be effected by visible light. Such an initiation would be expected to lead to some dependence of the overall kinetics on daylight, and a simple representation is shown in Scheme 3.5, together with the corresponding steady-state rate equation (5).

SCHEME 3.5.

Possible involvement of visible light in initiation.

$$AH_2 + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AH_2 \cdots\cdots O_2 \underset{k_{-2}}{\overset{h\nu,\ k_2}{\rightleftharpoons}} \overset{+}{AH_2}\cdots\cdots O_2^-$$

$$\overset{+}{AH_2}\cdots\cdots O_2^- \xrightarrow{k_3} AH. + HO_2.$$

$$AH_2 + HO_2. \longrightarrow AH. + H_2O_2$$

$$AH. + O_2 \xrightarrow{k_4} AHOO.$$

$$AH_2 + AHOO. \xrightarrow{k_5} AH. + AHOOH$$

$$AH. + AHOO. \xrightarrow{k_6} \text{non-radical products.}$$

Neglecting slow initiation, steady state rate equation is:

$$-\frac{d(O_2)}{dt} = \left[\frac{k_3 k_a}{2} \pm \left\{\frac{k_3^2 k_a^2}{4} + \frac{k_3 k_4 k_a}{4\,k_6}\right\}^{\frac{1}{2}}\right](AH_2)(O_2) \qquad (5)$$

where $k_a = \dfrac{k_1 k_2}{k_{-1}k_{-2} + k_{-1}k_3 - 2k_{-2} - k_3}$ and k_2 shows dependence on visible radiation.

(see Appendix D).

It can be seen that equation (5) still predicts a first-order dependence on oxygen, though it is possible that further complications may arise which account for the deviations. For instance, there may be some quenching by oxygen at high concentrations, or possibly a bimolecular displacement of oxygen from one of the contact complexes, resulting in an inverse dependence of k_1 or k_2 on oxygen pressure. So far, it has not been possible to rigorously test these suggestions or to derive the observed rate law from a reasonable mechanism.

To sum up then, we conclude that the complexity of oxygen dependence, the involvement of ground state oxygen, the observation of induction periods and inhibition by phenols may be taken as reasonable evidence for a free radical chain reaction mechanism. This is supported by the polarographic studies and e.s.r. data discussed in Chapter One, which indicate the presence of free radicals and therefore distinct one-electron steps.

Before concluding this section it may be mentioned that although quinone autocatalysis is observed in the autoxidation of hydroquinones[136,137], and such phenomena might be anticipated in the present reaction owing to the possible intermediacy of quinonoid dihydropterins, no evidence for its occurrence was found in these kinetic studies. Thus, slight reductions in rate are observed when the buffer concentration is decreased (Figs. 3.3 and 3.4), but since the rearrangement of quinonoid dihydropterin is buffer-catalysed (see Chapter One) its steady-state

concentration would increase as buffer concentration decreases. This would be expected to lead to increase in rate if quinone autocatalysis were occurring. Furthermore, the use of tetrahydropterins labelled at C(6) with deuterium would be expected to give increased concentration of quinonoid intermediate, leading to rate increases, and results in the next section show that the deuterated compounds react with the same velocity as non-deuterated compounds. Hence, these reactions are not autocatalysed by quinone intermediates.

In the foregoing discussion, no mention was made of the exact point in the tetrahydropterin molecule which undergoes attack by oxygen. The next section provides results which give strong indications that this process occurs in the N(5) - C(4a) region of the molecule, a proposal which has provoked some controversy in the literature.

The dependence of oxidation rates on functional group ionisation as a criterion for position of attack by molecular oxygen.

The effect on oxidation rate of changing solution pH has now been investigated over a wide range. Protonation of N(5) is known[83] to produce large decreases in rate of oxidation with ferricyanide, and results such as these have often been used as evidence that electron withdrawal occurs primarily from the lone pair on this nitrogen. This observation is now confirmed using THFA. The effect of pH has also been studied up to pH 13 using THFA, 6,7-dideuterio tetrahydrofolic acid, and tetrahydrobiopterin.

Results.

The region pH 1 to pH 7.

On increasing the solution pH in this region a concurrent increase in rate occurs in a sigmoid fashion as shown in Fig. 3.11. Unfortunately, the manometric technique could not give very accurate rates in this region, owing to the slowness of reaction giving time for comparatively large fluctuations in atmospheric pressure, this in turn necessitating large corrections in the reaction vessel pressure change. Two determinations were done at each pH, and Fig. 3.11 shows error bars corresponding to the range involved. This pH corresponds to the region in which N(5) of THFA is protonated (pKa = 4.82, estimated

FIG. 3.11

Initial rate dependence on pH for acidic region. Autoxidation of THFA (0.4 mM) under oxygen at 25°C. Error bars indicate ranges.

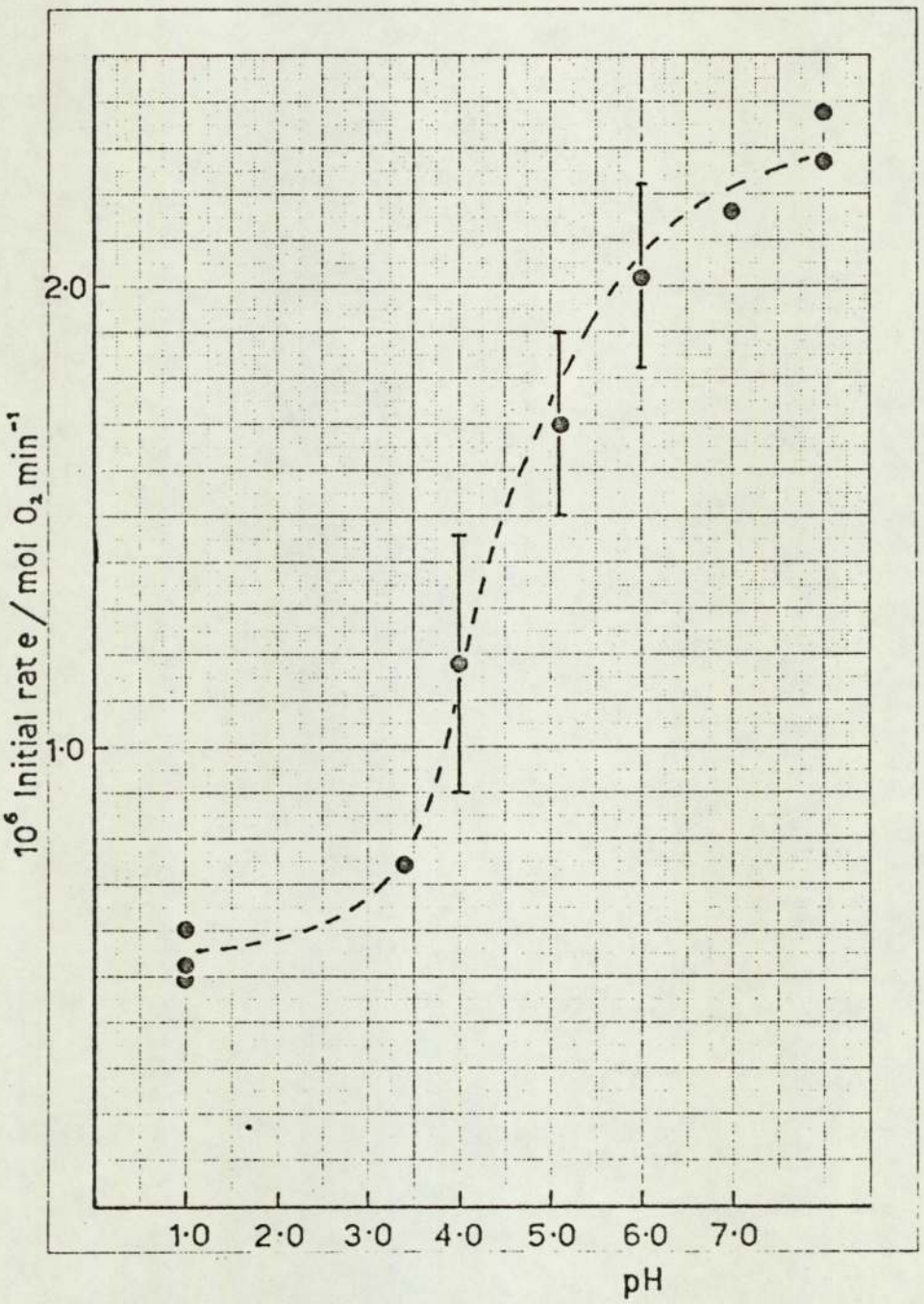
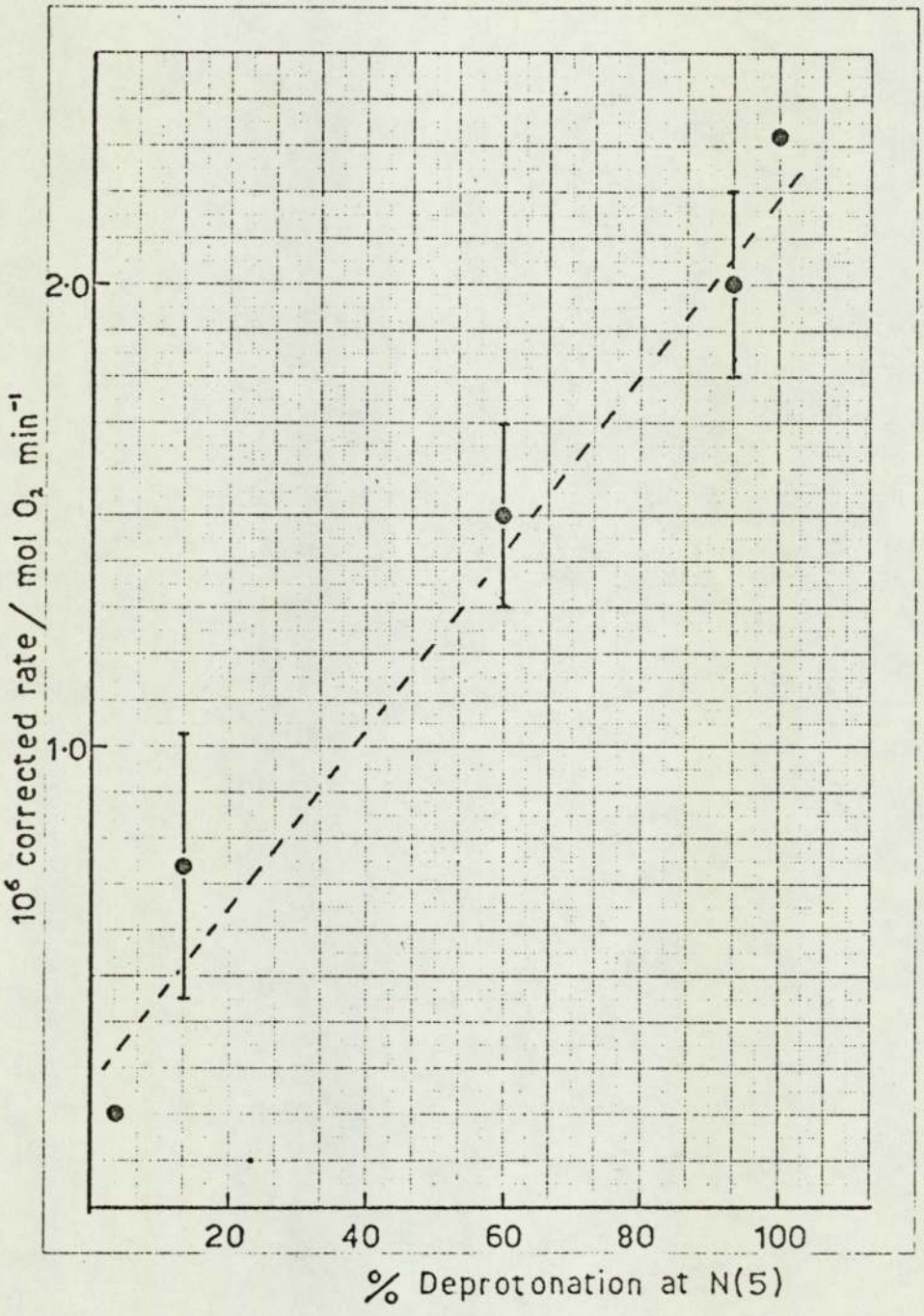


FIG. 3.12

Plot showing approximately linear correlation between corrected rate (text) and deprotonation of N(5) amino group for THFA/oxygen gas at 25°C. Error bars indicate ranges.



spectrophotometrically[138]), and the shape of the curve leads us to suppose that there is a relationship between the fraction of free base species present and the observed rate. The residual rate at pH 1 corresponds to oxidation of fully protonated THFA. The data may be analysed by proposing a rate law of the form (6).

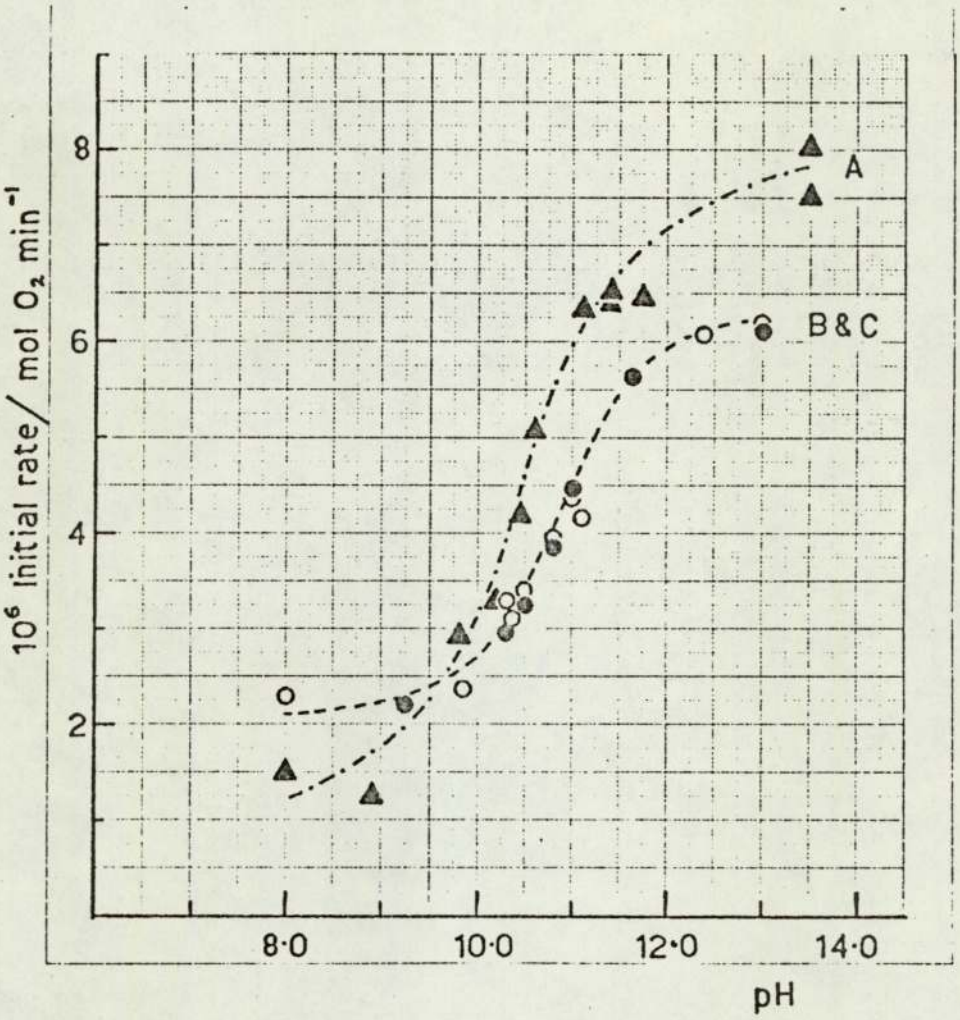
$$\text{Observed Rate} = k\left\{\begin{matrix}\text{fully protonated}\\ \text{THFA}\end{matrix}\right\} + k^{1}\left\{\begin{matrix}\text{Free base}\\ \text{THFA}\end{matrix}\right\} \qquad (6)$$

Now using the observed rate at pH 1, and a pK_a for N(5) of 4.82, it is possible to estimate the fractions of protonated and unprotonated forms at each pH, and hence obtain values for the first term on the right hand side in (6). If each value is then subtracted from the observed rate at the same pH, a set of corrected rates is obtained corresponding to a series of fractions of free base (at N(5)). A plot of this corrected rate v. % free base at N(5) should then be linear if (6) is valid. Such a plot is shown in Fig. 3.12, and does indeed show reasonable linearity, though a perfect straight line is not expected owing to the inaccuracy of the data in this region. Numerical values are derived in Appendix E.

These results show the correctness of literature reports[83] concerning the effect of N(5) protonation on oxidation rates.

FIG. 3.13

High-pH-dependence of initial rates of autoxidation under oxygen at 25°C.



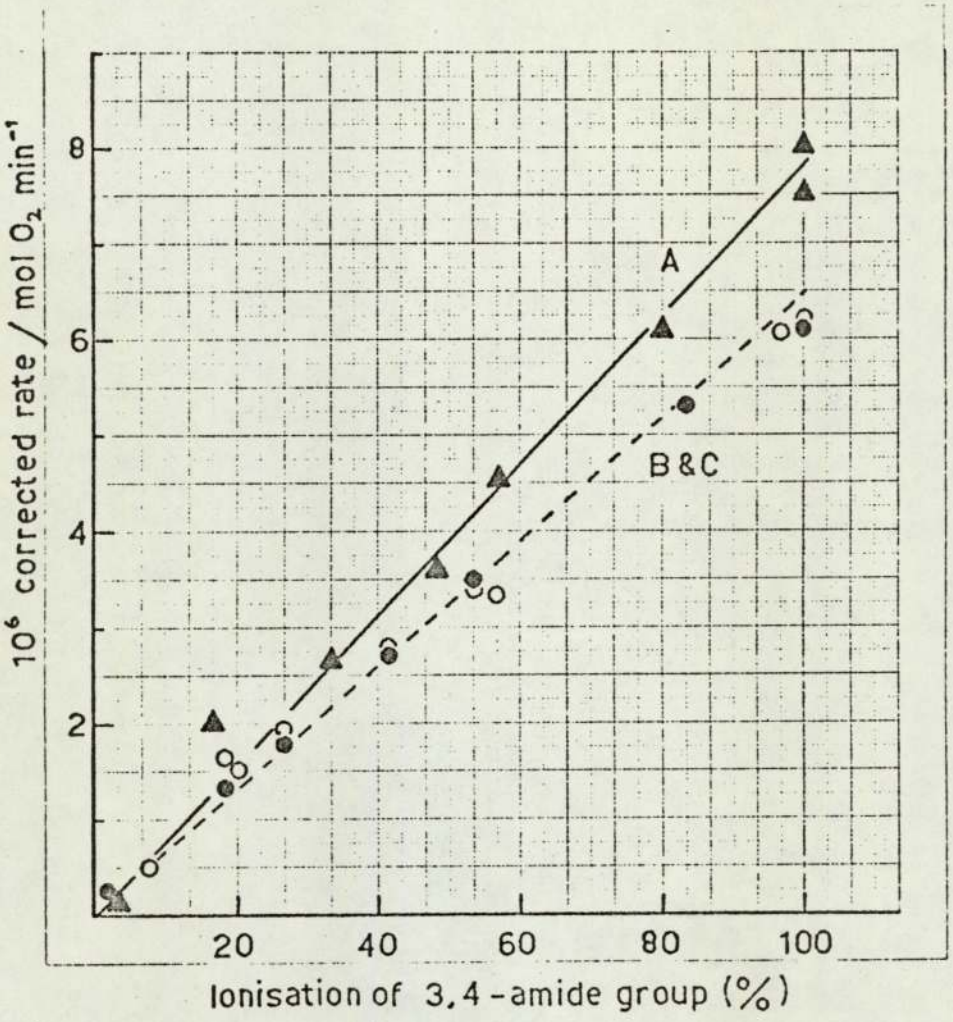
A) Tetrahydrobiopterin 0.312 mM.

B) (open circles) THFA, 0.4 mM.

C) (dark circles) 6,7-dideuteriated THFA, 0.4 mM.

FIG. 3.14

Dependence of corrected rate (text) on ionisation of 3,4 - amide group. Under oxygen at 25°C.



A) Tetrahydrobiopterin 0.312 mM.

B) (open circles) THFA, 0.4 mM.

C) (dark circles) 6,7-dideuteriated THFA, 0.4 mM.

The region pH 7 to pH 13.

Fig. 3.13 shows the behaviour of oxygen uptake rates over this pH range, and again a sigmoid curve is obtained for all compounds studied (tetrahydrobiopterin, THFA and 6,7-dideuterio tetrahydrofolic acid). The mid-point of the portion of curve showing increasing rate corresponds approximately to the pKa values for ionisation of the 3,4-amide group in the tetrahydropterin moiety (values usually ca. 10.5; see for example, Blakley[18] p. 62). Consequently, it appears that increasing concentration of the imino-enolate anion derived by loss of proton from N(3) leads to increase in rate. Using the rate equation (7) and treating the data in an analogous manner to the above section, i.e., estimating

$$\text{Observed Rate} = k^{1}\left(\begin{matrix}\text{neutral}\\ \text{tetrahydropterin}\end{matrix}\right) + k^{11}\left(\begin{matrix}\text{imino-enolate}\\ \text{anion}\end{matrix}\right) \qquad (7)$$

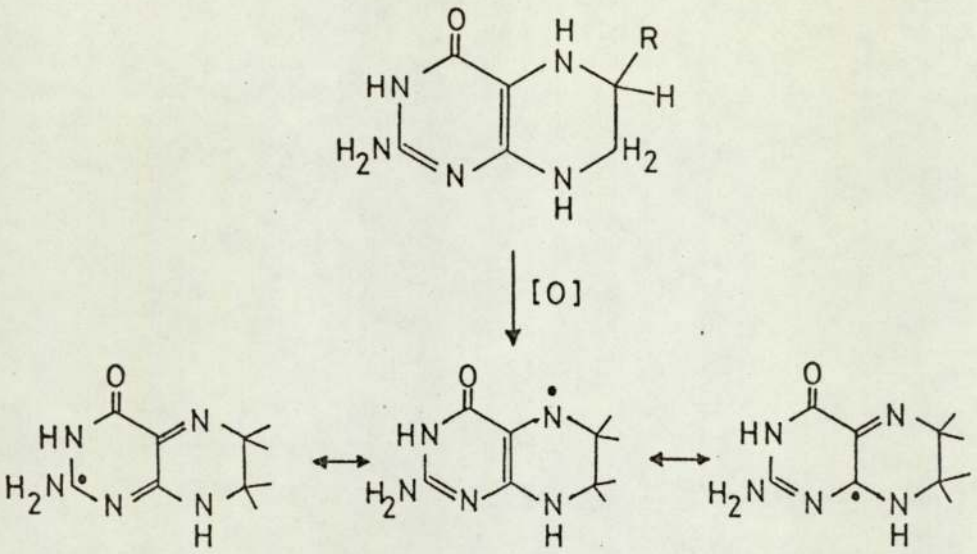
the first term on the right hand side for each pH and subtracting this from observed rate to give corrected rate, an excellent straight line is obtained (Fig. 3.14) when the corrected rate is plotted against percentage ionisation of the 3,4-amide group, showing the validity of (7). In deriving the data, independently estimated values of pKa for the amide group were used (determined spectrophotometrically, Appendix F). Numerical values of the derived rate data are given in Appendix E. It

may be noted that, owing to the higher rates involved, the data is more accurate in this region than for the lower pH region.

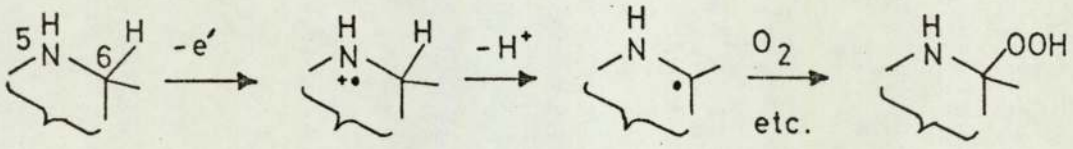
The above results show conclusively that there is a linear dependence of rate of oxidation on the percentage deprotonation of both the protonated N(5) amine group and the N(3) amide function for tetrahydropterins.

Discussion of pH-dependence.

At first sight, the observation that protonation of N(5) leads to parallel decrease in rate of oxidation leads one to suspect that oxygen attack occurs on this atom. This is certainly consistent with experience regarding hydrogen abstraction in the liquid phase by free radicals, where it is known that N-H, P-H and S-H bonds are particularly reactive, which seems to reflect the ability of the heteroatom to stabilise polar structures contributing to the transition state.[139] On this basis it is tempting to write the thermal-initiation and propagation steps as involving N-H homolysis at N(5), and this indeed seems to be the basis of the mechanism of autoxidation of tetrahydropteridines suggested by Mager and Berends (Chapter One and references therein). In such a scheme, canonical structures derived from the nitrogen radical might be expected to contribute to the resonance hybrid, as shown in Scheme 3.6, and addition of



SCHEME 3.6 Consideration of N-H(5) homolysis

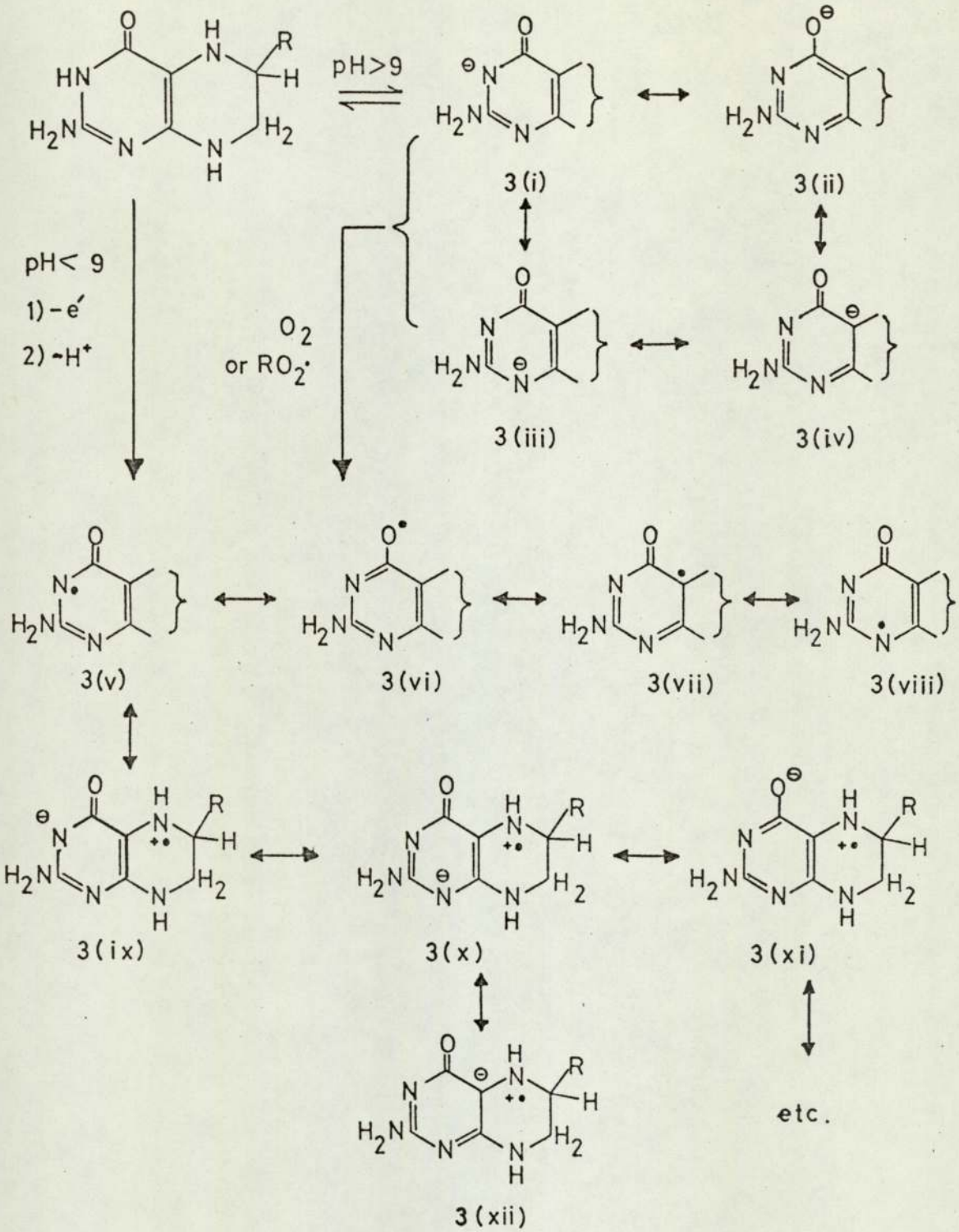


SCHEME 3.7 N(5) electron removal and 6-CH heterolysis according to Stocks-Wilson[58]

oxygen to C(8a) of the radical could occur as suggested by Mager and Berends. However, it is difficult to rationalise this suggestion in the knowledge that heterolysis of bonds is usually favoured over homolysis in solvents of high dielectric constant, whilst free radical chains are more probable in non-polar solvents.[140] Thus, it might be expected that N-H homolysis would be favoured in, say, ethanol compared to aqueous media (Dielectric constants[141]: ethanol 24.3, water 78.54). However, the reverse has been observed (see Table 3.4), suggesting the mechanism to be one of charge transfer (a type of heterolysis). This is also supported by the observation[143] that autoxidation of tryptophan, which involves N-H homolysis, is actually acid-catalysed due to the probable formation of weaker N-H bonds on protonation. So it appears that electron removal may be involved, and on the basis only of N(5) protonation effects one is tempted to suggest removal of a lone pair electron from this atom which would result in similar (protonated) canonical forms to those shown in Scheme 3.6. Such a mechanism is analogous to the suggestions by Bartholomew and Davidson[143] regarding photosensitised oxidation of aliphatic amines. These authors also suggested that proton removal from the α-carbon atom of the resulting radical-cation would give a carbon free-radical to which oxygen might add, resulting in hydroperoxide intermediates. Such an idea was used by Stocks-Wilson[58] in postulating that hydroperoxide formation at C(6) of the tetrahydropterin molecule

accounted for the products. The mechanism is shown in Scheme 3.7 (partial formulae). The isolation[144] of 1-hydroperoxy cyclohexylamine in yields up to 50% during photosensitised autoxidation of cyclohexylamine seems to support this type of mechanism. One might expect C(6) - hydroperoxides of tetrahydropterins to be as stable, and the failure to characterise such compounds even at low temperatures casts some doubt on the validity of the Stocks-Wilson mechanism.

These discussions are based only on the one observation of protonation effects, and other possibilities become apparent on examination of the imino-enolate anion dependence. If one considers the oxidation of this electron-rich species, one is immediately led to the conclusion that electron removal is the characteristic feature of the reaction. What is more, the increase in electron density in the pyrimidine part of the molecule which occurs on deprotonation at N(3) suggests that involvement of the N(5) lone pair is an oversimplification. Consider what happens during formation of the anion and subsequent electron transfer to oxygen or chain carrier, as shown in Scheme 3.8. Clearly, the anion may be written as a resonance hybrid of the canonical forms, 3(i) to 3(iv), and the derived semiquinone free radical is represented by the canonical forms 3(v) to 3(xii). The structures 3(ix) - 3(xii) show a separation of charge and are expected to be of higher energy, and therefore less important than 3(v) - 3(viii), although the participation of the N(5) lone pair in these clarifies



SCHEME 3.8 Canonical forms of imino-enolate anion and derived semiquinone radical.

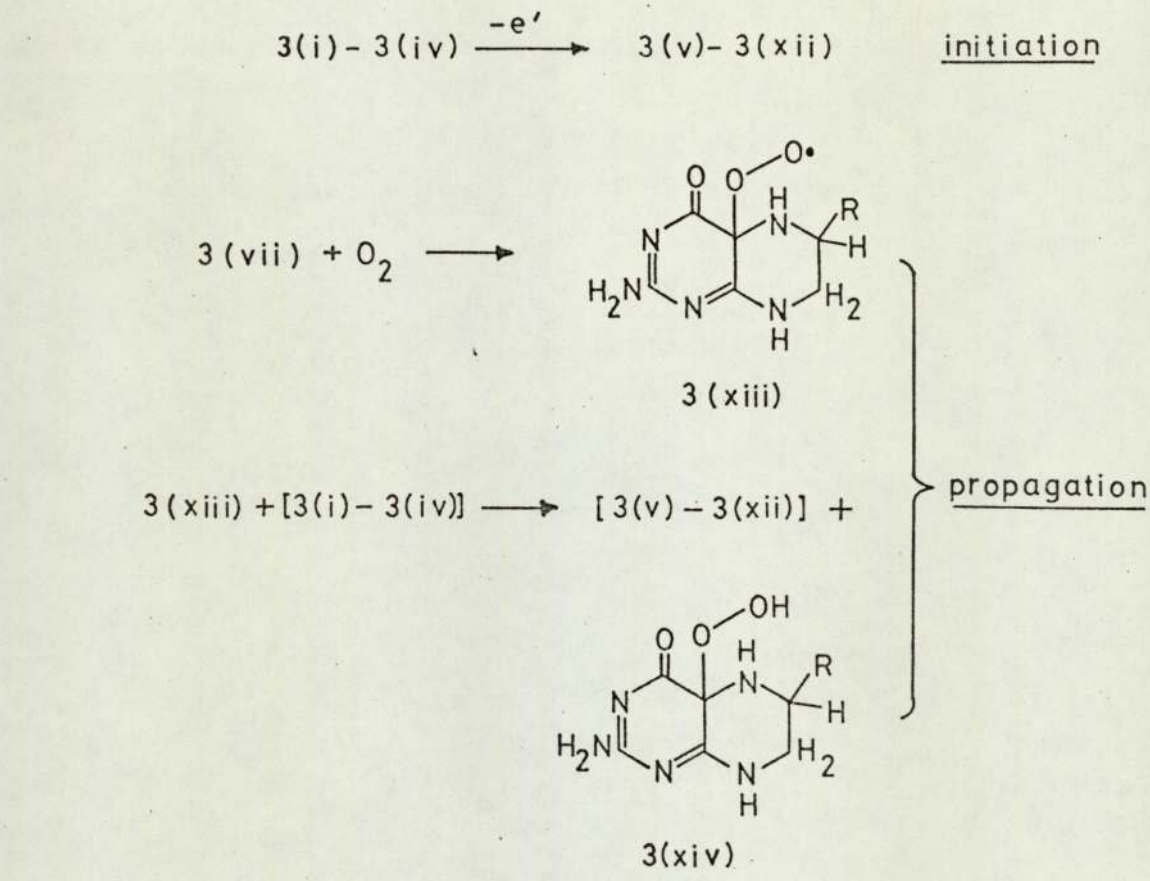
the role of this atom in facilitating oxidation. Factors affecting the stability of an intermediate may also be expected to affect the transition state for formation of that intermediate in a parallel fashion, and conjugation with the N(5) lone pair must stabilise the free radical. Further structures can be written in which the single electron from N(5) in 3(ix) - 3(xii) is placed at C(8a) and C(2). However, owing to the charge-separation involved and the high energy of 3(ix) - 3(xii), it is safe to omit these when discussing unpaired electron densities in the semiquinone radical.

Considering the forms 3(i) to 3(iv), it can be seen that the only carbon atom at which there is appreciable unpaired electron density is the angular position C(4a). Therefore, in view of the fact that oxygen is a triplet in the ground state and has some radical character, it might be expected that any bond formation between the semiquinone and oxygen would result in a C(4a) oxygenated intermediate, as suggested by the author elsewhere.[68] This is supported by the observation[145] that whereas 5-methyl tetrahydrofolic acid parallels the oxidation kinetics of THFA with regard to ionisation, etc., the observed rates are a factor of ten lower for this N(5) substituted compound. On the other hand, tetrahydropterins substituted at N(8) do not show suppressed oxidation rates[65]. These results may be rationalised if oxygen attack occurs at the C(4a) - N(5) portion of the molecule, when substitution at N(5) would result in

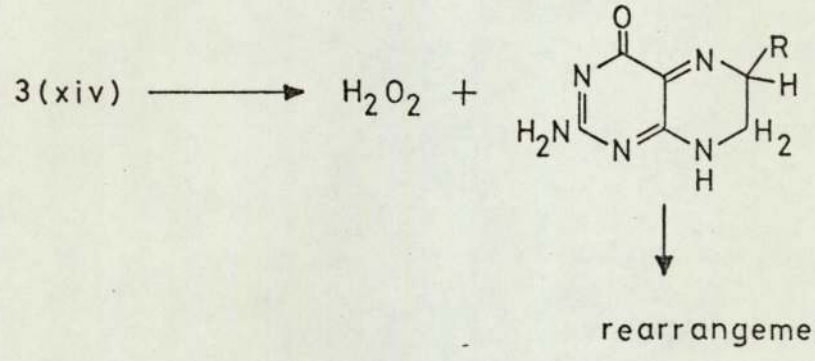
considerable steric hindrance to the approach of oxygen. The parallel between the canonical forms for imino enolate anion and semiquinone radical may be criticised in that electronegative substituents such as oxygen might be expected to have a greater share of electron density in conjugated anions than unpaired spin in free-radicals. However, the conclusion that C(4a) carries a large proportion of unpaired electron density, by comparison with the anion resonance structures, is justified by observations that base-catalysed autoxidations of carbonyl compounds,[146-148] phenols,[149-151] and compounds related to 9,10-dihydroanthracene[152] result in hydroperoxides formed by oxygen attack at the carbon atom carrying formal negative charge in the derived anions.

The observation that 6,7-dideuterio-THFA gives exactly the same pH-dependence for rates of autoxidation as does THFA renders obsolete the mechanistic suggestions of C(6) or C(7) attack proposed by Stocks-Wilson[58] and Hawkins[89], respectively, since primary isotope effects might be expected to occur in the event of these hypotheses being correct. The absence of such effects confirms the validity of the suggested involvement of imino-enolate anion, and this is difficult to rationalise with the mechanism proposed by Mager and Berends.

On the basis of the above unpaired spin assignments, a mechanism was suggested[68] involving C(4a) hydroperoxide intermediates, 3(xiv) as shown in Scheme 3.9. In the absence of any conflicting evidence, this is a useful working hypothesis, since it agrees with suggestions



Followed by :-



SCHEME 3.9 Possible 4a-hydroperoxide involvement

from molecular orbital data discussed later, and loss of hydrogen peroxide will lead to quinonoid dihydropterin, a recognised intermediate. However, oxygen addition to the intermediate free radical is not the only propagation step which can be conceived, there being two reasonable alternatives:

(a) Molecular oxygen may abstract hydrogen atom from N(5) of the radical to give a quinonoid dihydropterin intermediate (Chapter One), which then undergoes rearrangement to products.

(b) Molecular oxygen may abstract an electron from the radical to give protonated quinonoid dihydropterin, with subsequent loss of proton and rearrangement.

The latter seems an unusual possibility, since the Gibbs' Free Energy for the addition of electron to oxygen is positive[112].

There is insufficient evidence from the results so far that the chain carrier is in fact an alkylperoxy radical, 3(xiii). Some kinetic parameters are discussed in Chapter Four, which shed further light on the nature of the propagation. However, the above data does show that a quinonoid dihydropterin is an obligatory intermediate during autoxidation of tetrahydropterins, since electron withdrawal occurs initially in the pyrimidine moiety, whilst the observed products show an oxidised pyrazine ring. The only explanation for this phenomenon is that a quinonoid dihydropterin is formed, and this undergoes rearrangement to 7,8 dihydropterins and products of side-chain loss. A scheme is presented in Chapter Seven.

A brief study of the kinetics of autoxidation of Dihydrofolic acid.

Since dihydrofolic acid (DHFA) is one of the initial products from THFA oxidation, it was considered useful to investigate the kinetics of its autoxidation. This was not done with any view to elucidating the oxidation mechanism of 7,8-dihydropterins, but rather to verify that DHFA is oxidised much more slowly than THFA, thereby showing that no appreciable inaccuracy in the estimation of initial rates for the latter arise due to the autoxidation of DHFA produced. Quantitative rate data is also useful in considering the mechanism from a theoretical viewpoint, as discussed in Chapter Six. Therefore, the rate of oxidation and approximate stoichiometry in 0.1M-phosphate buffer were determined, and the pH-dependence was investigated in the 3,4-amide ionisation region.

Methods.

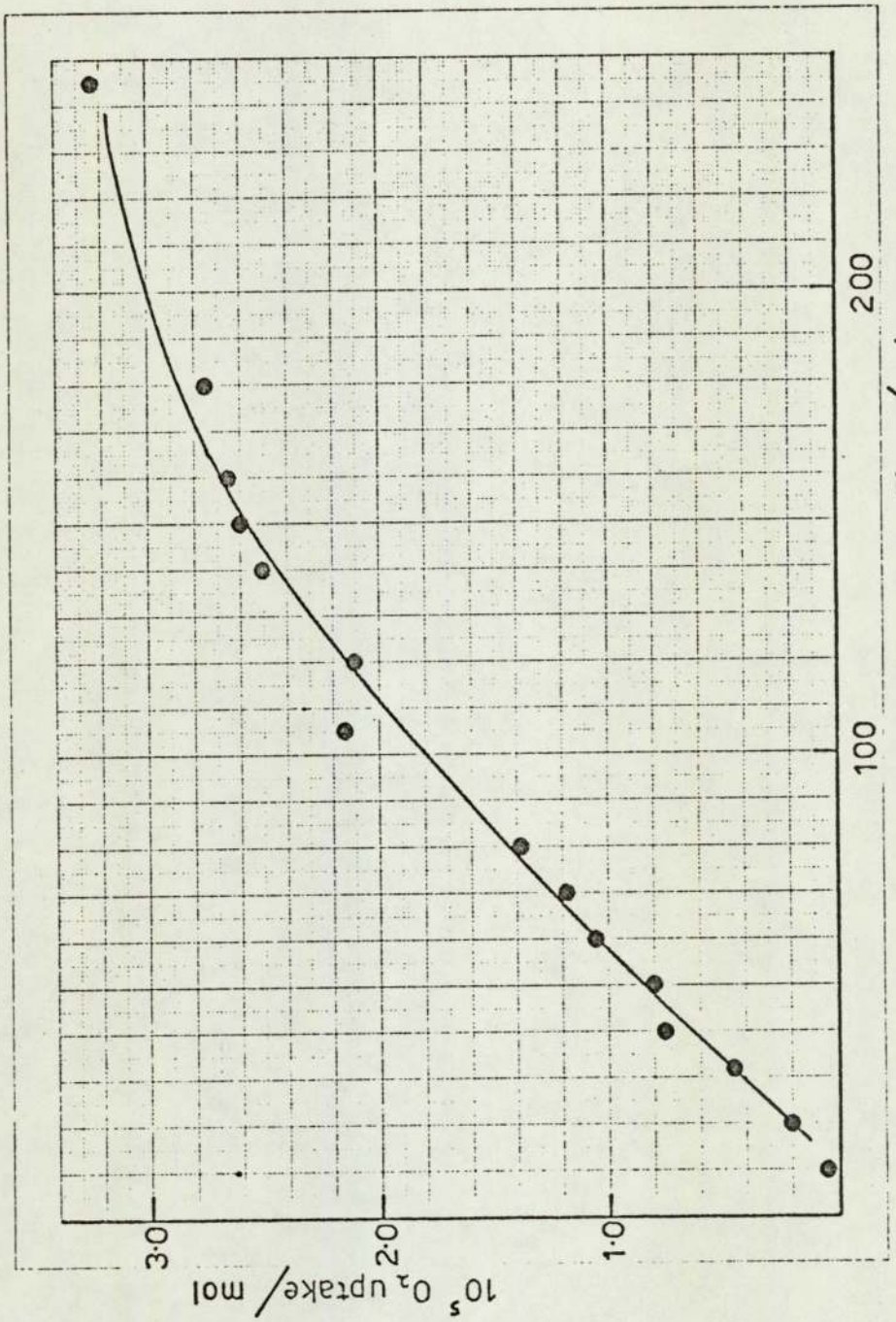
Rates of oxidation were determined manometrically, as previously described. The accuracy of the determinations is rather less than for the tetrahydro- compounds owing to the comparative slowness of the present reaction.

Results and Discussion.

The curve of oxygen uptake for a 0.45 mM-DHFA solution in 0.1M-phosphate buffer, pH 7, at 25°C and

FIG. 3.15

Oxygen uptake by 0.45 mM - dihydrofolic acid in 0.1M-sodium phosphate buffer under oxygen gas at 25°C.



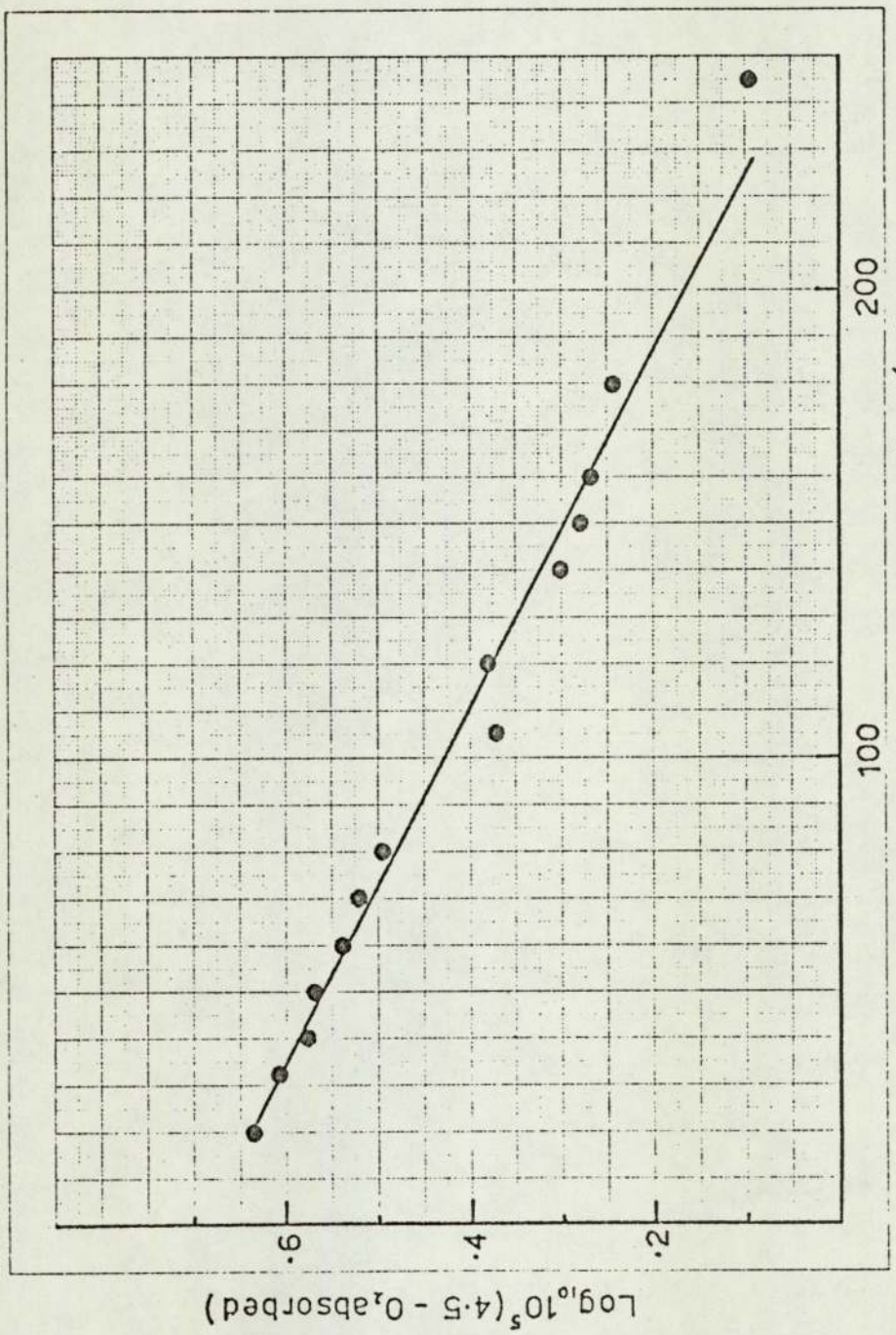


FIG. 3.16

First-order plot for data of Fig. 3.15.

under oxygen gas is shown in Fig. 3.15. It was found that a 1:1 stoichiometry for oxygen consumption was exceeded after about seven hours. After twenty-seven hours 7.3×10^{-5} moles of oxygen had been consumed (1:1 stoichiometry requires 4.5×10^{-5} moles), but further reaction was not followed owing to excessive barometric fluctuations. This excessive stoichiometry is consistent with the formation of compounds such as dihydroxanthopterin and 7,8-dihydropterin-6-carboxaldehyde as mentioned in Chapters One and Two. A 'one-shot' oxidation to folic acid, xanthopterin or pterin-6-carboxaldehyde would give unit stoichiometry. However, the secondary oxidation must be very slow (requiring a further 20 hours after apparent slowing down of the reaction) so it is possible to analyse the curve using 4.5×10^{-5} moles O_2 as infinity value and making the appropriate first order plot, which turns out to be linear (Fig. 3.16) showing first order dependence on DHFA concentration (oxygen concentration is effectively constant since the overall pressure change is very small,ca.0.5 cm Hg).

The initial rate of autoxidation under these conditions was found to be 1.8×10^{-7} moles oxygen per minute, compared to a value of ca. 3.5×10^{-6} for THFA oxidation at the same concentration. Thus, DHFA is autoxidised approximately 20 times more slowly than is THFA.

On increasing the solution pH in the region 10 to 14 there occurred a similar increase in rate as had been noted for the tetrahydropterins (Table 3.6).

TABLE 3.6.

Effect of pH on velocity constants for DHFA autoxidation in aqueous solution under oxygen gas at 25°C.

Solution pH	Pseudo first-order rate constant x 10^3 (min^{-1}).
9.6	1.34
10.3	0.89
11.1	1.06
11.2	1.04
11.6	1.10
11.6	1.68
12.3	1.38
12.5	2.23
12.6	2.56

Owing to the rather low accuracy of the data at such small rates no attempt was made to obtain a linear correlation between rate and ionisation of the 3,4-amide function.

These results once more indicate an electron withdrawal favoured by increasing electron density in the pyrimidine moiety, and show that the relatively slow rates of DHFA autoxidation will not lead to inaccuracies in the kinetic determinations for THFA. No activation parameters were estimated.

CHAPTER 4.

Some Kinetic Data concerning the nature of the Chain Carrier.

Evidence for a slow reaction of the semiquinone free radical intermediate with molecular oxygen.

During the attempts to isolate peroxidic materials from the low temperature oxidations of tetrahydrobiopterin described in Chapter Two, it was found that one of the reaction mixtures, on removal of solvent, left a residue having a strong reddish colouration. In view of the report by Bobst[85] that such colourations are due to semiquinone free radicals, an e.s.r. spectrum was run on the solid, when a broad signal was indeed obtained (Fig. 4.1). It was also observed that on dissolution of this solid in water no tetrahydrobiopterin was apparent (no rapid u.v. spectral change) but a fairly broad absorption was present in the visible region, with λ_{max} = 550 nm. Thus, the solid appeared to consist mainly of dihydrobiopterin and biopterin (t.l.c.) with small amounts of the semiquinone free radical. It was possible to observe the decay of the latter in water and thereby determine the kinetics for its reaction with molecular oxygen, which will now be described.

Method.

A solution of the residue containing free radicals was rapidly made up in water at room temperature (23°C) in the u.v. cell (ca. 1 mg/cm^3) and transferred to the spectrophotometer. The absorbance at 550 nm. was then determined at convenient intervals of time using water as blank and the data plotted graphically.

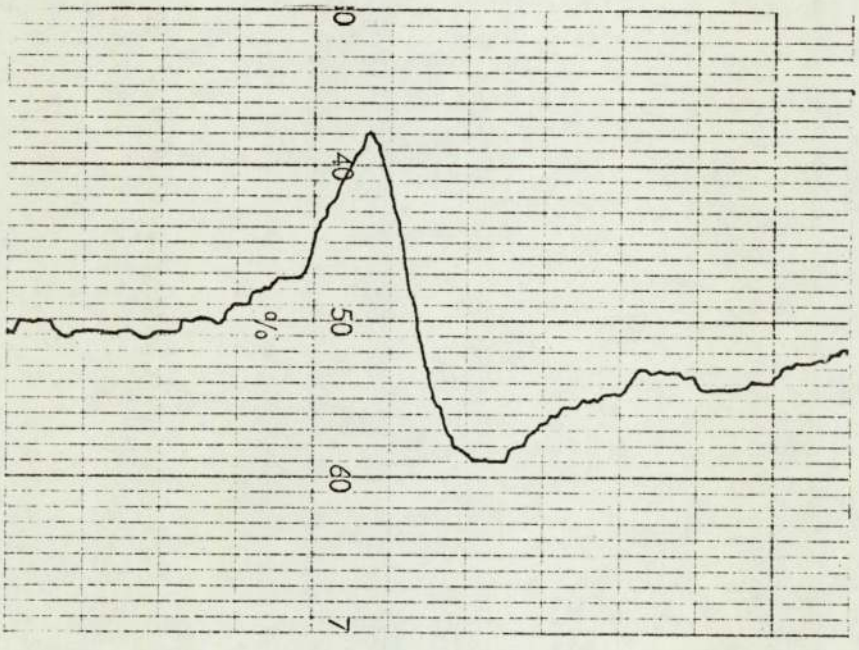
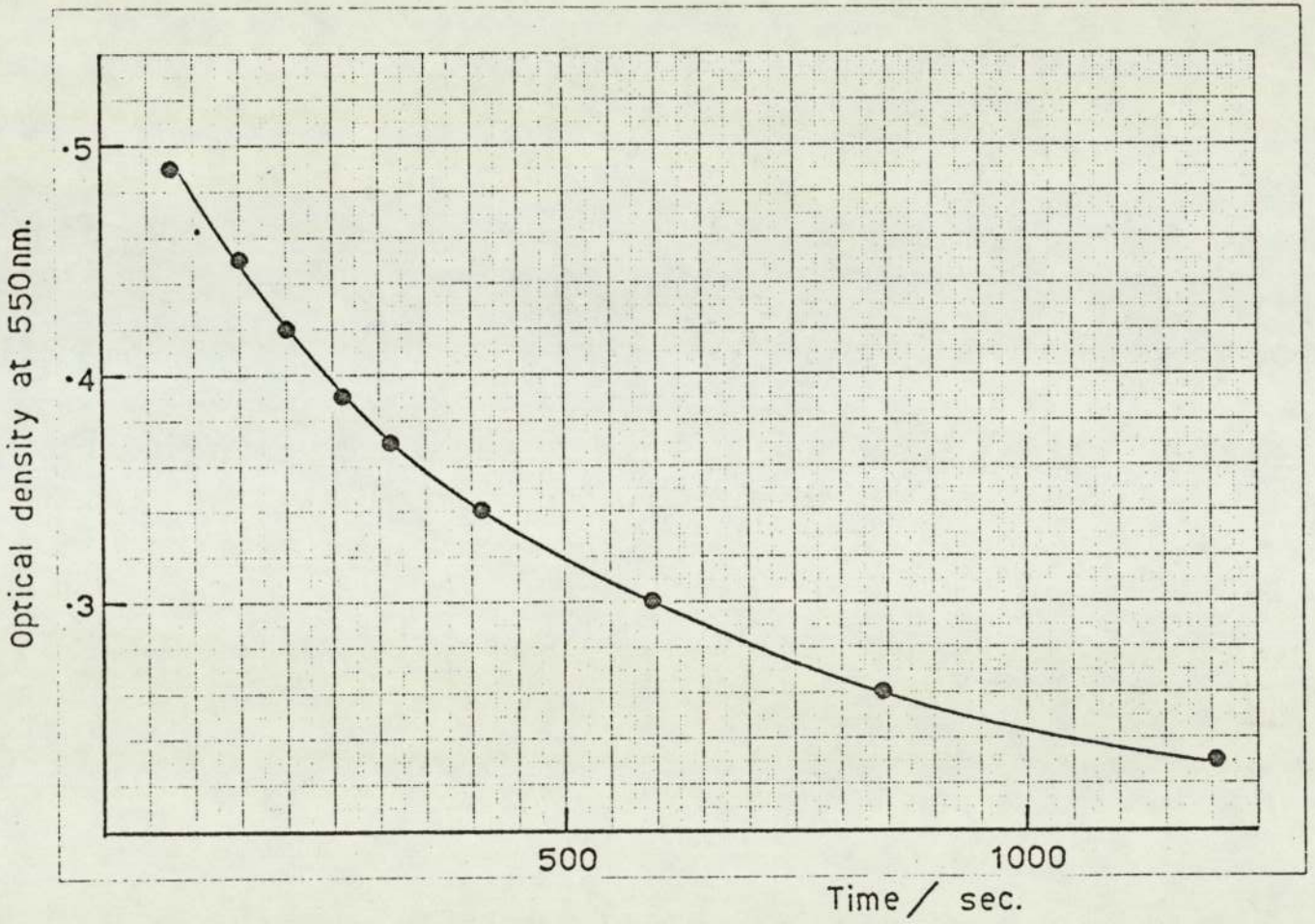


FIG. 4.1

E.S.R. Spectrum of purple solid isolated from low-temperature oxidation of tetrahydrobiopterin. Suspected semiquinone radical.

FIG. 4.2

Disappearance of coloured compound (free radical) from tetrahydrobiopterin oxidation. In distilled water under air at ambient temperature.



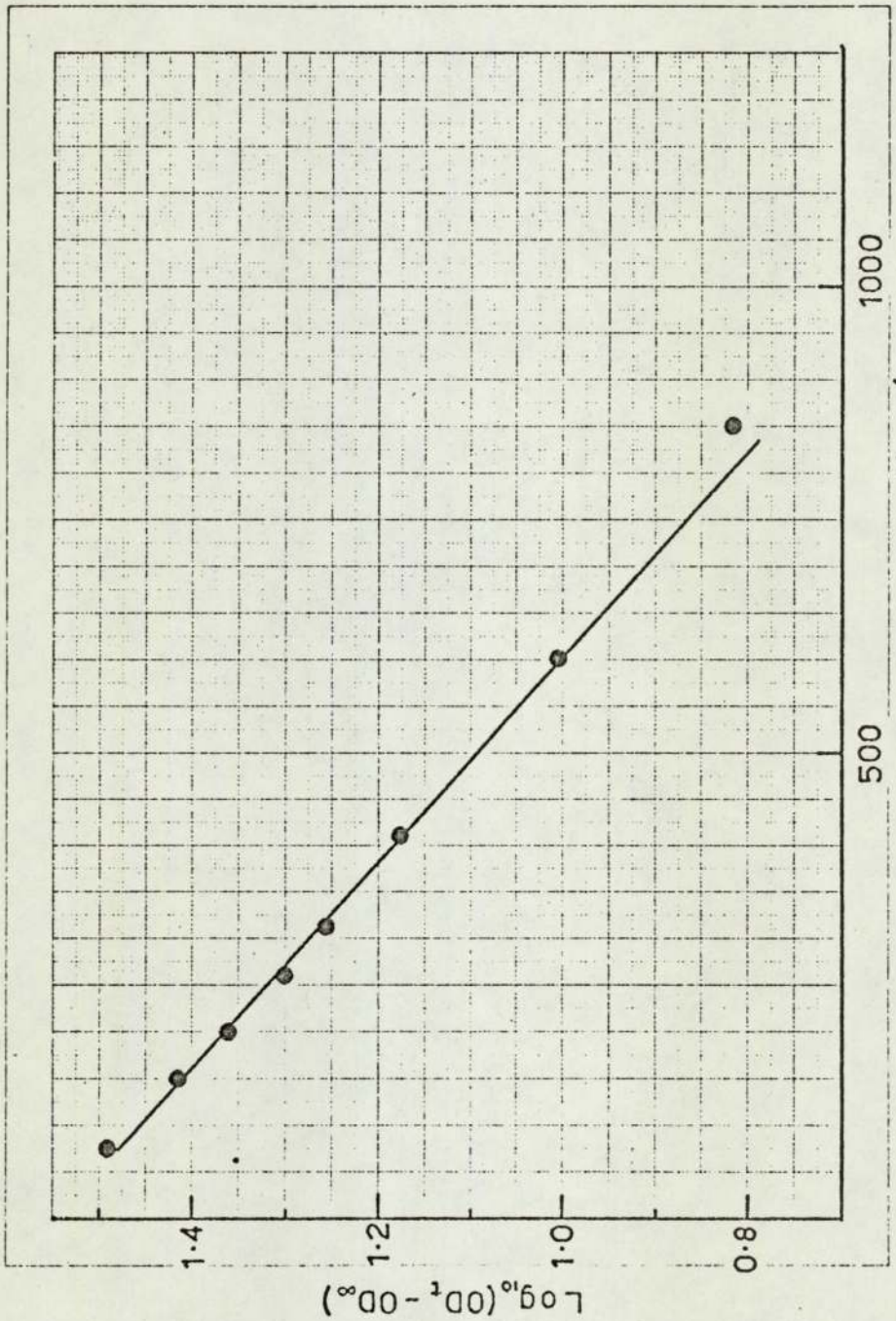


FIG. 4.3

First-order plot for data of Fig. 4.2

An infinity reading of optical density was taken after one hour ($>$ 10 half-lives). Sufficient material was available for only one experiment.

Results and Discussion.

The decay of free radicals with time is shown graphically in Fig. 4.2, and there appears to be an exponential approach to the infinity optical density value (0.19), the latter being due to "tail-off" from the spectrum of oxidation products already present. The decay is first order with respect to the intermediate, as shown by the excellent linearity of the first order plot (Fig. 4.3). From the slope of this plot of $\log_{10}(O.D_t - O.D_\infty)$ v. time it is possible to estimate a value for the pseudo first order rate constant for the decay, and this is $k_1 = 3.66 \times 10^{-4}\ s^{-1}$.

A reasonable assumption is that the radical is disappearing by reaction with the oxygen present in the solution, and that since there is no residual tetrahydrobiopterin present no radicals are being generated simultaneously. Thus, if we assume that the reaction of free radical with oxygen is first order in the latter, then the relationship (1) holds between the pseudo first order rate constant and the overall second order rate constant, k_2.

$$k_1 = k_2 (O_2) \qquad (1)$$

Hence it is possible to estimate a value for k_2 and, using a concentration of oxygen (in equilibrium with air) of 2.58×10^{-4}M [110], this turns out to be $k_2 = 1.415$ l.mol^{-1}.s^{-1}. Now, considering the fact that the process under observation is the reaction of oxygen with a free radical, this is a remarkably small rate constant. If this were a simple addition of O_2 to the semiquinone to yield an organic peroxy radical chain carrier, we should expect the process to be diffusion controlled. Diffusion controlled reactions usually have [140] $k_2 = 10^9 - 10^{11}$ l.mol^{-1} s^{-1}, suggesting that this assumption is not correct. This observation tempts us to go one stage further, and estimate a rough value of the activation energy for the reaction.

The Arrhenius equation (2) gives a relationship between rate constant k_2, activation energy Ea, and temperature, T.

$$k_2 = A\ e^{-Ea/RT} \qquad (2)$$

where A = pre exponential factor =
10^9 to 10^{11} l.mol^{-1}. s^{-1}

R = gas constant = 8.314 J. mol^{-1} K^{-1}

Usual values for the A-factor for bimolecular reactions (Laidler[140] p. 87) range from 10^9 to 10^{11} l. mol^{-1} s^{-1} for reasonably simple molecules. Using the

lower value (10^9), since one of the molecules involved (semiquinone radical) is a little more complex than normal, and substituting the values obtained into (2), an activation energy for the reaction can be estimated. This is found to be E_a = 50 kJ. mol^{-1}.

This value is exceptionally high for an addition of oxygen to a free radical, as such reactions are expected to be zero- or low- activation energy processes. Therefore, it is likely that the reaction is not an addition. However, owing to the rather crude preparation utilised, this observation needs to be confirmed by an independent determination.

<u>The Dependence of "Steady State" semiquinone concentration on reaction conditions as an aid to determining the nature of the chain carrier.</u>

Consider the general autoxidation process shown in Scheme 3.3. It can be seen that the only steps in which oxygen is consumed are the initiation (k_1), and propagation in which semiquinone radical reacts with molecular oxygen (k_2). If we can neglect the initiation, a reasonable approximation in most reactions where this step is slow, and apparently valid for the present light rates under air (owing to the relatively large zero-order dependence on oxygen), then the rate of oxygen uptake is given by (3).

$$\frac{-d(O_2)}{dt} = k_2 (AH.)(O_2) \qquad (3)$$

Here k_2 is the same rate constant as that estimated above. Now, if the concentration of semiquinone radical at steady state, $(AH.)_{SS}$, can be determined, then valuable information can be obtained regarding k_2, since the relationship (4) holds under these conditions.

$$k_2(O_2) = \frac{\text{Observed Rate}}{(AH.)_{SS}} \qquad (4)$$

On autoxidising tetrahydrobiopterin in 0.1M - phosphate buffer, pH 7, and in 0.1M - ammonium acetate buffer, pH 6.8, at concentrations of 1 mg. cm^{-3} it was observed that the characteristic semiquinone absorption, λ_{max} 550 nm, appeared. Thus, the relative concentrations of semiquinone may be determined under a variety of conditions by measuring the optical density at this wavelength. The same peak was produced during autoxidation of THFA in glacial acetic acid, though clean spectra in other media could not be obtained owing to absorptions in this region caused by "tailing off" from the UV spectrum of oxidation products. Utilising this, some kinetic characteristics of the semiquinone free radical were observed.

Methods.

Tetrahydrobiopterin. This compound (25 mg) was stirred very rapidly in 0.1M-ammonium acetate buffer solutions

(25 cm^3), pH 6.8 at each temperature required, with a continuous stream of air over the solutions. The optical density at 550 nm was determined for aliquots removed at 1 minute intervals, using a thermostatted cell holder at the appropriate temperatures, and plots of absorbance vs. time were recorded. Oxygen uptake curves were determined under the same conditions for solutions of 20 mg. tetrahydrobiopterin in 100 cm^3 buffer (since absolute values of k_2 were not required this was found convenient). The data was treated as described later.

The effects of small additions of Cu(II) were observed, together with the effect of small concentrations of EDTA, using the appropriate weights of cupric sulphate and EDTA, respectively.

Tetrahydrofolic acid. Determinations of steady state free radical concentrations at different temperatures were done as above for solutions of THFA in glacial acetic acid (2 mg. cm^{-3}).

Results and Discussion.

Fig. 4.4 shows typical plots of free radical concentration (as absorbance) vs. time. It can be seen that the concentration passes through a maximum and then decays. Some tailing off into the visible spectrum from the final products prevented accurate determinations beyond the times shown for curves B, C and D. It may be observed that the maximum concentration attained is

FIG. 4.4

Time-dependence of concentration of free radical intermediates during tetrahydrobiopterin autoxidation - typical examples.

A) In 0.1M-ammonium acetate/0.4 mM - EDTA, Air, 25°C.

B) As A, 35°C.

C) As A, 45°C.

D) In 0.1M- ammonium acetate/0.08 mM-$CuSO_4$, Air, 25°C.

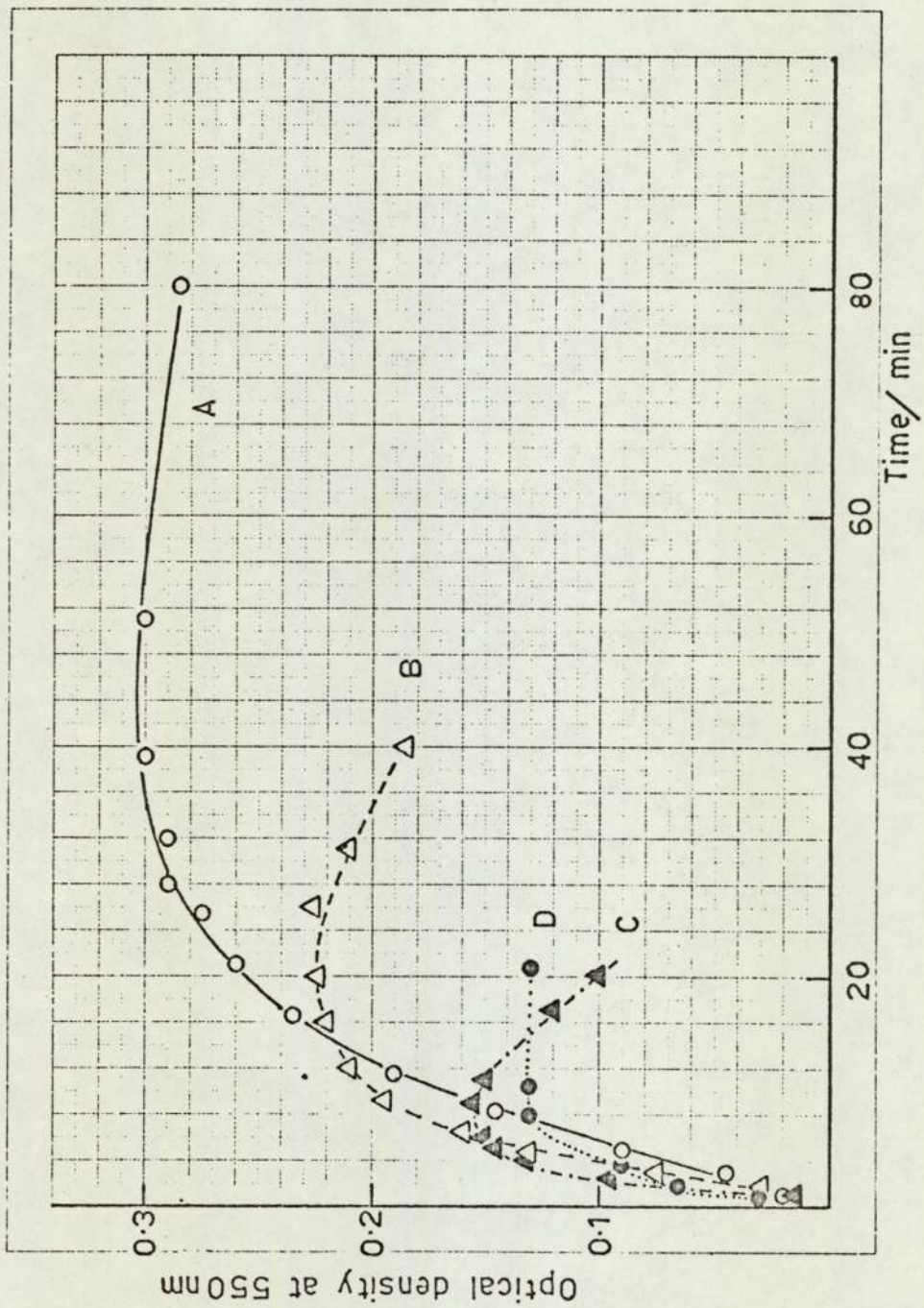


FIG. 4.4

considerably reduced as temperature is increased and also the corresponding decay rate is increased. Now, the build-up of free radical concentration is due to the rate difference between radical-producing and radical consuming reactions and at the maximum these are equal. It is reasonable to suppose that radical producing reactions involve relatively high activation energy, so that the rate will increase as temperature is raised. Now, if the usual assumption that radical consuming reactions (radical + oxygen propagation, and radical + peroxy radical termination) are zero- or low- activation energy addition processes[153] is applicable in the present reaction, then we should expect the radical disappearance rates to be approximately unaltered by increasing temperature. This would lead to an increase in the maximum radical concentration at higher temperatures. Clearly, this is not so, and the fact that the maximum drops indicates that radical consuming reactions have an activation energy comparable to radical forming reactions. This is supported by the observed increase in radical decay rates after the maximum as temperature is raised. An objection which may be raised against this qualitative argument is that the semiquinone maximum concentration is also dependent on the concentration of peroxy radicals. If we consider only the propagation steps of Scheme 3.3, repeated below:

$$AH. + O_2 \xrightarrow{k_2} AHOO.$$

$$AHOO. + AH_2 \xrightarrow{k_3} AH. + AHOOH$$

we can see that an increase in k_3 will lead to more rapid production of AH. (semiquinone) which will in turn increase the rate of formation of AHOO. proportionately (since k_2 is unaffected by temperature for this scheme). However, AHOO. is now being used more rapidly and this results in the dilemma that we cannot predict how its concentration will change with temperature. If this remains approximately constant or increases as temperature is raised, the above arguments regarding semiquinone concentration will be valid. If, on the other hand, AHOO. concentration decreases then semiquinone concentration might also decrease. Unfortunately the mathematics of this situation cannot be solved (which is the reason for making steady state approximations in treating chain reaction kinetics), and as yet peroxy radical concentrations have not been determined. However, an important observation is that rate of oxygen uptake increases as temperature is raised and this implies that the term k_2 (AH.)(O_2) increases. Again we conclude that the only way in which this can occur for a diffusion-controlled step is for the (AH.) term to increase. This is not observed, so we are forced to assume that k_2 increases as temperature is raised, thereby militating against the intermediacy of organic peroxy radicals (AHOO.). Numerical treatment is discussed later.

It is noted also that the addition of small concentrations of cupric ion results in a decrease of

TABLE 4.1. Kinetic characteristics of semiquinone radical formed during autoxidation of tetrahydrobiopterin and tetrahydrofolic acid.

Compound	Buffer	Gas Phase	Steady State Absorbance at 550 nm.	10^6 Steady State O_2 uptake rate (mol. min^{-1})	$k_2(O_2)$ (see text)	Temp. (°C).
Tetrahydro-biopterin.	0.1M-Ammonium acetate	Air	0.230	-	-	25
"	0.1M-Ammonium acetate	Pure oxygen	0.190	-	-	25
"	0.1M-Ammonium acetate + 0.08 mM-Cu(II)	Air	0.130	-	-	25
"	0.1M-Ammonium acetate; 0.08 mM - Cu(II) + 0.4 mM-EDTA	Air	0.300*	-	-	25
"	0.1M-Ammonium acetate + 0.4mM-EDTA	Air	0.300	0.40	1.33	25
"	"	Air	0.245	0.57	2.33	30
"	"	Air	0.220	0.70	3.18	35
"	"	Air	0.155	1.25	8.06	45
"	0.1M-Ammonium acetate in D_2O	Air	0.275	0.35	1.27	25
THFA	Glacial Acetic acid	Air	0.250	-	-	25
THFA	Glacial Acetic acid	Air	0.285	-	-	35

continued .....

TABLE 4.1. continued.

Compound	Buffer	Gas Phase	Steady State Absorbance at 550 nm.	10^6 Steady State O_2 uptake rate (mol. min^{-1})	$k_2(O_2)$ (see text)	Temp. (°C).
Tetrahydro-biopterin	0.1M-Ammonium acetate + 0.4 mM - EDTA + Catalase (0.1mg/cm^3)	Air	0.257*	-	-	25
Tetrahydro-biopterin	"	Air	0.155*	-	-	45

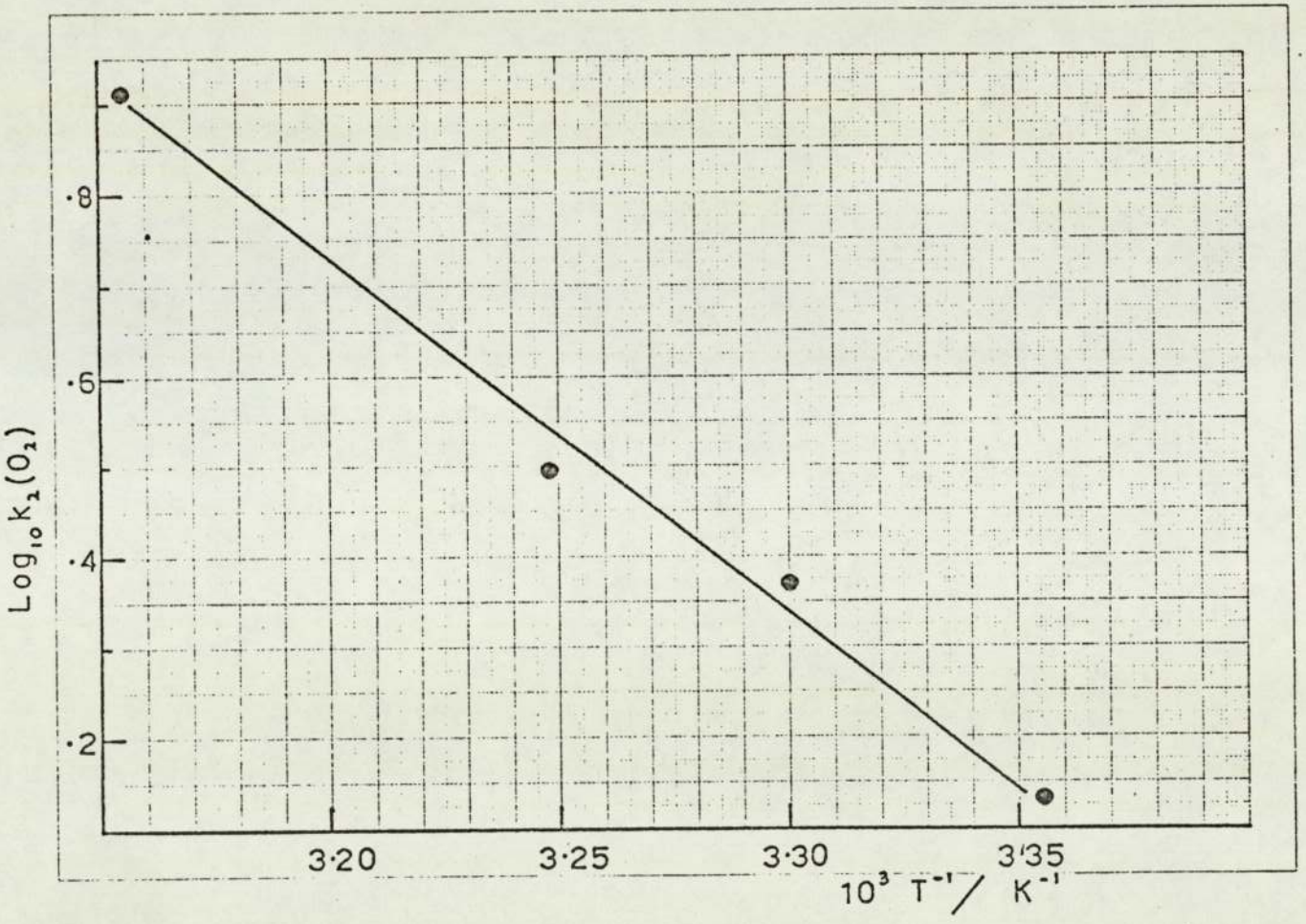
* These results indicate that the observed temperature effects are not due reaction of hydrogen peroxide with the semiquinone free radical.

the maximum semiquinone radical concentration (Fig. 4.4). It was found that addition of EDTA restored the maximum to its original value, and for this reason EDTA was included in all runs. The observation of scavenging effects by Cu(II) is quite important since we should expect the reaction between the metal and free radical to involve an appreciable activation energy. Scavenging of the oxygen reaction will then only occur if the latter also has an appreciable activation energy. Similar scavenging of organic radicals by Cu(II) has been observed[154] during hydroxyl radical reactions.

In order to treat the data obtained in the above way so as to obtain useful numerical data, it was assumed that the time period over which the semiquinone radical concentration went through its maximum represented the interval during which steady state approximation was reasonably valid, and the rate of oxygen uptake was given by equation (3). Thus, determination of this "steady state" concentration and the rate of oxygen consumption for the reaction at the steady state time allowed computation of the product $k_2(O_2)$ from equation (4). Appropriate values are recorded in Table 4.1 and it can be seen that raising the temperature leads to an increase in this product. Since oxygen concentration decreases slightly as temperature is raised in this region, this must imply that k_2 increases, and therefore has an appreciable activation energy. A quantitative estimate of this activation parameter is obtained from the corres-

FIG. 4.5

Arrhenius Plot for reaction of free radical with oxygen at steady state (text).



ponding Arrhenius plot (Fig. 4.5) in which oxygen solubility changes are neglected, since these are partially counterbalanced by the small reciprocity between semiquinone and oxygen concentrations (Table 4.1), and the smallness of oxygen solubility changes in this region[110]. The value for this apparent activation energy is 52 kJ. mol^{-1}, in excellent agreement with the value obtained in the previous section.

Unfortunately, owing to the slowness of reaction, the overall reaction rates for THFA in glacial acetic acid could not be reliably determined, so similar estimates are not possible. However, assuming an overall Q_{10} at these acidities of around 2.5, the use of the semiquinone concentrations in Table 4.1 leads to a similar value of activation energy for the radical + oxygen reaction.

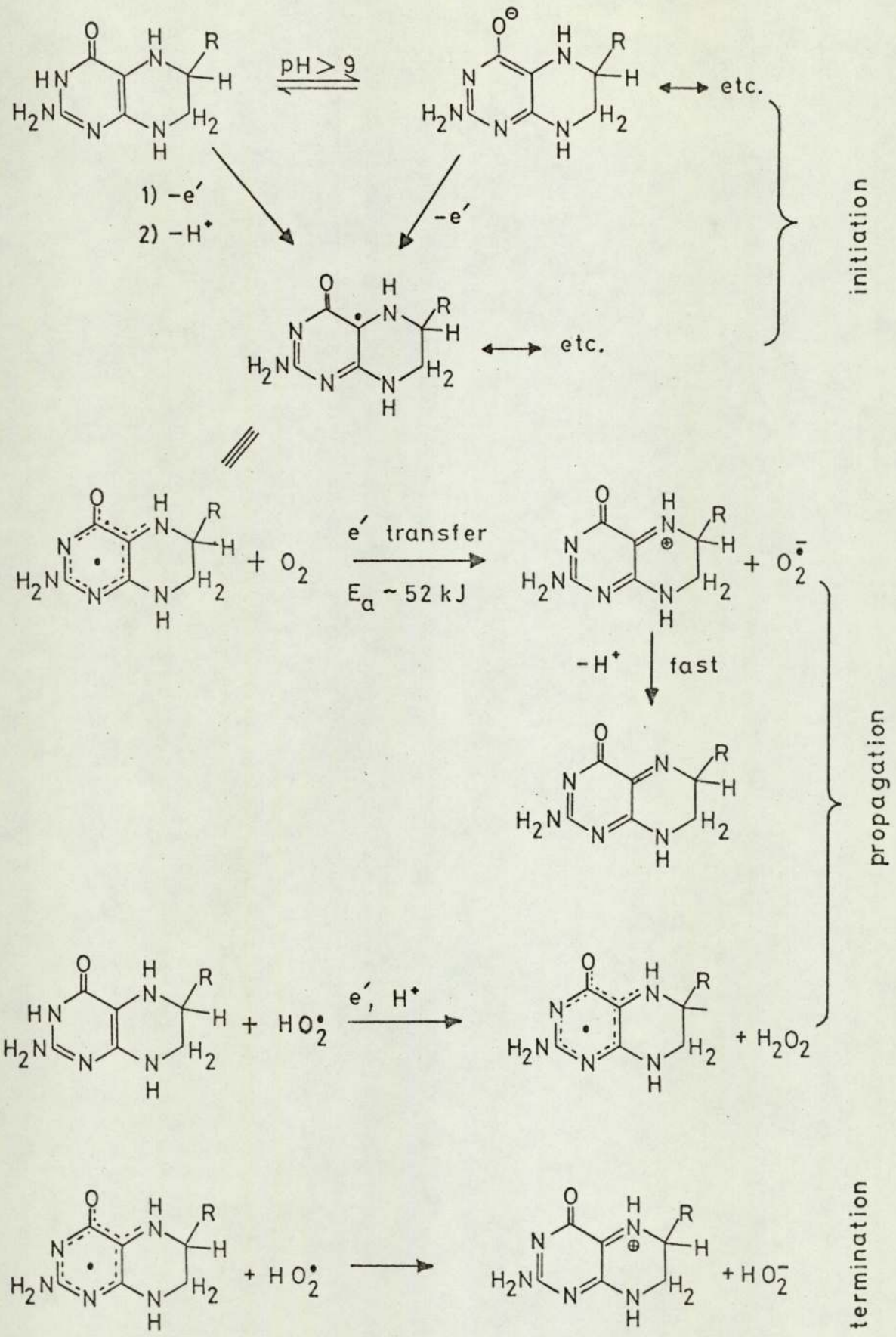
The lack of significant isotope effect in $k_2(O_2)$ when the determination is carried out in deuterium oxide (99.5 atom per cent deuterium) shows that no N - H bond homolysis is occurring in this step, so that the process must be one of electron transfer from semiquinone to oxygen. Hence the autoxidation occurs by consecutive electron transfer reactions leading to a chain process in which the chain carrier is superoxide anion or the hydroperoxyl radical (HO_2.) formed by protonation of the latter. This proposal is in complete agreement with the observations of Archer and Scrimgeour[83], who showed that oxygen is not an effective scavenger during ferricyanide oxidation of tetrahydropterins. In view of the high

activation energy for all the propagation steps, the chains are probably of short length. A reasonable mechanism is outlined in Scheme 4.1.

All attempts to generate the semiquinone radical *in situ*, free from unoxidised tetrahydro-compound, and thereby directly determine the kinetics of its reaction with oxygen, were unsuccessful owing to the similarity in reactivity of starting molecule and intermediate. Hence the "Steady-State" method provides the only means of determining the semiquinone reactivity.

The possibility of such electron transfer or H-abstraction sequences has been recognised for other autoxidations[155-164], but no evidence of the type presented here appears to have been discussed. Reduced flavins, of general structure 4(i), are readily autoxidised in aqueous media and a large volume of evidence for the involvement of superoxide anion has been presented[165-170], including the observation of its e.s.r. spectrum in a frozen matrix[171]. These compounds are structurally very similar to tetrahydropterins, and as such might be expected to show analogous behaviour towards molecular oxygen.

In addition, it is no novelty to observe a free radical which does not react rapidly with oxygen. The stability phenomenon appears to occur for highly conjugated radicals, e.g. α,γ -bisdiphenylene-β-phenylallyl[172] and 3,3',5,5' -tetra-t-butyl-4,4' -dihydroxydiphenyl-methane[173]. The extent of conjugation in the semiquinone



SCHEME 4.1 Final autoxidation mechanism — kinetics-based

radicals formed in tetrahydropterin autoxidation confers stability on these and no doubt reduces the reactivity towards addition of molecular oxygen owing to the molecular distortion and loss of resonance energy involved. However, the electron rich radical may easily lose electron which involves no such losses of resonance energy, etc., and is therefore the preferred reaction.

What is more, Scheme 4.1. is consistent with the observation made in Chapter 3 that the autoxidation reaction is considerably slower in non-polar media, since charge transfer and separation of the resulting ion pair is expected to be facilitated by solvents of high dielectric constant. In non-polar media a "back-transfer" of electron might occur before the ion-pair can dissociate. Furthermore, transition states showing charge separation might be stabilised by polar solvents.

Competition Studies with Superoxide Dismutase - a probe for superoxide radicals.

Superoxide Dismutase is an enzyme isolated by McCord and Fridovich[174,175] from bovine erythrocytes, which specifically catalyses the conversion of two superoxide radical anions to one molecule of oxygen and one molecule of hydrogen peroxide, according to equation (5).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow[\text{Dismutase}]{\text{Superoxide}} O_2 + H_2O_2 \qquad (5)$$

The enzyme has been extensively used to demonstrate the participation of superoxide anion in many processes (e.g. see review by Fridovich[176]) including its presence in the autoxidation of epinephrine, 4 (ii), the mechanism of which has been interpreted[177] in an analogous way to that presented in Scheme 4.1. for tetrahydropterins.

It has recently been noted by Kaufman[178], that tetrahydrobiopterin autoxidation shows a 50% reduction in rate when superoxide dismutase is present, although no details of conditions are given.

Since the pKa of the hydroperoxyl radical ($HO_2^{\cdot}$) is 4.8 [179], then at a pH of 7 or greater, we should expect the proposed chain carrier in tetrahydropterin autoxidation to be almost entirely in the ionised form. Therefore, an attempt was made to observe inhibition by superoxide dismutase, but in view of the fact that oxygen is liberated during the disproportionation reaction, the use of manometric techniques for rate determinations was abandoned in favour of spectrophotometric methods.

Method.

A solution of tetrahydrobiopterin (ca. 2mg) in 0.1M-sodium phosphate buffer, pH 7, (50 cm^3, previously equilibrated with air), was rapidly made up

and the absorbance at 300 nm was noted at convenient times, at ambient temperature (23°C). Since the reaction is known to be first order in tetrahydrobiopterin there was no need to use exactly the same weights in all experiments, and the pseudo first order rate constant for the reaction was derived from the appropriate first order plots (Fig. 4.6).

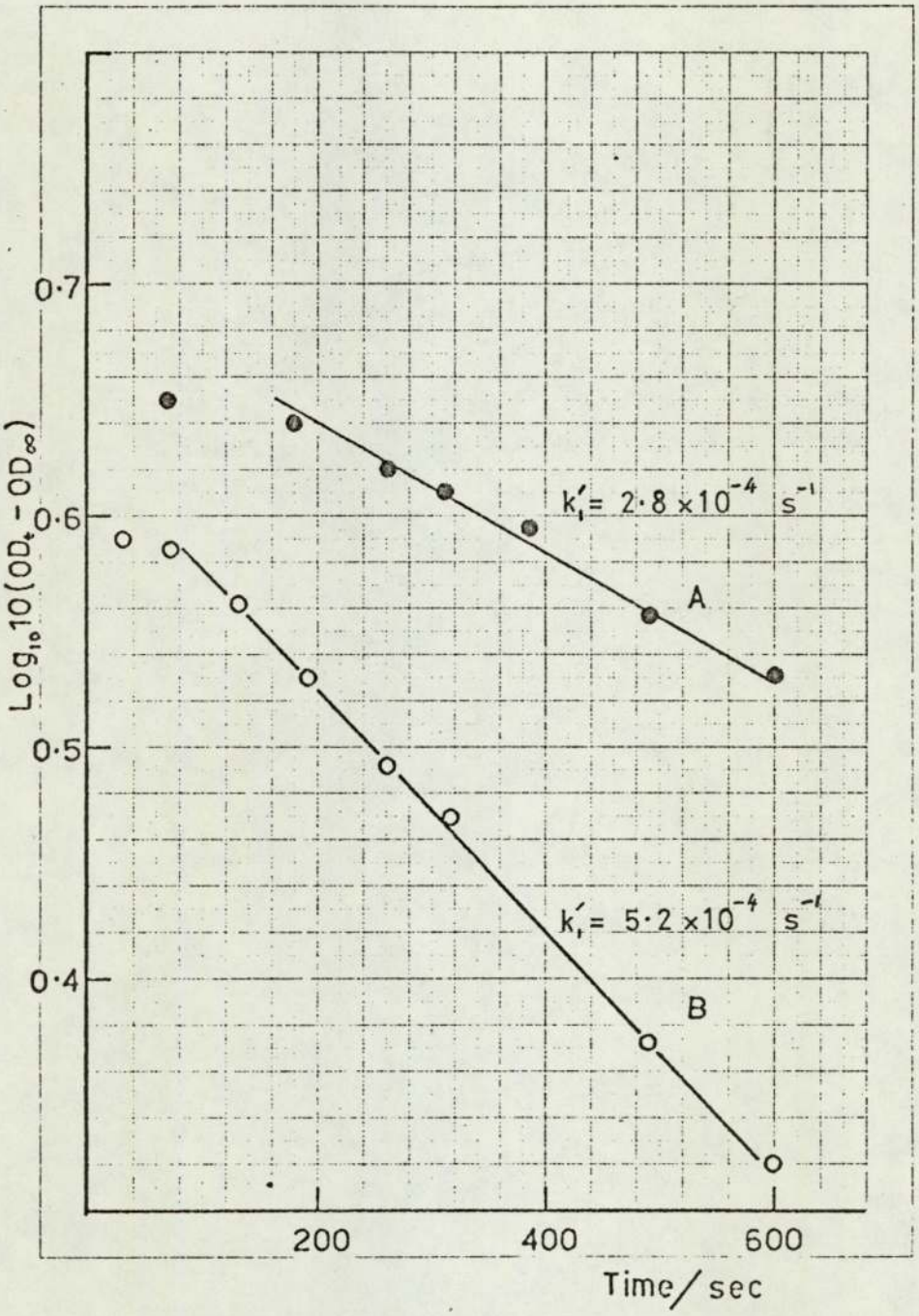
The experiment was repeated, using phosphate buffer containing superoxide dismutase at a concentration (4.4 mg/100 ml) which gave no absorption in the ultra-violet above 250 nm.

Results and Discussion.

The linear first-order plots are given in Fig. 4.6, and the presence of an induction period may be observed as with the oxygen uptake experiments. It can be seen that there is an approximately 50% inhibition of oxidation by the enzyme, thus confirming the observation of Kaufman[178] suggesting the involvement of superoxide radical. These results support the mechanistic interpretation presented in Scheme 4.1.

FIG. 4.6

Pseudo-first-order plots showing effect of Superoxide Dismutase on tetrahydrobiopterin autoxidation in 0.1M-sodium phosphate buffer/air. Spectrophotometric determination.

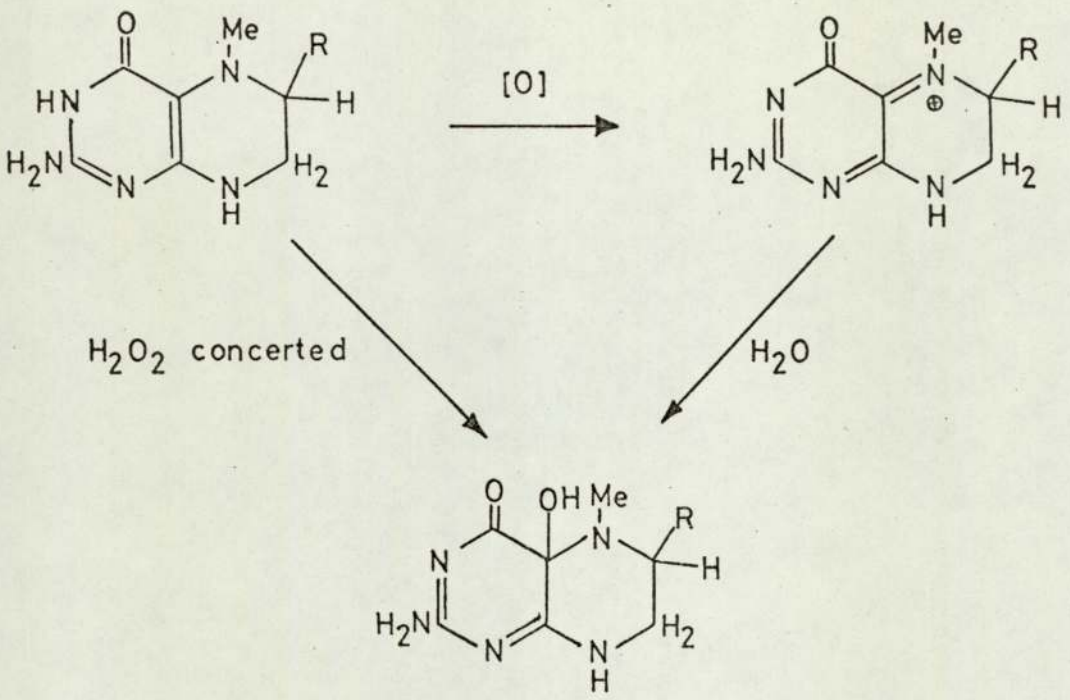


A) With Superoxide dismutase.

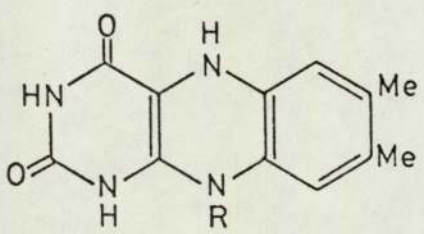
B) Without Superoxide dismutase.

Comment.

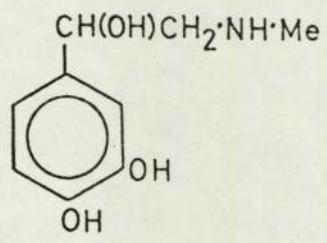
On the basis of the mechanism proposed above, there may appear to be a slight discrepancy with the literature. For instance, it has been reported[93,180,181] that oxidation of 5-methyl tetrahydropterins with either hydrogen peroxide or oxygen sometimes leads to a 4a-hydroxylated derivative. This observation can be very misleading in that such compounds might arise from decomposition of 4a-hydroperoxide intermediates formed during autoxidation. However, in only one case[181] have ^{18}O - labelling experiments been done and these have shown that oxidation with labelled H_2O_2 results in labelled 4a-alcohol. This does not necessarily show that molecular oxygen is incorporated via hydroperoxide intermediates, and it is likely that any alcohol formed in oxygen reactions is a pseudo-base formed by addition of water to a quaternary quinonoid dihydropterin cation, as shown in Scheme 4.2. Similar incorporation of oxygen from hydrogen peroxide into oxidation products from model tetrahydropteridines has been observed[114], in which there is no evidence for molecular oxygen incorporation, as discussed in Chapter Five, and it is proposed that a concerted reaction between "blocked" tetrahydropteridines and hydrogen peroxide might occur as a side reaction.



SCHEME 4.2 Possible 4a-hydroxylation modes



4 (i)



4(ii)

CHAPTER 5.

The autoxidative ring contraction of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione. Evidence for hydroperoxide intermediates?

In Chapter One it was mentioned that Mager and Berends have proposed a reaction mechanism for the autoxidation of model tetrahydropteridines involving intermediacy of hydroperoxides formed by oxygen attack at C(8a). The evidence for this was the ring contraction of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione to give 1',3',4,5,6-pentamethyl-3-oxopiperazine-2-spiro-5'-hydantoin (Scheme 1.9, Chapter One), in which the 3-oxo function is a new oxygen atom thought to have arisen from molecular oxygen.

It was decided to test this proposal by carrying out the ring contraction in solvents containing ^{18}O-labelled water.

Methods.

Owing to the small quantity of starting material available, it was not possible to carry out the ring contraction on a scale large enough to allow isolation and purification of the spiro hydantoin. Therefore, the following small scale method was devised to examine the incorporation of ^{18}O into product.

(a) The first experiment utilised a mixture of pyridine and 43.46% enriched ^{18}O-labelled water (10:1) as solvent. The blocked compound (10 mg) was dissolved in the pyridine/water (2 cm^3) and shaken under unlabelled oxygen gas for two days at ambient temperature. A sample of the solution was removed with a hypodermic

syringe and introduced into a small melting point capillary, sealed at one end. This was then placed in a desiccator which was evacuated until all solvent had been removed. The mass spectrum of the residue was then determined by inserting the sample capillary into the mass spectrometer.

(b) A second series of experiments was run, in which pyridine was dispensed with and the possibility of secondary reactions with hydrogen peroxide generated by autoxidation was examined. The blocked tetrahydropteridine (3 mg) was shaken in labelled water (0.5 cm^3; 43.46% enriched) under the following conditions: (1) under unlabelled oxygen gas for four days at room temperature; (2) as in (1) but including catalase (4 mg) in the reaction mixture; (3) under argon for 5 h, using water previously de-oxygenated with a stream of dry argon, and in the presence of 2 x 10^{-5}M- unlabelled hydrogen peroxide (20 µl of 3% H_2O_2). After the time indicated the product from (2) was dried in vacuo, and the catalase was removed by extraction of the spirohydantoin with chloroform (0.5 cm^3). The solutions were then all treated as in (1) for mass spectrum determination.

Mass spectral examination of the products obtained above showed the presence of both starting material and 1',3',4,5,6-pentamethyl-3-oxopiperazine-2-spiro-5'-hydantoin. The incorporation of ^{18}O label,

and the percentage introduction of water as the 3-oxo-group, were calculated from the ratio of the spiro-hydantoin M and M+2 peak intensities (at $m/_e$ 254 and 256 respectively), after correction for the natural abundance M+2. 1,3,6,7,8-Pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione had $m/_e$(%) 238 (100), 223 (54), 209 (24) 195 (27), 181 (19), 166 (18), 140 (60), 138 (19), 123 (16), 109 (14), 97 (30), 95 (35), 83 (38), and 69 (57), M* 170.5 (223 ⟶ 195) (loss of CO from M-15 fragment).

Unlabelled 1'3',4,5,6-pentamethyl-3-oxopiperazine-2-spiro-5'-hydantoin (cf. Mager and Berends[97]) had $m/_e$(%) 254 (100), 239 (25), 225 (2), 196 (4), 169 (17), 154 (15), 142 (85), 141 (25), 127 (44), and 86 (15).

Results and Discussion.

The results of these experiments are shown in Table 5.1. It can be seen that in all experiments where catalase was not included there is a high incorporation of label from the water, the remaining 3-oxo function apparently being derived from molecular oxygen. However, it is also apparent that reaction of the starting tetrahydropteridine with unlabelled hydrogen peroxide in labelled water results in rapid formation of spiro hydantoin carrying very little label. Inclusion of catalase in the reaction mixture for autoxidations yields spiro hydantoin in which all the

oxygen in the 3-oxo group arises from water. That label introduction was not due to exchange in the product was shown by the fact that labelled product does not lose its label when dissolved in unlabelled water. This indicates that the apparent molecular oxygen incorporation results from a side reaction with hydrogen peroxide generated during autoxidation, which is consistent with the stoichiometry results of Chapter Three. An alternative mechanism for ring contraction has been suggested by the author[114, 182], and is reproduced in Scheme 5.1. This mechanism does not require the intermediacy of organic hydroperoxides and is consistent with all the observations recorded in the present dissertation. The mechanism of Mager and Berends does not fit in at all well with the kinetics of autoxidation for tetrahydropterins, particularly the dependence of rate on imino enolate anion concentration, but it may be argued that the methylated 2,4-dioxo compound does not furnish a good analogue. For instance, it has an oxo-function at C(2) instead of amine, and the presence of the 1-, 3- and 8- methyl substituents undoubtedly forces the ring system out of planarity. Thus, the derived semiquinone radical may not resemble that from, say, tetrahydrobiopterin and might behave rather differently. Nonetheless, the earlier results have prompted Mager and Berends to stress the detail[183] in their mechanism, in which ionisation of the 8a-hydroperoxide intermediate is proposed to

TABLE 5.1. Incorporation of ^{18}O during autoxidative ring contraction of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione using ^{18}O-labelled water. (43.46% enriched).

Reaction conditions	Ratio of (M+2):M peaks in spirohydantoin mass spectrum.	Incorporation of oxygen from water (%)
4 days; unlabelled oxygen gas, unlabelled water.	0.019	
2 days; unlabelled oxygen gas, pyridine/labelled water 10:1	0.606*	87
4 days; unlabelled oxygen gas, labelled water.	0.535*	80
4 days; unlabelled oxygen gas, labelled water + excess of catalase	0.766*	100
5h; unlabelled H_2O_2, labelled water, anaerobic.	0.073*	1.57

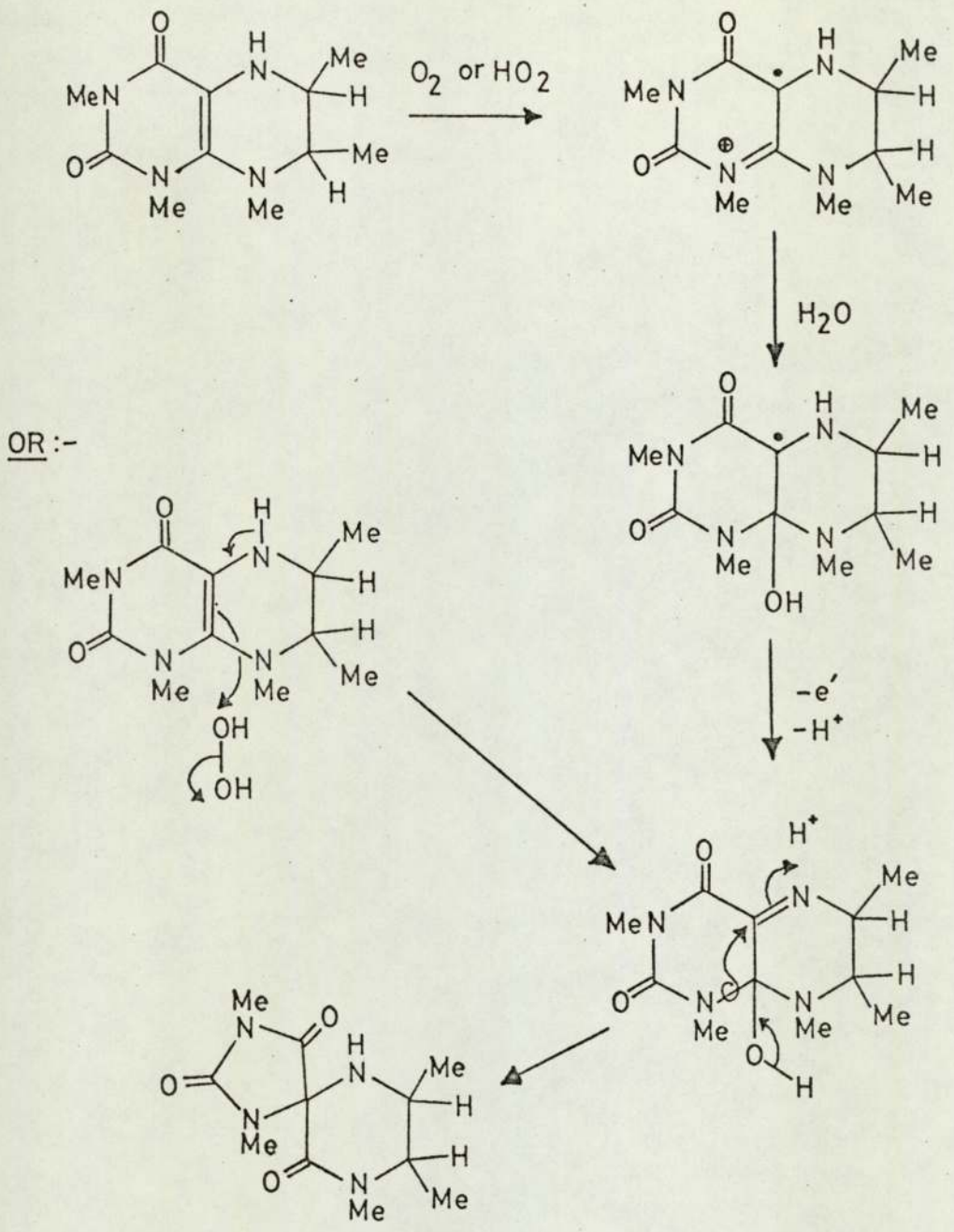
* After correction for natural (M + 2) peak (first result in Table).

TABLE 5.2.

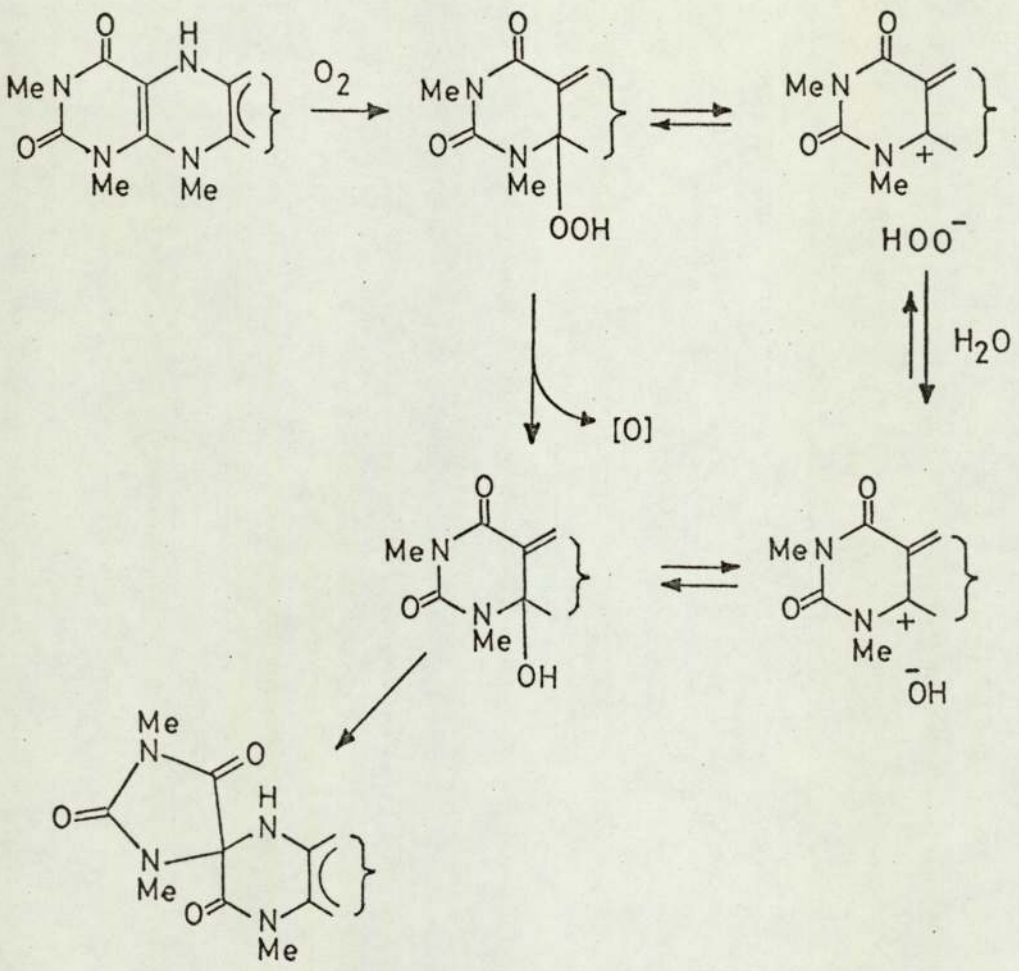
Comparison of rate of appearance of spiro-hydantoin in pyridine, pyridine/water and water solvents.

Reaction conditions.	Ratio of $^{m}/_{e}$ 254 to $^{m}/_{e}$ 238 (i.e. molecular ions of product and starting material).
5 days; dry pyridine (over $MgSO_4$) unlabelled oxygen gas.	0.0053 *
5 days; pyridine/unlabelled water 10:1 unlabelled oxygen gas.	0.145
5 days; unlabelled water, unlabelled oxygen gas.	2.22

* No other oxidation products apparent in mass spectrum.



SCHEME 5.1 Most probable mechanism for autoxidative ring contraction



SCHEME 5.2 Mager & Berends' detailed ring contraction scheme

account for the incorporation of water into product, as shown in Scheme 5.2. This mechanism might predict a lower incorporation of water in the pyridine/water system as opposed to pure water solvent, owing to the rather lower polarity of the former, and this was not observed in our experiments. Mager reports high incorporation of label from oxygen gas when benzene is used as solvent, but it is likely that this may be due to the hydrogen peroxide side-reaction mentioned above becoming predominant in solvents with low moisture content. An interesting observation is that the reaction slows down as the solvent polarity is reduced, as shown by the results of Table 5.2. If the ratio of molecular ion intensities for starting material (m/e 238) and spiro hydantoin (m/e 254) in the mass spectrum of products is compared at similar time intervals for oxidation in 'moist' pyridine (anhydrous $MgSO_4$ dried), pyridine/water (10:1) and pure water, it is observed that spiro hydantoin is produced more quickly in the media containing higher concentrations of water. This agrees well with the observed slowness of oxidation of tetrahydrobiopterin in aqueous ethanol and again suggests an electron transfer sequence.

In conclusion, it must be realised that although these experiments do not prove that organic hydroperoxide intermediates are <u>not</u> formed, they show

that the ring contractions studied cannot be offered as evidence for the structure of such species. Moreover, their existence need not even be invoked to explain the products of autoxidation.

CHAPTER 6.

Molecular orbital calcutions for 'semiquinone' free radicals from a variety of common reduced pterins.

A formulation of the transition state for electron transfer to oxygen.

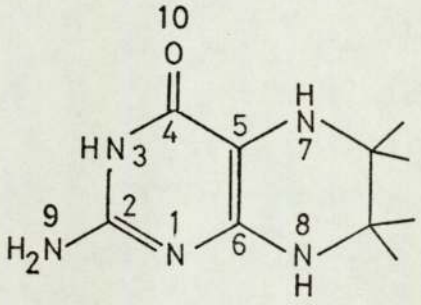
Although several molecular orbital calculations on various pterins and reduced pterins have appeared in the literature[184-187] these have all centred around determination of total electron density, resonance energy, electronic transition energies and group transfer potentials. No details of the results, such as Huckel coefficients for the highest occupied molecular orbital or the effect of choice of Huckel parameters for hetero-atoms on these coefficients, has been reported. Consequently, a brief study of these molecules, using Huckel molecular orbital (HMO) theory, was undertaken in order to examine the distribution of unpaired electron density in the free radicals resulting from one-electron oxidation, and also to correlate the ease of oxidation with the energies of the highest occupied molecular orbital (HOMO).

The unpaired electron distribution might be expected to give valuable information concerning possible points of oxygen attack on the free radical during the propagation steps of autoxidation. In view of the preceding evidence that this reaction is an electron transfer process, and not bond formation with oxygen, any such discussion of spin distribution may seem superfluous. However, owing to the fact that molecular oxygen is not a particularly good electron acceptor[112], the process of charge-transfer might be a relatively gradual process in which the transition-state resembles a <u>partially bonded</u> peroxy radical. The point of oxygen

attack on the free radical has been proposed in previous chapters as being most probably at C(4a), and it would be satisfying to support this with theoretical predictions. Such correlation is possible only if the unpaired electron density distribution can be calculated and compared with the results of e.s.r. experiments. Attempts at such a rationalisation are now described.

Method.

The methods of molecular orbital theory and Huckel approximation are adequately described in text books on quantum chemistry such as Streitwieser[188] and will be discussed no further here. Suffice it to say that the secular determinant was written for the tetrahydropterin molecule using the numbering shown in 6(i) which, although different from the conventional system, was found to be convenient for the present purposes. Any ambiguity is adequately removed later when discussing unpaired electron densities. Application of Huckel approximation results in the modified determinant



6 (i)

FIG. 6.1. Modified secular determinant for tetrahydropterins (6(i)) after Huckel approximation.

$$
\begin{vmatrix}
(\alpha_1-E) & \beta_{12} & 0 & 0 & 0 & \beta_{16} & 0 & 0 & 0 & 0 \\
\beta_{21} & (\alpha_2-E) & \beta_{23} & 0 & 0 & 0 & 0 & 0 & \beta_{29} & 0 \\
0 & \beta_{32} & (\alpha_3-E) & \beta_{34} & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & \beta_{43} & (\alpha_4-E) & \beta_{45} & 0 & 0 & 0 & 0 & \beta_{410} \\
0 & 0 & 0 & \beta_{54} & (\alpha_5-E) & \beta_{56} & \beta_{57} & 0 & 0 & 0 \\
\beta_{61} & 0 & 0 & 0 & \beta_{65} & (\alpha_6-E) & 0 & \beta_{68} & 0 & 0 \\
0 & 0 & 0 & 0 & \beta_{75} & 0 & (\alpha_7-E) & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & \beta_{86} & 0 & (\alpha_8-E) & 0 & 0 \\
0 & \beta_{92} & 0 & 0 & 0 & 0 & 0 & 0 & (\alpha_9-E) & 0 \\
0 & 0 & 0 & \beta_{104} & 0 & 0 & 0 & 0 & 0 & (\alpha_{10}-E)
\end{vmatrix}
$$

FIG. 6.2. Huckel determinant for tetrahydropterin system (6(i)).

$$
\begin{vmatrix}
x+h_1 & k_1 & 0 & 0 & 0 & k_1 & 0 & 0 & 0 & 0 \\
k_1 & x & k_3 & 0 & 0 & 0 & 0 & 0 & k_9 & 0 \\
0 & k_3 & x+h_3 & k_3 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & k_3 & x & 1 & 0 & 0 & 0 & 0 & k_{10} \\
0 & 0 & 0 & 1 & x & 1 & k_7 & 0 & 0 & 0 \\
k_1 & 0 & 0 & 0 & 1 & x & 0 & k_8 & 0 & 0 \\
0 & 0 & 0 & 0 & k_7 & 0 & x+h_7 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & k_8 & 0 & x+h_8 & 0 & 0 \\
0 & k_9 & 0 & 0 & 0 & 0 & 0 & 0 & x+h_9 & 0 \\
0 & 0 & 0 & k_{10} & 0 & 0 & 0 & 0 & 0 & x+h_{10}
\end{vmatrix}
$$

(Fig. 6.1) which still cannot be solved owing to the presence of the heteroatom coulomb- (α) and resonance- (β) integrals. This is overcome by making the approximations shown in (1) and (2) relating heteroatom to carbon parameters:

$$\alpha_x = \alpha_c + h\beta_{cc} \qquad (1)$$

$$\beta_{cx} = k\beta_{cc} \qquad (2)$$

for hetero atom X, where h and k are numerical coefficients.

Application of this approximation, dividing through by β_{cc} and putting $\frac{\alpha_c - E}{\beta_{cc}} = x$ we obtain the Huckel determinant (Fig. 6.2). Solution of the latter after substituting appropriate h and k values was achieved using the ICL 1905 computer and employing a modification of the programme used by Wheway[189] which uses the Jacobi method of matrix diagonalisation. This gives directly results of Huckel coefficients for all atoms in all energy levels, the relative values of the latter in units of β_{cc} and the values of Huckel spin density for all atoms in the radical formed by removal of electron from the highest occupied molecular orbital. These values have been shown[189] to agree very well with e.s.r. data for a number of phenolic semiquinone radicals.

The h and k parameters chosen for each hetero-atom in a particular molecule are given in the results section for that compound. Since all e.s.r. spectra of tetrahydropterins reported in the literature (see Chapter One) show a high unpaired electron density on atom 7 (equivalent to N(5)) it was decided to vary the h and k values for this systematically and note the effect. Then, a choice of spin density distribution can be exercised which gives the experimental value for this particular atom.

Results and Discussion.

(1) The tetrahydropterin system, 6(i).

The results of Huckel spin densities for the derived semiquinone radical and the effect of atom 7 (N(5)) parameter variation are shown graphically in Fig. 6.3 for atoms having appreciable spin density and including atom 6 (C(8a)). Other parameters used are shown in Table 6.1. Most notably, the effect of h variation is more pronounced than k variation.

Now, Bobst has shown[87] in e.s.r. studies that the hyperfine coupling constant for N(5) in these radicals is 9.5 $\pm$ 1 gauss, and that for the hydrogen atom on N(5) is 10.5 $\pm$ 1 gauss, and these agree with Ehrenberg *et al*[86], who give 10 $\pm$ 1 gauss for all values. The relationships between coupling constant (a) and unpaired π- electron density (ρ) on the i^{th} nitrogen are given in (3) and (4) for nitrogen and hydrogen coupling,

respectively:

$$a_i^N = Q_N^N \rho_{N_i}^{\pi} \qquad (3)$$

$$a_i^H = Q_{NH}^H \rho_{N_i}^{\pi} \qquad (4)$$

from Bobst[87].

Normally, it is found[190] that $|Q_N^N|$ is 25-36 gauss and $|Q_{NH}^H|$ is 35-39 gauss. Substituting these values into (3) and (4) and using Bobst's values of a^N and a^H for N(5) results in an unpaired electron density of 0.25 - 0.3 for this atom. From Fig. 6.3 it is found that the corresponding spin density for C(4a) lies in the range 0.21 - 0.28, whilst that for C(8a) is 0.06 - 0.09. This is in complete agreement with the qualitative predictions made from the kinetics discussed in Chapter Three. The parameters which give useful values are h = 1.0 and k = 0.90, which is between the accepted values[188] for a pyrrole-type nitrogen ($\succ\ddot{N}-$, h = 1.5 k = 1.0) and pyridine-type nitrogen ($-N=$ h = 0.5, k = 0.8). This might have been anticipated on the grounds that partial removal of electron from the lone pair of this atom causes a deviation from the conjugative properties of pyrrole-types towards those of pyridine-types. Using these parameters, the coefficients of all atoms in the complete set of energy levels is included for reference in Appendix G. The relative energy levels are given also in Table 6.2.

TABLE 6.1.

Heteroatom parameters chosen for atoms in tetrahydropterin system. 6 (i).

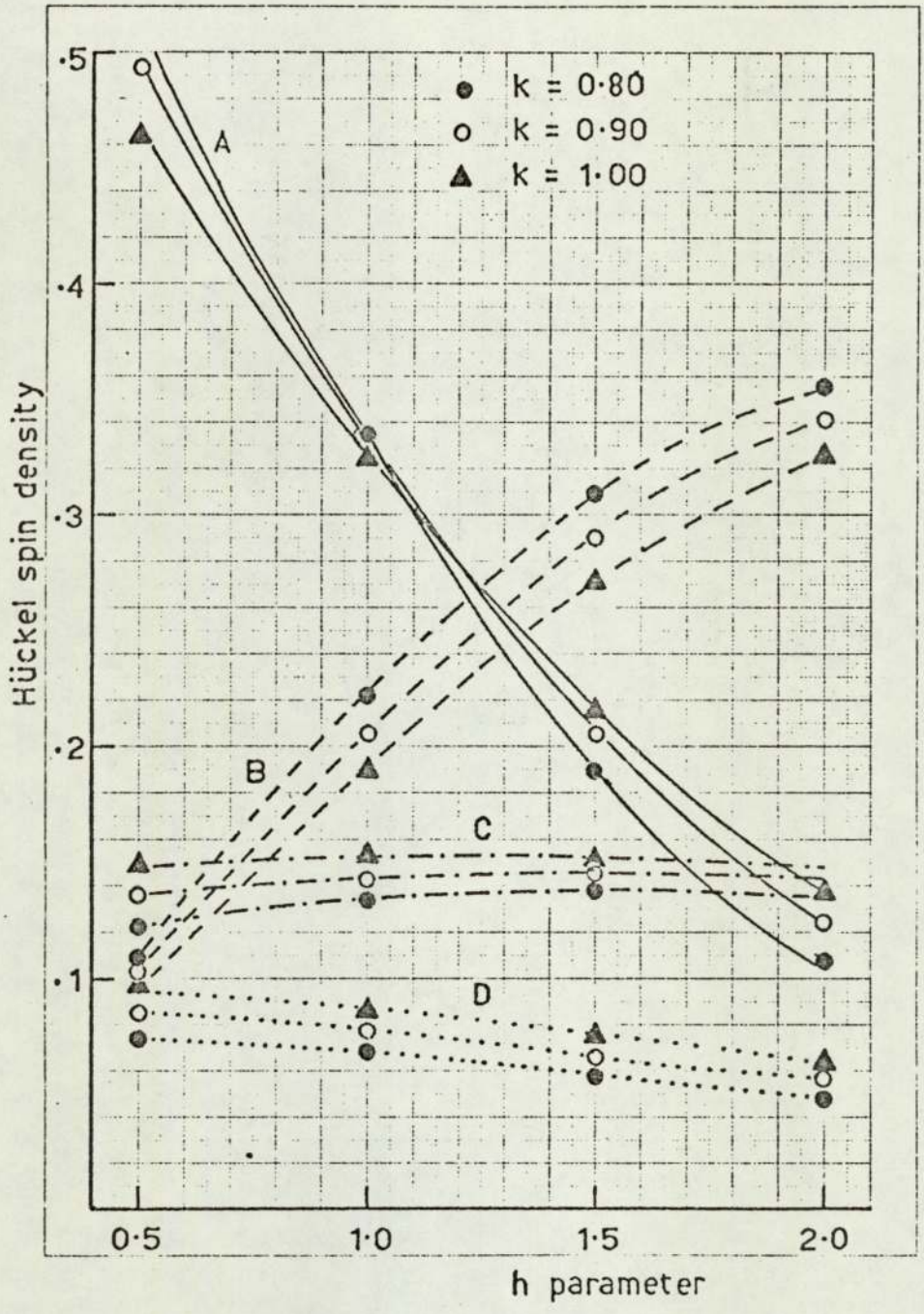
Atom		h	k
1	(≡ N(1))	0.50	0.80
3	(≡ N(3))	1.50	1.00
7	(≡ N(5))	variable	variable
8	(≡ N(8))	1.50	1.00
9	(≡ 2-amino nitrogen)	1.50	1.00
10	(amide oxygen)	2.00	1.00

Spin densities using N(5) parameters h = 1.00, k = 0.90:

Atom		Huckel spin density.
1	(N(1))	0.0311
2	(C(2))	0.0540
3	(N(3))	0.0145
4	(C(4))	0.1426
5	(C(4a))	0.2057
6	(C(8a))	0.0771
7	(N(5))	0.3355
8	(N(8))	0.0531
9	(2-amino N)	0.0372
10	(amide 0)	0.0491

FIG. 6.3

Effect of heteroatom parameter variation for N(5) on Huckel spin densities for tetrahydropterin-derived semiquinone radical.



A) Atom N(5)

B) Atom C(4a).

C) Atom C(4)

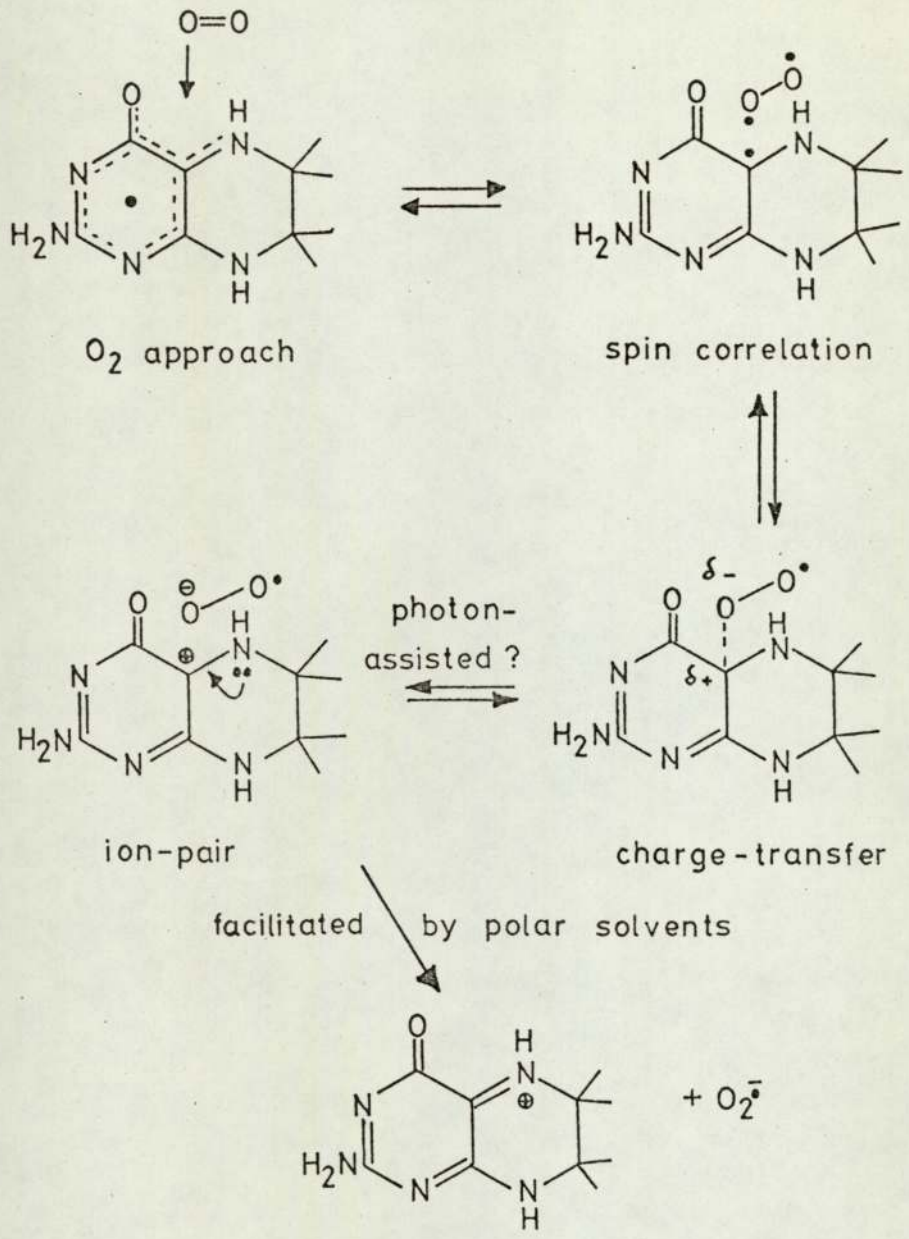
D) Atom C(8a)

TABLE 6.2.

Relative energy levels for π-molecular orbitals of semiquinone from tetrahydropterin.

Energy level	electronic occupation
$\alpha + 2.8942\ \beta$	↑↓
$\alpha + 2.3080\ \beta$	↑↓
$\alpha + 2.2274\ \beta$	↑↓
$\alpha + 1.6597\ \beta$	↑↓
$\alpha + 1.5161\ \beta$	↑↓
$\alpha + 0.9240\ \beta$	↑↓
$\alpha + 0.2953\ \beta$	↑
$\alpha - 0.8397\ \beta$	empty (antibonding)
$\alpha - 1.0673\ \beta$	empty (antibonding)
$\alpha - 1.9176\ \beta$	empty (antibonding)

These results of unpaired electron density are very interesting. Suppose an oxygen molecule undergoes collision with the semiquinone radical, and suppose also that the transfer of electron from the latter is a gradual process (on the atomic time scale). In order that an effective flow of charge should occur, let us imagine that a partial bond is formed between oxygen and the semiquinone. This is quite reasonable, since oxygen has two unpaired electrons in the ground state and pairing may occur between one of them and the odd electron on the semiquinone. Furthermore, it is normally supposed that bond formation in a radical + oxygen reaction is preferred at a carbon atom, rather than nitrogen. Thus, the above partial bond formation is preferred at carbon, and moreover will occur only at a carbon atom having an appreciable share of the odd electron. On this basis the oxygen must attack at C(4a) for the most effective electron transfer, and the possible steps involved in the process are shown in Scheme 6.1. In forming the transition state the odd electron is localised at C(4a) and this leads to some loss of resonance energy. This has not been estimated, but it will no doubt contribute to the activation energy for electron transfer. Also, the addition of electron to oxygen is a process involving a positive Gibbs Free energy change. George[191] quotes the value of -0.45 volts for the equilibrium of equation (5) at pH 7, which corresponds



SCHEME 6.1 Transfer of electron from semiquinone to oxygen

to $\Delta G^{o} = +43.3$ kJ. mol^{-1}, a value rather close

$$O_2 + e^- \rightleftharpoons O_2^- \qquad (5)$$

to the observed apparent activation energy of 52 kJ.$mol.^{-1}$ Now, it may seem that the process is thermodynamically unfavourable. However, Archer and Scrimgeour[81] quote a value for the redox potential of the equilibrium (7) at pH 7 of + 0.15 volts.

$$\text{Tetrahydropterin} \rightleftharpoons \text{quinonoid dihydropterin} + 2e' + 2H^+ \qquad (7)$$

Assuming that the semiquinone radical intermediate is of rather higher energy than tetrahydropterin (if this were not so it would probably be much less easily oxidised and might be observed in higher concentration) then the corresponding potential for radical conversion to quinonoid dihydropterin might be rather more positive. This would largely offset the endothermicity of oxygen reduction and may well result in a thermodynamically favourable reaction. On the other hand, addition of oxygen to the free radical to give organic peroxy radical is not thermodynamically favourable, since the same loss of resonance energy would be involved and also a re-hybridisation of C(4a) to a tetrahedral configuration would be required as opposed to the relatively planar structure being maintained

during electron transfer. Such a process involves considerable molecular reorganisation and might lead to an overall increase in Gibbs Free Energy even though σ-bond formation would normally be exothermic. Hence the interpretation of the phenomena observed in Chapter Four in terms of activation parameters is rationalised, and it is seen that here we have a delicate balance in which a high activation energy, thermodynamically favoured process is preferred over a lower activation energy but thermodynamically unfavourable reaction. That is to say, the reaction of semiquinone with oxygen exhibits thermodynamic rather than kinetic control. At this point, it may also be noted that the potential for reduction of superoxide to hydrogen peroxide is + 0.98 volts[191] at pH 7, for equilibrium (8):

$$O_2^- + 2H^+ + e^- \rightleftharpoons H_2O_2 \qquad (8)$$

This species would therefore abstract electron from tetrahydropterin more easily than oxygen, thereby justifying its appearance in the propagation reactions of Chapter Four (Scheme 4.1). The corresponding reaction with semiquinone is a chain termination step. This will be facile, and will probably lead to rather short chains.

Another point which arises from these considerations is that the transition state in Scheme 6.1 can be looked upon as a kind of transient charge-transfer complex. Consequently, it might be expected that absorption of

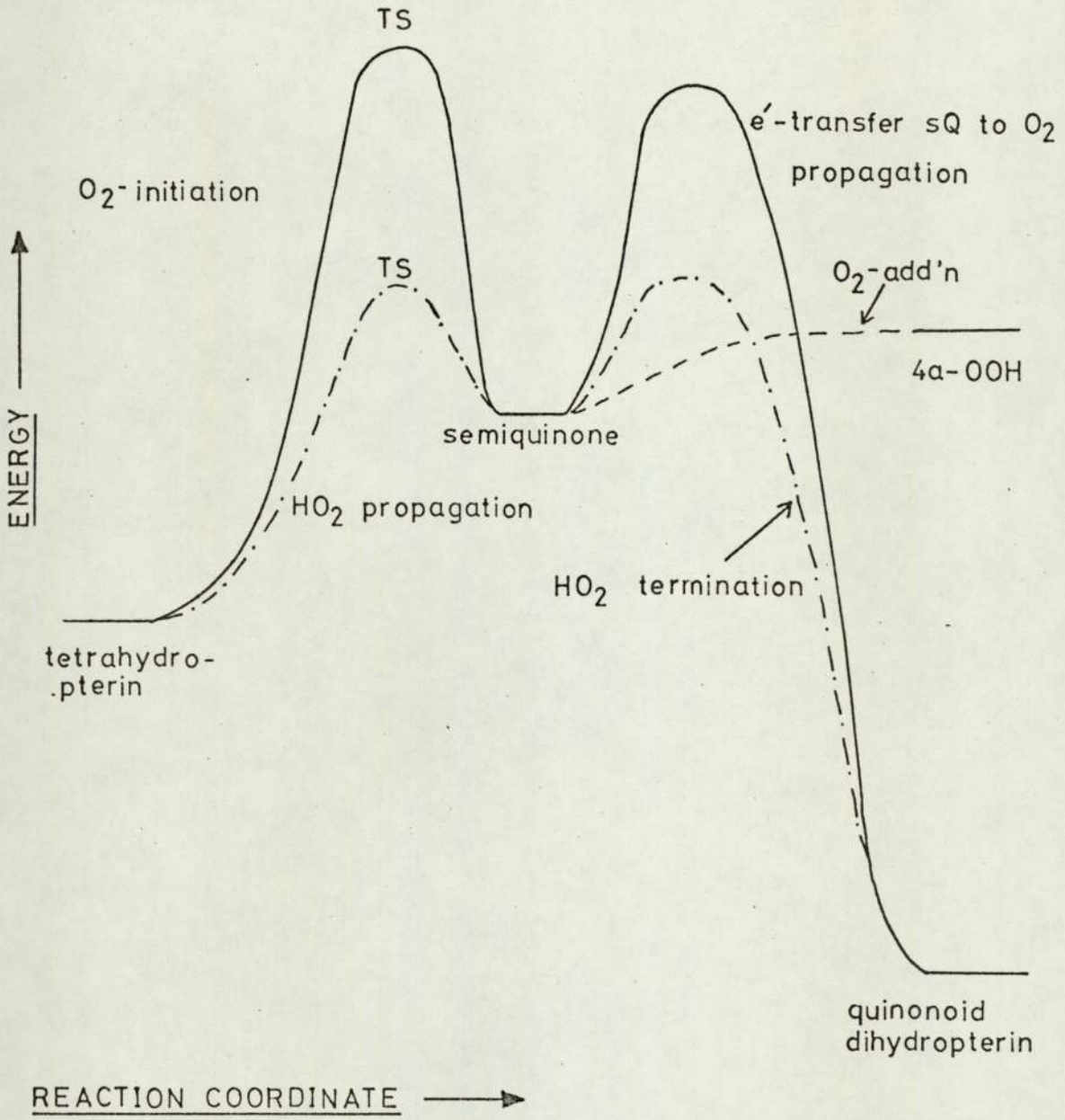


FIG. 6.4 Proposed reaction profiles to account for preference of electron transfer

visible light will assist the process of electron transfer, and this might explain the effects of daylight noted in Chapter Three equally as well as a light-assisted initiation. The probable endothermicity of an oxygen initiation step also justifies the omission of this in steady-state rate equations.

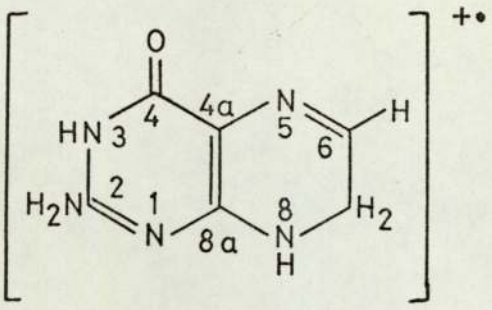
A possible reaction profile has been drawn in Fig. 6.4 which shows the above effects diagrammatically, although it must be admitted that this is rather speculative.

(2) Unpaired electron density distributions in other reduced pterin free radicals.

Huckel calculations have been done for the free radicals derived by electron abstraction from (a) 7,8-dihydropterin, (b) 5,8-dihydropterin, (c) 5,6-dihydropterin, (d) dihydroxanthopterin and (e) dihydroisoxanthopterin. The heteroatom parameters chosen are shown together with the results given in Tables 6.3 to 6.7. The choice of parameters was rather arbitrary, based on the nature of the groups involved (e.g. whether nitrogen is pyrrole- or pyridine- type in the starting non-oxidised molecule).

It is apparent from these results that C(4a) always has a large share of the unpaired electron, and this is only delocalised on to either C(6) or C(7) when

TABLE 6.3. Unpaired electron densities in the radical derived from 7,8-dihydropterin.

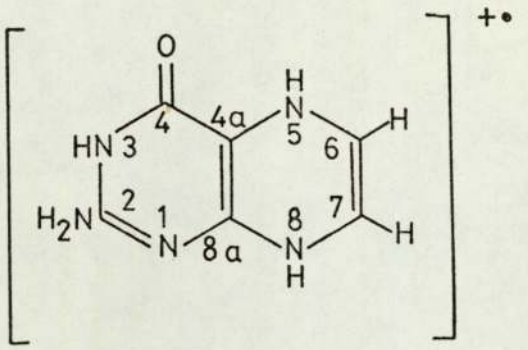


Heteroatom parameters:

	h	k
N(3), N(8), 2-amino N	1.50	1.00
N(1), N(5)	0.50	0.80
Amide oxygen	2.00	1.00

Atom	Huckel spin density.
N(1)	0.0592
C(2)	0.0416
N(3)	0.0140
C(4)	0.1026
C(4a)	0.2495
N(5)	0.1067
C(6)	0.2562
N(8)	0.0408
C(8a)	0.0396
2-Amino N	0.0430
Amide oxygen	0.0466

TABLE 6.4. Unpaired electron densities in the radical derived from 5,8-dihydropterin.



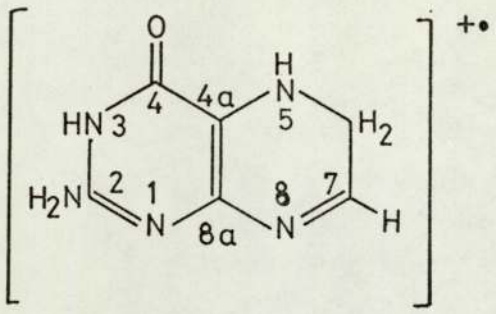
Heteroatom parameters.

	h	k
N(1)	0.50	0.80
N(3), N(5), N(8), 2-amino N	1.50	1.00
Amide oxygen	2.00	1.00

Spin densities.

Atom	Huckel spin density.
N(1)	0.0039
C(2)	0.0194
N(3)	0.0202
C(4)	0.1249
C(4a)	0.1007
N(5)	0.2066
C(6)	0.1340
C(7)	0.2053
N(8)	0.1353
C(8a)	0.0100
2-amino N	0.0086
amide oxygen	0.0311

TABLE 6.5. Unpaired electron densities in the radical derived from 5,6-dihydropterin.



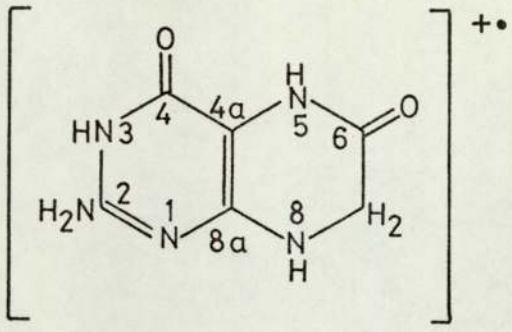
Heteroatom parameters.

	h	k
N(1), N(8)	0.50	0.80
N(3), N(5), 2-amino N	1.50	1.00
Amide oxygen	2.00	1.00

Spin densities.

Atom	Huckel spin density
N(1)	0.0930
C(2)	0.0644
N(3)	0.0096
C(4)	0.1292
C(4a)	0.2276
N(5)	0.1956
C(7)	0.0728
N(8)	0.0202
C(8a)	0.0805
2-amino N	0.0553
Amide oxygen	0.0518

TABLE 6.6. Unpaired electron densities in the radical derived from dihydroxanthopterin.



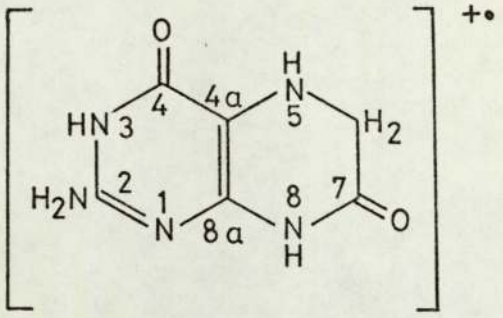
Heteroatom parameters.

	h	k
N(1)	0.50	0.80
N(3), N(5), N(8), 2-amino N	1.50	1.00
Amide oxygens	2.00	1.00

Spin densities.

Atom	Huckel spin density
N(1)	0.0730
C(2)	0.0532
N(3)	0.0179
C(4)	0.1319
C(4a)	0.3156
N(5)	0.0932
C(6)	0.0671
N(8)	0.0528
C(8a)	0.0519
2-amino N	0.0541
4-oxo O	0.0593
6-oxo O	0.0302

TABLE 6.7. Unpaired electron densities in the radical derived from dihydroisoxanthopterin.



Heteroatom parameters.

	h	k
N(1)	0.50	0.80
N(3), N(5), N(8), 2-amino N	1.50	1.00
Amide oxygens	2.00	1.00

Spin densities.

Atom	Huckel spin density.
N(1)	0.0869
C(2)	0.0661
N(3)	0.0115
C(4)	0.1394
C(4a)	0.2467
N(5)	0.2089
C(7)	0.0183
N(8)	0.0200
C(8a)	0.0835
2-amino N	0.0560
4-oxo O	0.0554
7-oxo O	0.0073

conjugation with these atoms occurs via N(5) (compare the 7,8- and 5,8- dihydro- systems with the 5,6-dihydro- system). The spin density on N(5) appears to be rather more variable, and C(4) always retains an appreciable share.

Although no details of mechanism can be inferred, such as whether oxygen will add to or abstract electron from the radical, it is clear that in none of these systems does C(8a) show an appreciable spin density relative to C(4a), and on this basis it is unlikely that addition of oxygen molecule would occur at this centre. Thus, the calculations provide no basis for rationalisation of the mechanism proposed by Mager and Berends with the kinetic data recorded in Chapter Three.

(3) The relative energies of the highest occupied molecular orbitals of reduced pterins in relation to their ease of oxidation.

Table 6.8 shows the relative energy levels for all the preceding pterin derivatives studied, in units of β_{cc}, the resonance integral for carbon. The hetero-atom parameters are as used in the last section except that the N(5) parameters chosen for tetrahydropterin are h = 1.50, k = 1.00 since this is expected to be a pyrrole-type nitrogen in the non-oxidised molecule. The filled energy levels are starred, and for convenience the

TABLE 6.8. Relative energy levels for π-molecular orbitals of reduced pterin derivatives.

Tetrahydropterin	7,8-dihydro-pterin	5,6-dihydro-pterin	5,8-dihydro-pterin	dihydro-xanthopterin	dihydro-isoxanthopterin
+2.9143*	+2.8865*	+2.9028*	+2.9664*	+2.9520*	+2.9328*
+2.3142*	+2.3077*	+2.3134*	+2.6105*	+2.6581*	+2.6973*
+2.2955*	+2.2095*	+2.1495*	+2.3273*	+2.3079*	+2.3134*
+1.8840*	+1.6401*	+1.6221*	+2.1334*	+2.2405*	+2.2120*
+1.6088*	+1.4385*	+1.5462*	+1.6195*	+1.9159*	+1.9084*
+0.9329*	+0.9301*	+0.9067*	+1.2733*	+1.6141*	+1.6097*
+0.3796*	+0.5164*	+0.4212*	+0.7314*	+0.9404*	+1.0113*
-0.8397	-0.5673	-0.3966	-0.0037*	+0.5084*	+0.4134*
-1.0671	-0.8398	-0.9676	-0.7979	-0.7474	-0.5728
-1.9226	-1.0947	-1.0720	-1.0367	-0.8399	-1.0111
	-1.9271	-1.9256	-1.3344	-1.1071	-1.0780
			-1.9891	-1.9430	-1.9365

← (E - α) in units of β_{cc}

Negative values are antibonding orbitals, positive values bonding orbitals.

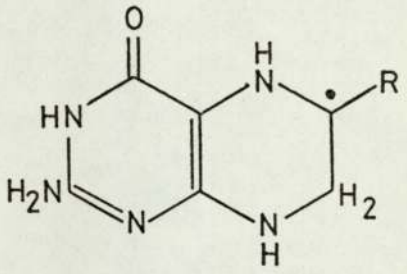
highest occupied levels are underlined. For an oxidation involving electron withdrawal, this will occur from the highest occupied molecular orbital, so that the higher is the energy level of the latter then the easier will be electron abstraction. On this basis we would predict the order of reactivity:

5,8-dihydropterin > tetrahydropterin > dihydroisoxanthopterin > 5,6-dihydropterin > dihydroxanthopterin > 7,8-dihydropterin

This sequence has not been subjected to rigorous testing, since some of the compounds (5,6- and 5,8-dihydropterin) are not readily available. However, the results of Chapter Three showed that THFA is oxidised much more rapidly than is DHFA under the same conditions, and it is known that dihydroxanthopterin is oxidised very slowly[192], although accurate rates were not obtained. Thus, the above suggestion is partially borne out in practice, and it would appear that oxidation does involve electron abstraction from the highest occupied M.O. in complete agreement with the suggestions made earlier.

(4) A reason for the absence of C-H bond homolysis at C(6) in tetrahydropterins.

Following the mechanistic suggestion of Stocks-Wilson in which the radical 6(ii) was proposed as being formed by a nett homolysis of the C-H bond at C(6), kinetic evidence was obtained which militated against the proposal. It is interesting to speculate about the reasons why this does not occur, from a molecular orbital theory viewpoint. Using the heteroatom parameters shown in Table 6.9, the energy levels and spin densities shown were calculated.



6 (ii)

It can be seen that the unpaired electron is strongly localised at C(6) with some delocalisation onto N(5), so if this radical were formed one might expect reaction with oxygen to occur at C(6). However, the odd electron is placed in an orbital which is markedly

TABLE 6.9. HMO calculations on Stocks-Wilson's C(6) free radical.

Heteroatom parameters:

As for tetrahydropterin Table 6.1 except

N(5) h = 1.50, k = 1.00

Spin densities

Atom	Huckel spin density.
N(1)	0.0002
C(2)	0.0412
N(3)	0.0001
C(4)	0.0513
C(4a)	0.0001
N(5)	0.1665
C(6)	0.6786
N(8)	0.0087
C(8a)	0.0347
2-Amino N	0.0103
Amide oxygen	0.0082

Energy levels	electron population.
$\alpha + 2.9334\ \beta$	↑↓
$\alpha + 2.4346\ \beta$	↑↓
$\alpha + 2.3065\ \beta$	↑↓
$\alpha + 2.0520\ \beta$	↑↓
$\alpha + 1.6180\ \beta$	↑↓
$\alpha + 0.9460\ \beta$	↑↓
$\alpha + 0.5752\ \beta$	↑↓
$\alpha - 0.4953\ \beta$	↑
$\alpha - 0.8398\ \beta$	empty
$\alpha - 1.0907\ \beta$	empty
$\alpha - 1.9399\ \beta$	empty

anti-bonding, and this is a strong destabilising factor. Although the bonding orbitals appear to show a slight lowering of energy relative to the "unoxidised" molecule (compare Table 6.8), the total drop is only 1.0708 β, and this is hardly sufficient to compensate for the energy required to break the C-H bond and place the odd electron in an orbital which is anti-bonding by 0.4953 β. This process might well require an energy greater than 4-5 β units. Thus, it would appear that such a step is thermodynamically unfavourable compared to the more facile electron abstraction.

CHAPTER 7.

Recapitulation; the completion of the autoxidative breakdown of tetrahydropterins.

Rearrangement of quinonoid dihydropterin intermediates.

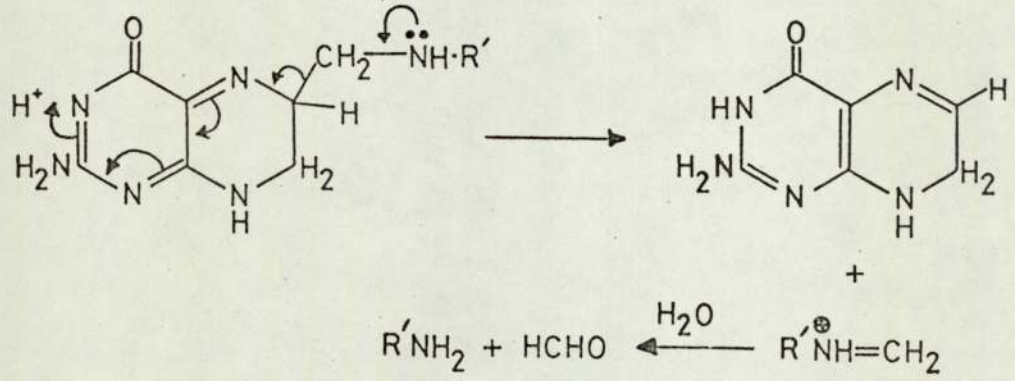
Suggestions for further work.

In the foregoing discussion it was seen that electron withdrawal during oxidation of tetrahydropterins occurs primarily in the electron rich pyrimidine moiety of the molecule. The involvement of a quinonoid dihydropterin is seen to be obligatory, owing to the fact that the stable end-products show oxidation (apparent dehydrogenation) of the 5,6 N-C bond. This is in complete agreement with most of the literature discussed in Chapter One. Thus, the final products must arise by rearrangement of the quinonoid intermediate. Using THFA as an example we can now rationalise the products formed under different conditions of pH with acid- and base-catalysed rearrangements of such a structure as in Scheme 7.1. At low pH, protonation at N(3) occurs and there is a fragmentation of the molecule due to the involvement of the N(10) lone pair leading to a pull-push movement of electrons. This will lead to 7,8-dihydropterin which is then oxidised to pterin, the observed major product under acid conditions. As the pH is raised protonation becomes less important and a base-catalysed removal of proton from C(6) predominates. This leads to the formation of 7,8-dihydrofolic acid, the observed first product at high pH. Oxidation of the latter probably involves similar electron removal, addition of hydroxide at C(6) and a quinonoid rearrangement in which the C(6) - C(9) bond is cleaved by the "electron push" effect from the lone pair of N(10) as shown in Scheme 7.2. This leads to dihydroxanthopterin

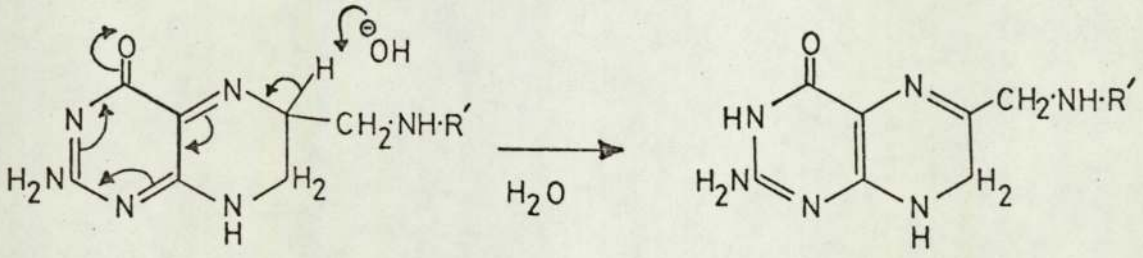
and, by further oxidation, to xanthopterin, as observed. Such nitrogen-assisted rearrangements cannot occur with compounds such as 6,7-dimethyltetrahydropterin, and this explains the absence of xanthopterin noted in the oxidation of this compound in Chapter Two. If the nitrogen function is replaced by another atom carrying a lone pair of electrons, e.g. oxygen as in tetrahydrobiopterin, loss of side chain is still possible. Presumably, this is of high activation energy for the tetrahydrobiopterin reaction, which explains the apparently lower yield of xanthopterin at normal temperatures.

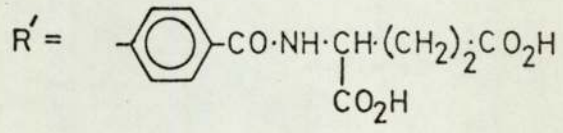
Hence, these simple considerations allow us to explain the apparently different fragmentation patterns observed at high and low acidities.

Acid-catalysed: low pH

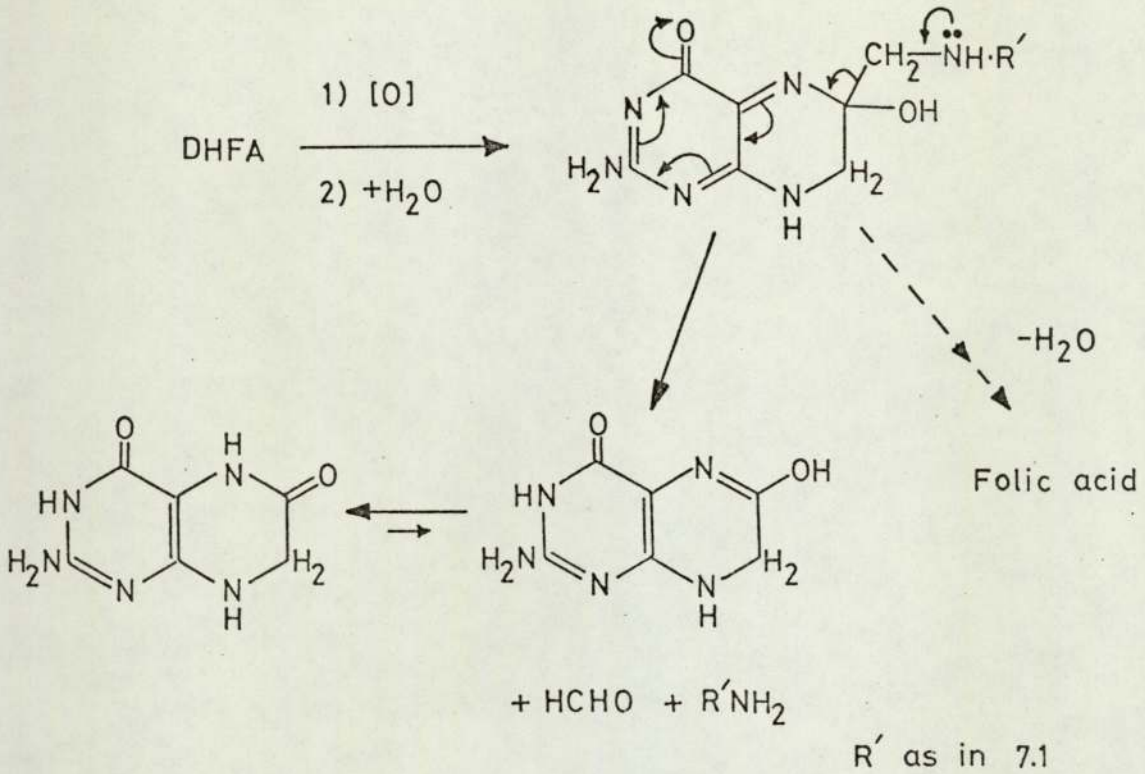


Base-catalysed: high pH





SCHEME 7.1 Rearrangements of quinonoid dihydrofolate



SCHEME 7.2 Possible mechanism of dihydroxanthopterin formation

So far as further experimental work is concerned the following suggestions might make useful and fulfilling exercises:

a) Extension of the work to other tetrahydropterins, particularly compounds carrying substituents at N(5) and N(8) thereby yielding useful information on steric effects. Studies might well involve independent confirmation of the results reported here, including estimates of activation energy for the free radical + oxygen propagation step.

b) Accurate determination of overall energies of activation for other reduced derivatives, and correlation with the results of molecular orbital calculations.

c) Synthesis of tetrahydropteridines which are soluble in non-polar solvents, and a complete study of their autoxidation kinetics with particular reference to the effect of solvent polarity. This should include attempts to rationalise any mechanistic ideas with the observations.

For the investigations (c) a useful compound would be the derivative described in Chapter Five, since a description of the synthesis is available in the literature, and it is known to be soluble in a variety of organic media.

CHAPTER 8.

The tetrahydrobiopterin-mediated non-enzymatic hydroxylation of phenylalanine. Some preliminary determinations of product yields.

Introductory Preamble.

Enzymatic aromatic hydroxylation reactions.

In Chapter One, mention was made of phenylalanine hydroxylase and its requirement for tetrahydrobiopterin as cofactor. In fact, this is just one of many aromatic hydroxylases which have been described by Neujahr and Gaal[195] as enzymes which "share the property of incorporating one atom of molecular oxygen into their aromatic substrate while the second oxygen atom is reduced to water by an appropriate hydrogen donor, different for different enzymes. Reduced pyridine nucleotides or tetrahydropterins are examples of such donors". Despite much intensive research in this field, the mechanism of activation of molecular oxygen is still obscure.

Some essential properties of the phenylalanine hydroxylase system are now well documented. Kaufman's group have recently shown[196] that rat liver enzyme in an essentially pure form contains 1 - 2 moles of iron per mole of enzyme (assuming a molecular weight for the latter of 100,000). Their e.s.r. studies showed that, in equilibrium with oxygen, the iron is present as the high spin ferric form. The addition of phenylalanine and 6,7-dimethyltetrahydropterin causes the signal for this form to disappear, and this may be due to either reduction to ferrous or a change to low spin ferric caused by a change in the ligand field. Removal of the

iron leads to loss of enzyme activity, which is restored by addition of ferrous chloride but not by addition of mercurous, nickelous, cobalt, manganese, cupric, chromium, cadmium or zinc ions. Hence it is concluded that the iron is essential for the enzyme's catalytic activity. It has also been found[197,198] that the enzyme shows some specificity as regards cofactor. For instance, L-erythro-tetrahydrobiopterin is the most active, and D-erythro-tetrahydrobiopterin is the least active optical isomer, whilst erythro tetrahydro-7-biopterins are completely inactive[197], indicating that the biopterin side chain is involved in binding at the enzyme active site. With reference to other tetrahydropteridines, Ayling *et al*[198] have shown that 2-amino-4-keto-6,7-dimethyltetrahydro-pteridine, 2,4-diamino-6,7-dimethyltetrahydropteridine, and 2-amino-4-keto-tetrahydropteridine are all active, whilst 4-amino- and 4-keto-tetrahydropteridine, 4-keto-6, 7-dimethyltetrahydropteridine, and 2-amino-4-keto-6,7-diphenyltetrahydropteridine all inhibit the enzyme. The same authors appear to believe that the hydroxylation reaction involves ternary complex formation between enzyme, phenylalanine and tetrahydropterin molecules.

A tetrahydropterin-requiring enzyme which hydroxylates p-tyrosine to 3,4-dopa (3,4-dihydroxypheny-lalanine, 8(i)) has been successfully isolated from adrenal and brain tissues by Ikeda *et al*[199], and has been shown to be effective for the phenylalanine to p-tyrosine conversion, but with a lower activity than

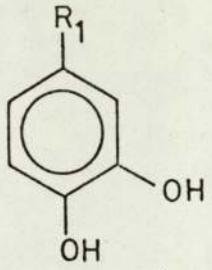
the "liver" enzyme. Chelating agents which have high affinities for divalent iron have been found[200,201] to inhibit tyrosine hydroxylase, thus indicating that it, too, is probably a ferrous-containing enzyme. The kinetics of the system have been studied in detail by Udenfriend's group[202] and are consistent with a "ping-pong" mechanism involving the sequential steps:

(a) reduction of an oxidised form of the enzyme by tetrahydropterin cofactor, followed by dissociation of oxidised pteridine from the enzyme, and

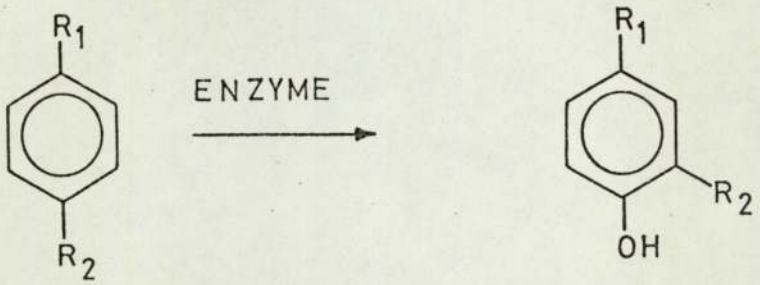
(b) aerobic oxidation of tyrosine to dopa plus oxidised enzyme.

In view of the similarity of phenylalanine- and tyrosine- hydroxylases with regard to substrate, cofactor, etc., it seems likely that similar mechanisms might apply, in which case there appears to be some conflict between the latter authors' interpretation and those of Ayling *et al*[198] and Fisher *et al*[196].

With regard to the activation of oxygen by these enzymes, and in view of their requirement for iron, it seems likely that the involvement of an iron-hydroperoxy complex (Fe^{II}-OOH) suggested for steroid hydroxylase reactions[203] is a possibility worth considering. The idea of tetrahydropterin hydroperoxides suggested earlier (see Chapter One) seems



8(i)



SCHEME 8.1 N.I.H. shift during enzymatic hydroxylation

R_1 = $CH_2 \cdot CH(NH_2) \cdot CO_2H$ R_2 = ^3H or ^2H

unlikely in view of the results of Chapter Four, though it must be remembered that enzyme-bound reactions may have features not exhibited by the simple chemical system.

In the course of investigating the enzymatic hydroxylation reactions, many simpler models have been suggested in the literature (discussed presently) which incorporate some features of the enzyme system. In assessing their resemblance to the latter, the important discovery of the N.I.H. shift (National Institute of Health shift) during enzyme-mediated reaction[204,205], must be considered. This consists in the migration of a para-substituent (e.g. tritium or deuterium) during para-hydroxylation, as shown in Scheme 8.1. Thus, any chemical system proposed as a model for aromatic hydroxylases must show a similar N.I.H. shift to its enzymatic counterpart.

As a preliminary to the experimental work described later, some of the models which have been proposed in the past will now be considered.

Fenton's Reagent.

It is well-known that reduction of hydrogen peroxide by ferrous ion in the presence of an aromatic compound results in non-specific hydroxylation of the latter. The decomposition of hydrogen peroxide by ferrous ion was proposed by Haber and Weiss[206] to

proceed according to Scheme 8.2, a mechanism now widely accepted. The hydroxylating entity is known[207-210] to be the hydroxyl radical, which oxidises the benzene ring as outlined in Scheme 8.3, a mechanism confirmed by the findings of Dorfman *et al*[211] that hexadeuterobenzene reacts with OH. radicals with the same specific rate as does benzene itself. The addition of OH. to the benzene ring is a rapid, diffusion-controlled, reaction (rate constant $4.3 \pm 0.9 \times 10^9$ $l.mol^{-1}$ s^{-1}).

Homolytic aromatic substitution, particularly with hydroxyl radicals, is known[212,213] to show electrophilic character, though the differences displayed in the ortho-, meta-, and para- positions to a substituent are less accentuated in homolytic than in 'heterolytic' substitution reactions. In the former, *all* substituents both activate the nucleus and are ortho- / para- directing, but the influences controlling these phenomena are free from Coulombic forces and are therefore less powerful than those which operate in 'heterolytic' substitution reactions.

The oxidation of phenylalanine by Fenton's reagent has been examined by Nofre *et al*[214, 215], who have reported the products to be o-, m-, and p- tyrosines; 2,3-, 2,5-, and 3,4- dihydroxyphenylalanines (dopas); aspartic acid, alanine, glycine and three unknown compounds. Mono-hydroxylation products were predominant. Wilson[216] has reported a rate constant of $3.3 \pm 0.3 \times 10^9$ $l.mol.^{-1}$ $s.^{-1}$ for the addition of OH. to the aromatic ring of phenylalanine.

$$Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + OH\bullet + OH^-$$

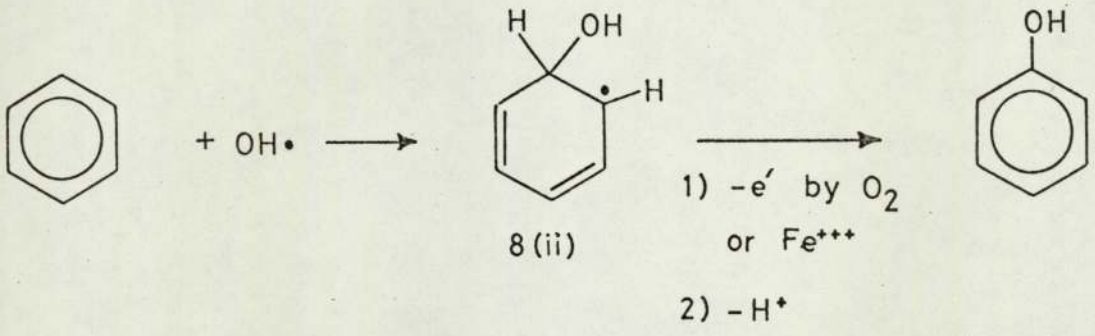
$$Fe^{+++} + H_2O_2 \longrightarrow Fe^{++} + HO_2\bullet + H^+$$

$$Fe^{++} + OH\bullet \longrightarrow Fe^{+++} + OH^-$$

$$Fe^{+++} + HO_2\bullet \longrightarrow Fe^{++} + O_2 + H^+$$

$$OH\bullet + H_2O_2 \longrightarrow H_2O + HO_2\bullet$$

SCHEME 8.2 H_2O_2 decomposition (Haber & Weiss)



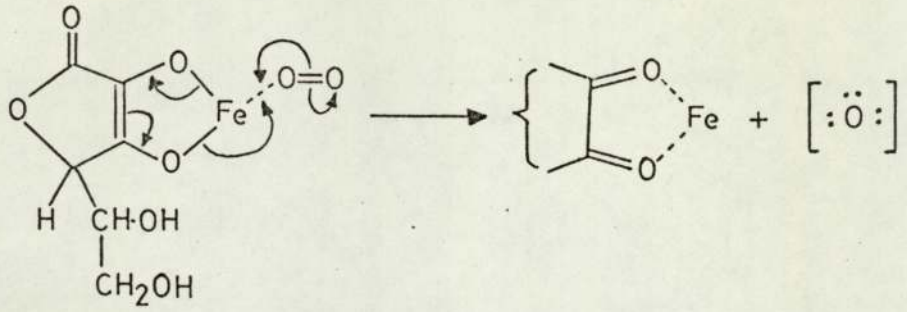
SCHEME 8.3 Aromatic hydroxylation by Fenton's reagent

The fact that Fenton's reagent does not give a good mechanistic model for aromatic hydroxylases is inferred from its small N.I.H. shift relative to the enzyme[217].

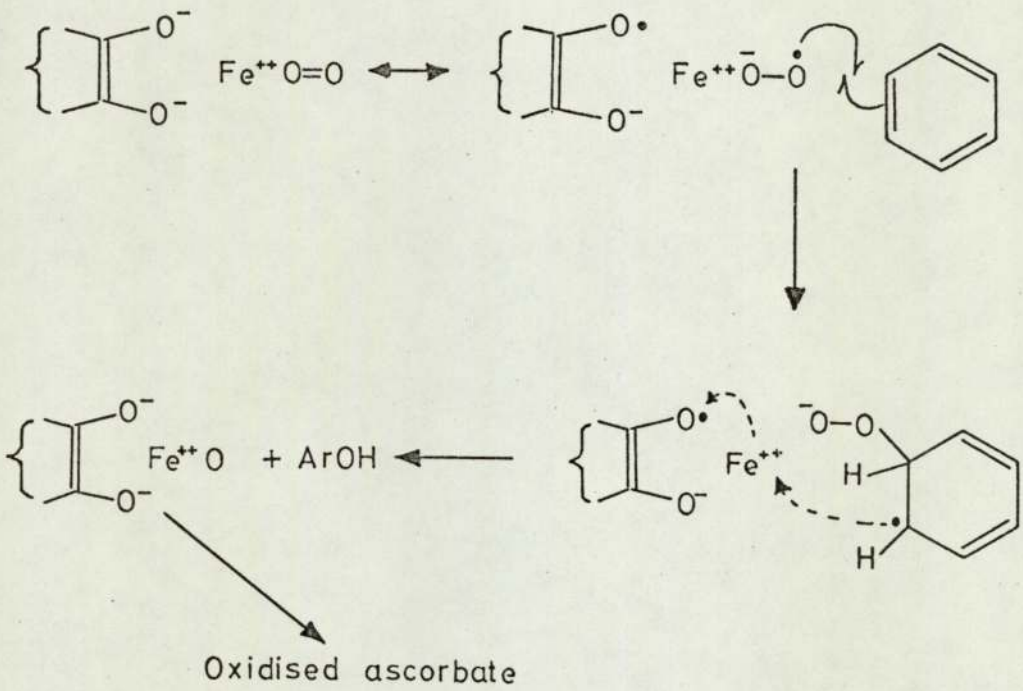
The Udenfriend System.

Udenfriend et al[218] demonstrated that an aqueous solution of ascorbic acid, ferrous sulphate and EDTA is capable of hydroxylating aromatic substrates in the presence of oxygen, and observed a preference for attack at electronegative sites. Later, Mason and Onoprienko[219] showed, using ^{18}O-labelled gas, that the hydroxyl group introduced during this reaction is derived almost entirely from molecular oxygen and proposed that the attacking species is not free-radical in nature, though Mason later suggested[220] that the reaction was related predominantly to homolytic rather than heterolytic processes. Breslow and Lukens[221] suggested that hydrogen peroxide, generated during ascorbate autoxidation, was decomposed to hydroxyl radicals, these being the predominant hydroxylating species, but Grinstead[222] and Hamilton et al[223] showed that inclusion of catalase in the system did not reduce the total hydroxylation yield. The former did suggest at least partial participation of hydrogen peroxide since a change in relative yields of different products was observed.

A mechanism put forward by Hamilton[224] is shown in Scheme 8.4, and it is suggested that an



SCHEME 8.4 Udenfriend system — Hamilton's mechanism



SCHEME 8.5 Norman & Lindsay-Smith mechanism

ascorbate-iron-oxygen complex undergoes cleavage to oxene, :Ö:, an analogue of carbene, ascorbic acid undergoing simultaneous oxidation. However, reaction of oxene with an aromatic ring (or alkene) might result in transient epoxide formation, and this would be expected to lead to significant N.I.H. shift (see later, Scheme 8.6). Jerina *et al*[217] report a noticeable lack of this rearrangement during Udenfriend system hydroxylation of 4-^{3}H-acetanilide to p-hydroxy-acetanilide, which also shows that the reaction is not a particularly useful model for hydroxylases. (All of the authors considered so far clearly treat the reaction as a working model for the enzyme).

An alternative mechanism, which is free-radical in nature, has been proposed by Norman and Lindsay Smith[225], and this is shown in Scheme 8.5. Again, this involves ascorbate-iron-oxygen complexes, which might be considered unlikely in the presence of a powerful chelating ligand such as EDTA. However, it is known[226,227,228] that EDTA is frequently unable to completely encompass the metal ion to form a true octahedral complex, so that co-ordination positions may be exposed. This still leaves the problem that a bulky ligand such as EDTA might severely hinder approach of, say, ascorbate to the central metal ion and thereby prevent complex formation. Norman and Radda[213] did conclude, however, that OH. radicals are not involved, by comparing product distribution from the normal Udenfriend system with that given when oxygen is

replaced by hydrogen peroxide and the system deoxygenated with nitrogen gas. The latter would lead to production of OH. radicals, but the possible effect of oxygen on the reactions of this species was not considered, and questions arise as to the validity of the conclusion.

Reports have appeared on the hydroxylation of phenylalanine by the ascorbate system[229,230,231] and Dalgliesh[231] has characterised o- and p- tyrosines, 2,3-, 2,5-, and 3,4- dihydroxyphenylalanines, hydroquinone and possibly catechol among the products. Bobst and Viscontini[232] and Coulson *et al*[102] showed that ascorbate could be replaced by tetrahydropterins in the Udenfriend hydroxylation of phenylalanine, but only measured the p-tyrosine yields. Woolf *et al*[233] report that hydroxylation by this system in the absence of added ferrous ion is completely eliminated by catalase, which seems to conflict with the earlier reports concerning its effect on the ascorbate reaction.

Thus, the mechanism of Udenfriend-type reactions is not well understood and provides an interesting chemical problem. Some investigations into the somewhat similar tetrahydrobiopterin-mediated hydroxylation of phenylalanine have been made, and are described later. Apart from providing mechanistic information, these are useful in giving quantitative data on the non-enzymatic reactions and also safeguard against spurious observation of "new" enzymatic products.

Chemical models which give significant N.I.H. shift.

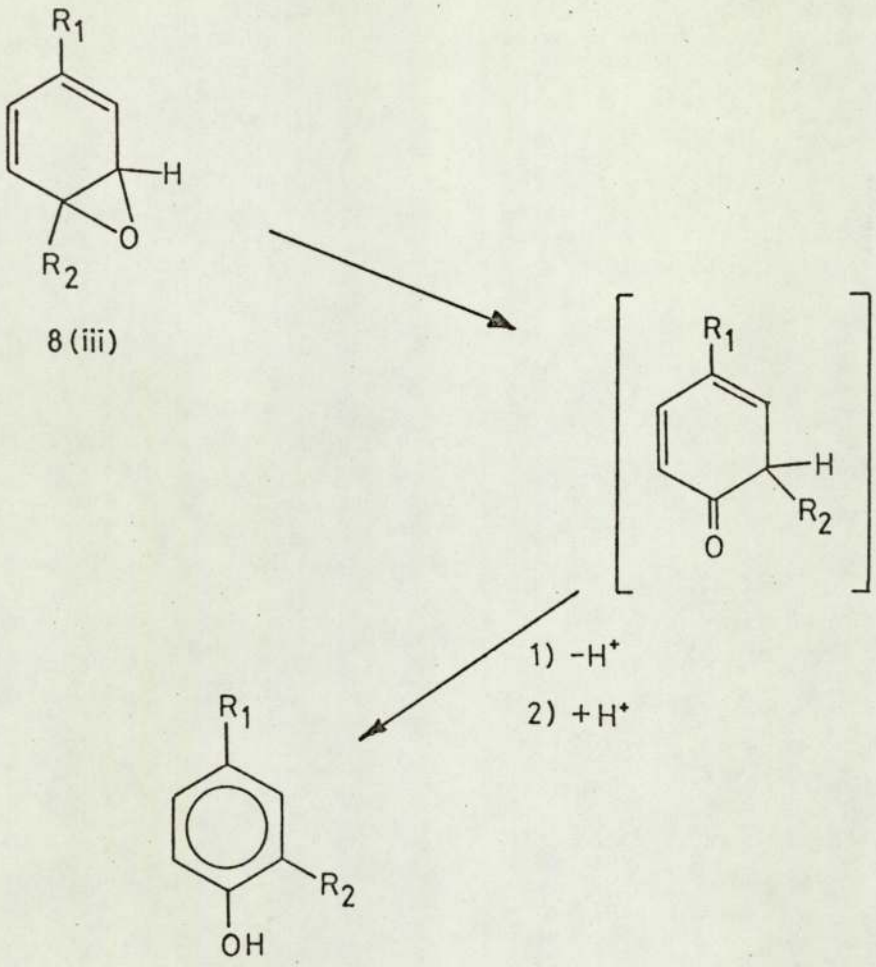
It is unfortunate that the model systems which utilise similar conditions as does the enzymatic reaction (ferrous ion, reducing agent and oxygen in aqueous media) do not imitate the latter in its ability to promote substituent migration. As fate would have it, the only chemical systems which do lead to any degree of N.I.H. shift use reaction conditions which are far removed from any pertaining to the hydroxylase reactions.

It is known that reactions which are capable of generating the electrophile "$OH^{\oplus}$" (not necessarily as a discrete entity) generally lead to N.I.H. shift. The Jerina-Daly research group have demonstrated that peroxytrifluoroacetic acid[217,234-238], irradiated pyridine-N-oxide[239] and t-butyl hydroperoxide/molybdenum hexacarbonyl[239] all hydroxylate aromatic rings with concurrent substituent migration, whilst Hart[240] reports similar effects using peroxytrifluoroacetic acid/boron trifluoride mixtures. The degree of tritium or deuterium retention in para-hydroxylation of para tritiated (deuteriated) aromatics depends upon the other substituent present. For example, peroxytrifluoroacetic acid hydroxylation of 4-^{3}H-acetanilide leads to 9.6% tritium retention[217], whereas 4-^{2}H-toluene retains 68% of its deuterium[238] when para-hydroxylated.

A substantial amount of work by the original discoverers of the effect shows that arene oxide intermediates, 8(iii), are generated during such reactions,

and it is the rearrangement of these which lead to N.I.H. shift[241-247]. This conclusion is supported by the work of Fehnel[248] and the kinetic studies of Kasperek and Bruice[249,250], and the accepted mechanistic interpretation is outlined in Scheme 8.6.

Reactions which do not implicate $OH^{\oplus}$ do not lead to N.I.H. shift and do not provide useful models for hydroxylase systems. In this context, and for future reference, it may be noted that enzymatic hydroxylation of 4-^{3}H-phenylalanine yields p-tyrosine in which there is a 95% retention of tritium[237].



SCHEME 8.6 Substituent migration during arene oxide rearrangement

Independent investigation of tetrahydrobiopterin-mediated, non-enzymatic hydroxylations of phenylalanine.

Methods.

Liquid scintillation counting for ^{14}C and ^{3}H determination was done with a Nuclear Enterprises NE 8305 counter, using Unisolve 1 Scintillator (Koch-Light, Ltd.) Where samples were burned in order to determine radioactivity, this was done with a Beckman biological material oxidiser. Microdensitometer traces were recorded with a Joyce-Loebl microdensitometer. All radiochemicals were purchased from the Radiochemical Centre, Amersham, and used without further purification, and non-radioactive materials used in synthesis and as t.l.c. standards were purchased from Phase Separations, Koch-Light and B.D.H. Ltd. Inorganic materials were Analytical Reagent grade. Tetrahydrobiopterin and superoxide dismutase were gifts from Roche Products, Ltd., and Dr. A. M. Stokes (Oxford University), respectively. Since 2,3- and 2,5- dihydroxyphenylalanines were not commercially available, and were required as t.l.c. standards for product characterisation, they were synthesised by the Erlenmeyer azlactone reaction using the method described by Lambooy[251] and characterised by comparison of m.pts. with literature values [251,252,253] and spectroscopic methods. This is described in Appendix H.

Hydroxylation reactions.

Solutions of ferrous ammonium sulphate, EDTA (where present) and phenylalanine (containing ca 4μCi. cm^{-3} of carboxyl-^{14}C-labelled phenylalanine) were mixed to give the concentrations shown in the results section (Table 8.2) in a final volume of 1 cm^3, and the temperature adjusted to 40^o by immersion in the water bath. Catalase and superoxide dismutase, where used, were contained in the phenylalanine solution at the appropriate concentration. For the Fenton reactions 100 μl of hydrogen peroxide solution at the appropriate concentration (standardised by iodide method) was added to give the concentrations of Table 8.2, and for the tetrahydrobiopterin reactions the correct weight of this compound was introduced as a freshly made solution in 100 μl water. The former were incubated for 3 h. in equilibrium with air, and the latter for 3 h. with the passage of a steady stream of oxygen gas.

Yield determinations.

After incubation, the solutions (10 μl) were applied to a cellulose layer (MN300, 0.1 mm, Polygram foil without fluorescent indicator) alongside appropriate authentic materials as standards, and eluted with solvent system (1) of Table 8.1. The foils were dried, marked with radioactive materials at appropriate points not coinciding with chromatograms, exposed to Ilford X-ray

film in the dark for 3-7 days and the autoradiograms developed. At this point, non-radioactive standards were located with ninhydrin spray, after marking off the product chromatograms (to avoid loss of $-CO_2H$ and, therefore, label). Bands corresponding to reaction products and unreacted phenylalanine were located by aligning the radioactive markers on the plate and autoradiogram, each was carefully removed by sectioning the plate, and extracted overnight with a known volume of 4% aq. ammonia (10 cm^3). 5 cm^3 of extracts were thoroughly mixed with scintillator (10 cm^3) and counted. Background count was obtained from a mixture of 4% aq. ammonia (5 cm^3) and scintillator (10 cm^3). Total recovery in all cases was found to be ca 95% by comparison with count rates of unchromatographed reactant solutions (10 µl in 5 cm^3 4% ammonia, plus scintillator), and yields are based on total recovered radioactivity.

Tritium retention by p-tyrosine - N.I.H. shifts.

The incubation procedure was as above except that ^{14}C-phenylalanine was replaced by para-tritiated compound (0.2 mCi. cm^{-3}). Concentrations were as in C and F of Table 8.2. After incubation each reaction mixture was introduced into boiling water saturated with an accurately known weight (1.5 - 2.0 g.) of non-radioactive p-tyrosine, and the latter crystallised out by cooling. It was then recrystallised from water eight times and a known weight finally purified by

t.l.c. on cellulose layer (with fluorescent indicator). The p-tyrosine band was located as absorbing material under UV (254 nm), removed and extracted into 2M-hydrochloric acid (2 cm^3). This solution could not be counted directly owing to interference from the fluorescent indicator which appeared to extract into the hydrochloric acid. Therefore, 100 µl samples were passed through the biological material oxidiser in the usual way, the tritiated water collected in a dry ice/acetone trap, taken up with scintillator (10 cm^3) and counted. In this way the <u>apparent</u> yield of p-tyrosine could be computed and the tritium retention values (%) obtained by comparison with the ^{14}C experiments. On passing unreacted phenylalanine through the above procedure radioactive p-tyrosine was found at an impurity level of 0.295 %. This could not be removed without risk of contamination by other materials (e.g. from chromatography procedures) which might upset the reactions and significantly alter yields. Therefore, all N.I.H. shift values were corrected for this, even though tyrosine might also react to some extent. The retention figures are possibly over-corrected and therefore represent minimum values. (A personal communication with the Radiochemical Centre, Amersham, revealed that no tritium is detectable in a position other than para- in the starting material, thereby ruling out errors due to non-specificity of labelling).

Results and discussion.

Products and yields.

Table 8.1. shows the R_f values of standard materials in two solvent systems which give reasonably good separation. System (1) was chosen for yield determinations because:

(a) it separated products into types, e.g. mono-hydroxylated, di-hydroxylated and fragmentation, and

(b) although in certain cases it appears to give poorer separation than (2), the spots in the latter were actually more diffuse and sometimes very distorted, particularly at high R_f values (near the solvent front).

The products retaining carboxyl group observed in the ^{14}C experiments were ortho- and para- tyrosines, 3,4-dihydroxyphenylalanine, and aspartic acid. Meta-tyrosine could not be detected by autoradiography even after prolonged exposure so any minor yield was included in that of o-tyrosine. 2,3-Dihydroxyphenylalanine was not detected, and 2,5-dihydroxyphenylalanine chromatographed too closely to the 3,4-isomer to allow separate determinations, hence all three were measured as a combined yield (dopas). The presence of 2,5-dopa was inferred from the relatively intense dopa spot on autoradiograms from hydroxylation of 4-^{3}H-phenylalanine. An unidentified compound, eluting between aspartic acid and dopa was

TABLE 8.1.

Typical R_f values for possible products of phenylalanine hydroxylation.

Compound (authentic)	R_f in solvent indicated	
	(1)	(2)
Phenylalanine	0.66	0.74
o-tyrosine	0.57	0.73
m-tyrosine	0.51	0.64
p-tyrosine	0.47	0.62
2,3-dihydroxyphenylalanine	0.43	0.47
2,5-dihydroxyphenylalanine	0.38	0.38
3,4-dihydroxyphenylalanine	0.36	0.30
Aspartic acid	0.27	0.15
Alanine	0.27	0.57
Glycine	0.26	0.36

Solvent Systems:

(1) n-butanol-acetic acid-water 4:1:5 v/v organic phase after 24 h. equilibration.

(2) Phenol-water 9:1 w/v.

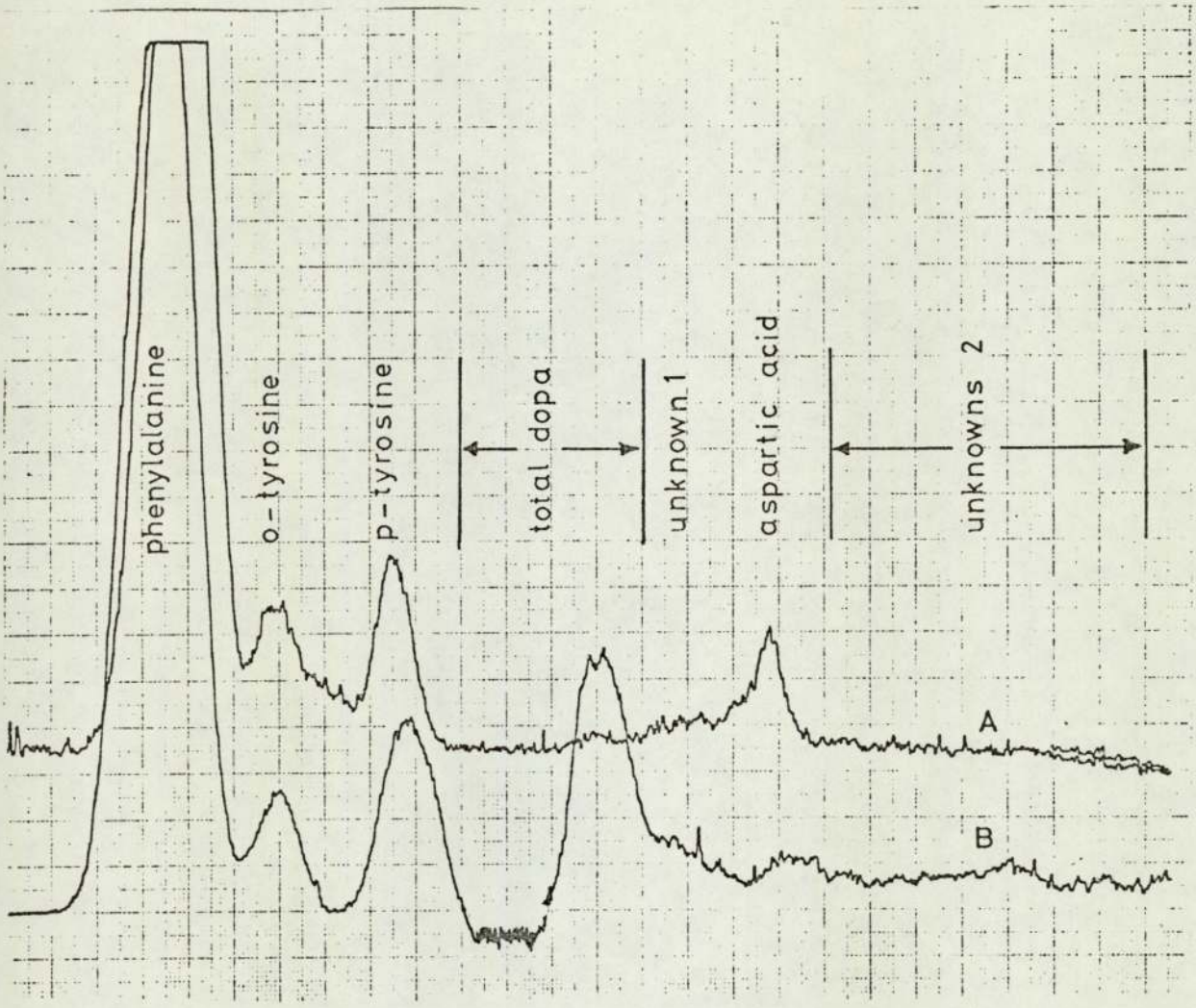


FIG. 8.1

Microdensitometer trace for ^{14}C-phenylalanine hydroxylations. Chromatography in solvent (1), followed by autoradiography.

A) Reaction F.

B) Reaction C.

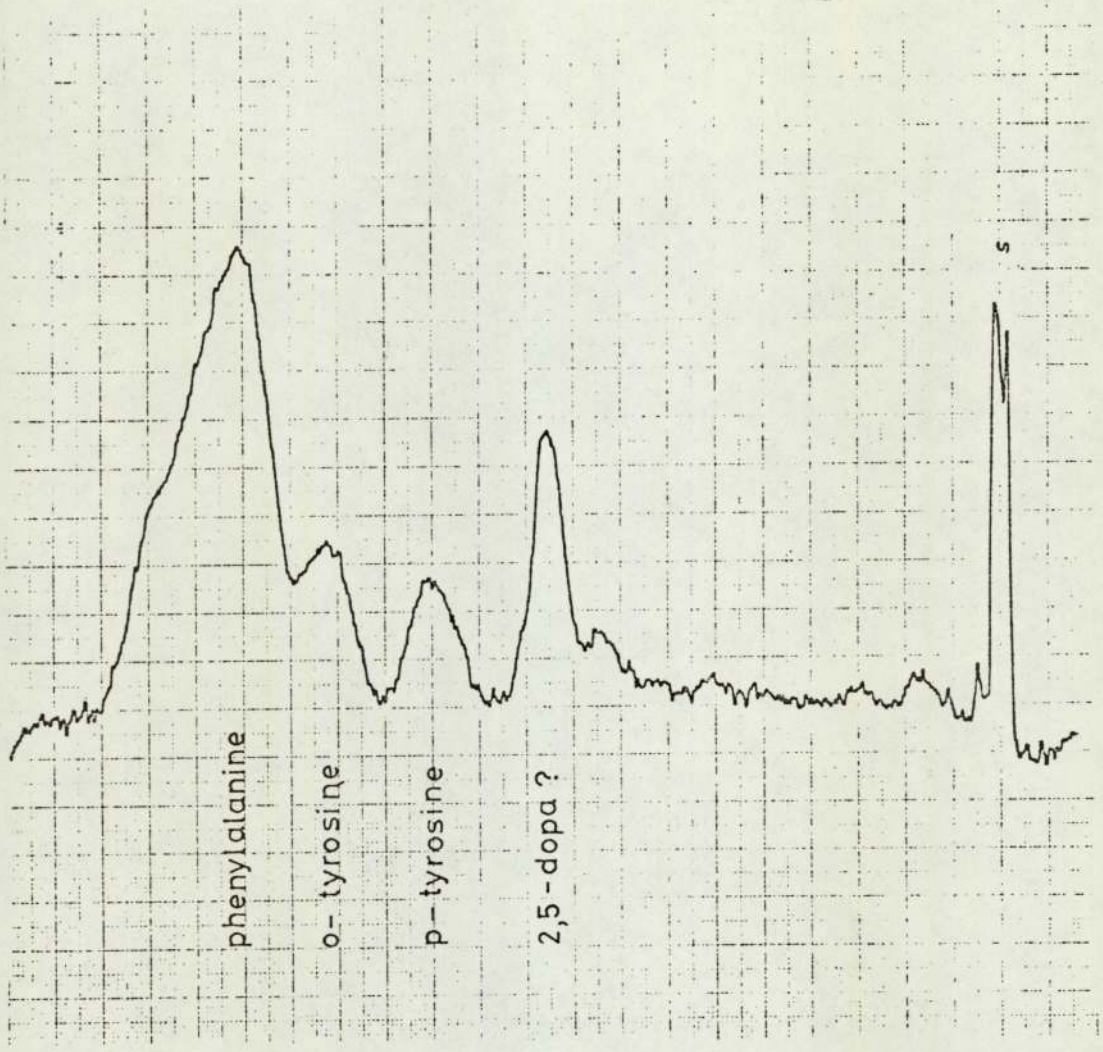


FIG. 8.2

Microdensitometer trace for p-^{3}H-phenylalanine hydroxylation, reaction C.

observed and determined, and radioactivity was also found at R_f values below aspartic acid. Some of the better autoradiograms indicated possibly up to three unidentified products in this region, and these were determined as a combined yield.

Microdensitometer traces of typical autoradiograms from t.l.c. in solvent (1) are shown in Figs. 8.1 and 8.2 (note inference of 2,5-dopa in 8.2.).

The yields of products, including unreacted phenylalanine, are presented in Table 8.2 for the Fenton reaction and tetrahydrobiopterin system under a variety of conditions. It can be seen that the relative yields are susceptible to slight changes in reaction conditions. Reaction I showed aspartic acid as the major product and its identity was confirmed, using this reaction, by chromatography in solvent (2) which separates it from alanine and glycine (these three elute very closely in solvent (1)). This variation in relative yields makes it impossible to identify the reactive entity in the tetrahydrobiopterin system by direct comparison with Fenton's reagent (OH. radicals) or any other well-characterised reaction. However, the addition of catalase gives rise to some interesting effects. Hydroxylation by Fenton's reagent is completely eliminated by the enzyme (reaction G), any minor yields probably being due to radiochemical impurities. On the other hand, there is a slight increase in total hydroxylation by the tetrahydrobiopterin system, together with a significant

TABLE 8.2. Analysis of reaction mixtures from phenylalanine hydroxylation with tetrahydrobiopterin system and Fenton's reagent.

(a) Tetrahydrobiopterin system.

Product \ Reaction	A*	B*	C**	D***	E*
Unreacted phenylalanine	59.4 ± 2.5	53.4 ± 2.0	57.3 ± 0.6	69.3 ± 0.6	85.5 ± 1.5
o-tyrosine	8.0 ± 1.5	5.2 ± 1.0	5.7 ± 0.5	3.7 ± 0.3	3.3 ± 1.0
p-tyrosine	5.7 ± 0.4	9.2 ± 0.5	7.9 ± 0.2	5.5 ± 0.1	3.4 ± 0.3
Total dopas	3.9 ± 0.3	13.8 ± 0.6	10.4 ± 0.4	7.9 ± 0.4	2.6 ± 0.2
Unknown 1	1.9 ± 0.4	4.8 ± 1.0	3.6 ± 0.2	2.6 ± 0.1	1.6 ± 0.4
Aspartic acid	6.2 ± 0.6	4.0 ± 0.5	4.2 ± 0.2	2.8 ± 0.3	1.5 ± 0.3
Unknowns 2	14.8 ± 0.6	9.1 ± 0.5	11.0 ± 0.1	8.2 ± 0.3	2.0 ± 0.3

Reaction conditions at end of table

* Mean of two determinations ± range

** Mean of four determinations ± S.E.M.

*** Mean of three determinations ± S.E.M.

continued ....

TABLE 8.2. (continued)

(b) Fenton reactions.

Product \ Reaction	F*	G****	H*	I*
Unreacted phenylalanine	83.5 ± 2.7	98.2	37.3 ± 0.5	3.0 ± 0.2
o-tyrosine	5.5 ± 1.5	0.9	18.1 ± 0.8	12.1 ± 1.0
p-tyrosine	4.2 ± 0.7	0.3	13.4 ± 0.5	3.5 ± 0.3
Total dopas	2.8 ± 0.4	0.4	9.0 ± 0.4	6.4 ± 0.4
Unknown 1	0.8 ± 0.1	0.1	2.2 ± 0.2	2.6 ± 0.3
Aspartic acid	2.0 ± 0.4	0.1'	10.3 ± 0.4	52.1 ± 0.5
Unknowns 2	1.6 ± 0.1	0.1	9.3 ± 0.4	20.4 ± 0.5

*Mean of two determinations ± range

**** One determination only

Reaction conditions overpage.

TABLE 8.2. (continued)

Reaction conditions: (A) 1.56 mM - phenylalanine, 0.6 mM - Fe^{2+}, 5 mM - EDTA, 12.8 mM - tetrahydrobiopterin;

(B) As A + catalase (0.2 mg. cm^{-3});

(C) As A without EDTA;

(D) As C + catalase (0.2 mg. cm^{-3});

(E) As C without added Fe^{2+};

(F) 1.56 mM - phenylalanine, 0.6 mM - Fe^{2+}, 5mM - EDTA, 6.75 mM - H_2O_2;

(G) As F + catalase (0.2 mg. cm^{-3});

(H) 0.69 mM - phenylalanine, 0.67 mM - Fe^{2+}, 5 mM - EDTA, 13.0 mM-H_2O_2;

(I) As F but using 0.5 M - H_2O_2.

TABLE 8.3. Effect of catalase on product distribution from tetrahydrobiopterin-mediated phenylalanine hydroxylation.

Product \ Reaction	Yields relative to p-tyrosine			
	A*	B*	C**	D***
p-tyrosine	1.00	1.00	1.00	1.00
o-tyrosine	1.40 ± 0.31	0.57 ± 0.10	0.72 ± 0.07	0.67 ± 0.06
Total dopas	0.68 ± 0.07	1.50 ± 0.08	1.32 ± 0.06	1.44 ± 0.08
Unknown 1	0.33 ± 0.07	0.52 ± 0.11	0.46 ± 0.03	0.47 ± 0.02
Aspartic acid	1.09 ± 0.14	0.44 ± 0.06	0.53 ± 0.03	0.51 ± 0.06
Unknown 2	2.60 ± 0.23	0.99 ± 0.06	1.39 ± 0.04	1.49 ± 0.07

Reaction conditions as in Table 8.2.

* mean of two determination ± range.

** mean of four determinations ± S.E.M.

*** mean of three determinations ± S.E.M.

change in product ratios, in the presence of EDTA, whilst in the absence of the ligand a reduction in total yield occurs without significant change in relative yields (Table 8.3 shows yields relative to p-tyrosine). These effects are not yet fully understood, and the role of EDTA requires more rigorous investigation. Although the solutions are unbuffered (pH ca. 2.5) the addition of the small amount of catalase used results in a negligible pH shift (< 0.1 pH units), so that the effects observed are not due to changes in acidity. It is clear that the hydrogen peroxide generated by tetrahydrobiopterin autoxidation is partially responsible for hydroxylation, via a Fenton-type reaction (OH. radicals). Comparison of total yields from C and D indicate that this represents approximately 30% of the total hydroxylation in the absence of EDTA. It is possible, however, that hydroxyl radicals may be generated in an alternative way and, even though catalase is known[254,255] to scavenge this species, its involvement cannot be discounted on the basis of incomplete suppression of hydroxylation by the enzyme.

It was concluded in Chapter 4 that the chain carrier during autoxidation of tetrahydrobiopterin is the hydroperoxyl radical (at low pH) or the superoxide anion radical (at neutral pH and higher). Although this species is reported[210] to be relatively unreactive in aromatic hydroxylation, it may be implicated in other ways, e.g. as a complex with the ferrous ion. In order to eliminate this possibility, the effect of superoxide dismutase on total hydroxylation yields was determined, the results being listed in Table 8.4. Under no conditions was any inhibition observed, but rather a slight enhancement which is possibly due to slowing-down of the tetrahydrobiopterin autoxidation reaction, thereby leading to its increased availability in the hydroxylation reaction. In particular, reactions (5) and (6) in Table 8.4. utilised conditions of pH and oxygen concentration under which the enzyme was observed to inhibit the autoxidation, so that superoxide does not appear to be active in the hydroxylation reaction. This conclusion is partly supported by the observation that phenylalanine causes no reduction in oxygen uptake rates by tetrahydrobiopterin and therefore does not effectively scavenge the chain carrier (numerical data in Appendix C).

TABLE 8.4.

Effect of superoxide dismutase
on total hydroxylation yields.

Reaction conditions	Total hydroxylation %
(1) 1.56 mM - phenylalanine, 0.6 mM - Fe^{2+}, 12.8 mM - tetrahydrobiopterin, oxygen, unbuffered	42.7
(2) As (1) + Superoxide Dismutase (1 mg. cm^{-3})	42.9
(3) As (1) without added Fe^{2+}	16.4
(4) As (3) + Superoxide Dismutase (1 mg. cm^{-3})	24.7
(5) 1.56 mM - phenylalanine, 12.8 mM - tetrahydrobiopterin in 0.05 M - phosphate buffer, pH 7 under air at 40°C overnight.	10.9
(6) As (5) + superoxide dismutase (1 mg. cm^{-3})	12.5

N.I.H. shift as a criterion for free-radical hydroxylation.

The tritium retentions, after correction for radiochemical impurity, which were observed for the Fenton- and tetrahydrobiopterin- systems are given in Table 8.5. It is known (see previous literature discussion) that the 'heterolytic' and enzymatic hydroxylation of 4-^{3}H-phenylalanine produce p-tyrosine showing ca. 95% tritium retention. The values for both reactions studied are appreciably lower than this (by about the same factor as observed for other substrate molecules[217]), and are almost identical in these determinations (but probably a fortuitous result - the error involved in the estimation is ca. 30% of the quoted values). This indicates that the reactive entity in the tetrahydrobiopterin system is free radical in nature.

TABLE 8.5.

N.I.H shift during Fenton reagent - and tetrahydrobiopterin mediated hydroxylation of para - ^{3}H - phenylalanine to p-tyrosine.

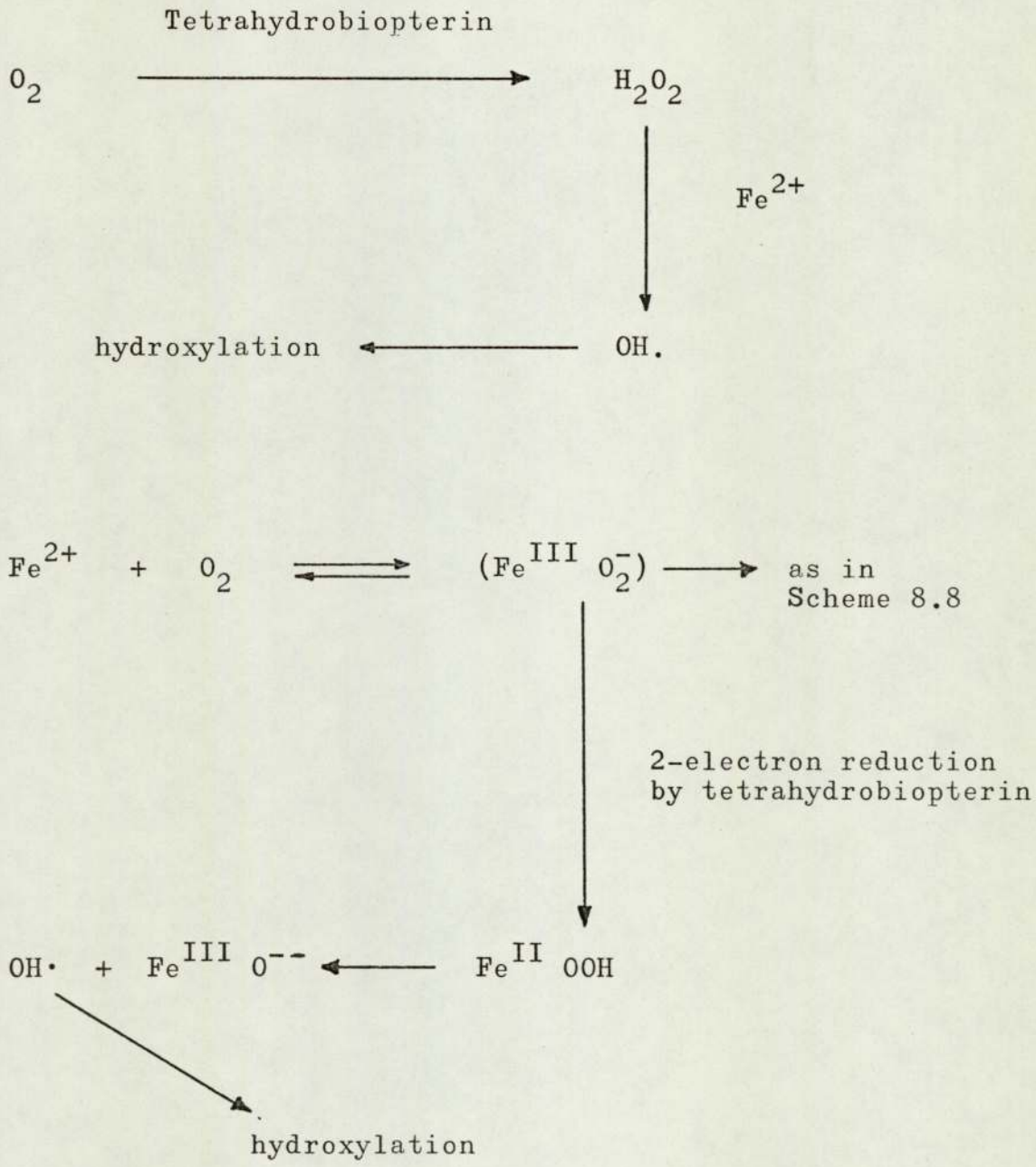
Reaction System*	Retention of tritium in p-tyrosine % **
Fenton	14 ± 5
Tetrahydrobiopterin	14 ± 5

* Conditions as in experimental.

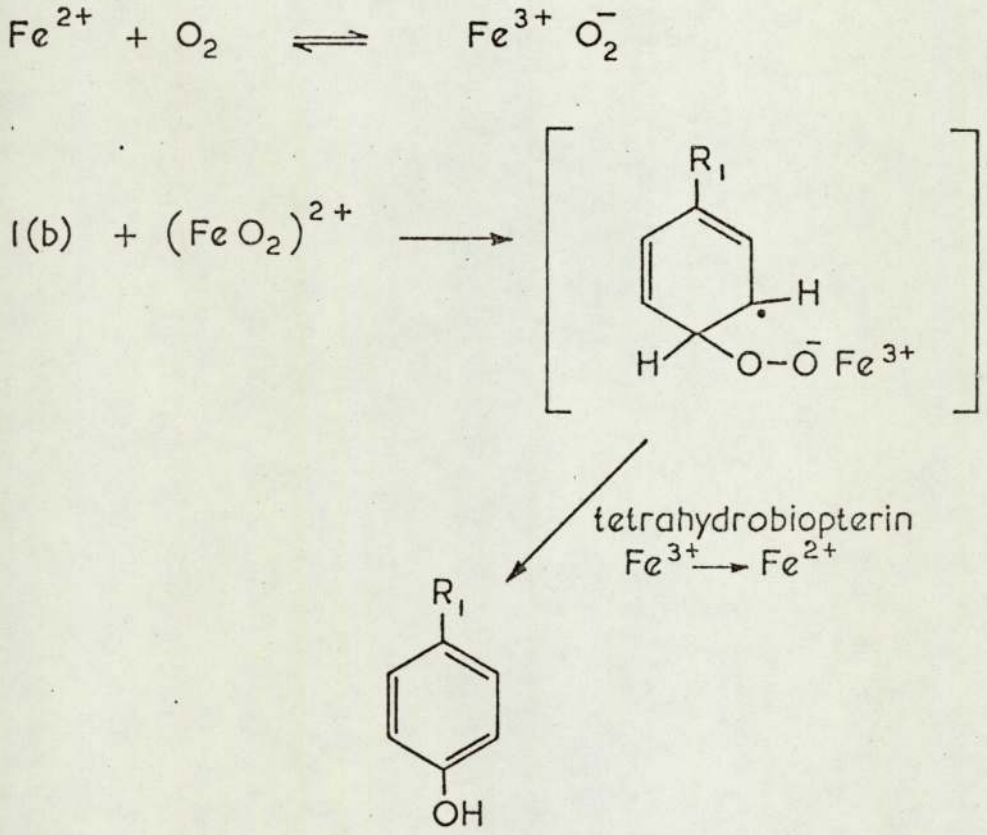
** Mean of three determinations ± S.E.M.

Conclusions.

The results show that non-enzymatic tetrahydropterin-mediated aromatic hydroxylations are extremely complex, and involve free radical species, though the hydroperoxyl radical chain carrier for tetrahydropterin autoxidation appears to be relatively unreactive toward the aromatic nucleus. It is known[256] that autoxidation of ferrous ion induces aromatic hydroxylation, and in the light of evidence to date ferrous ion - oxygen complexes may be implicated both in the presence and absence of reducing agents. Such a proposal is not inconsistent with the knowledge[257-259] that complexes exist, e.g. for cobalt, in which electron transfer occurs from metal ion to oxygen. The rather lower hydroxylation yields observed in the absence of added ferrous ion is possibly due to the catalytic effect of trace metal impurities in the water and starting materials which cannot be completely removed. The iron-oxygen complexes might well effect free-radical hydroxylation directly, or might be reduced by tetrahydropterin, leading ultimately to hydroxyl radicals. Possible mechanisms are summarised in Schemes 8.7 and 8.8.



SCHEME 8.7 Possible mechanism for generation of hydroxylating entities.



SCHEME 8.8 Possible hydroxylation mechanism involving $Fe-O_2$ complexes

Suggestions for further work.

Udenfriend-type hydroxylations are shown above to be exceptionally complex reactions. However, the radiochemical method described represents a fairly accurate method of analysis of the reaction mixture, and may now be used routinely in order to determine the effects of reaction conditions. The following problems can now be tackled:

1) The role of metal ions, i.e., the dependence of total hydroxylation on metal ion concentration, particularly at impurity levels.

2) The role of chelating ligands, e.g., EDTA.

3) The effect of tetrahydrobiopterin concentration, and oxygen tension in the solution.

4) The effect of temperature on product distribution, and possible simplification of the reaction by use of lower temperature.

5) The effect of pH on the reaction.

Owing to the complexity, it is probable that the mechanistic details derivable from product analysis are rather limited. However, refinement of the method might well allow an intensive study of the reaction pathway, and a determination of the detailed kinetics for the system.

APPENDICES.

APPENDIX A.

Materials used in Chapters 2 - 5.

All pH's were determined on an E.I.L. pH meter (model 23A), U.V. and visible spectra were recorded with a Perkin-Elmer 137 UV spectrophotometer, n.m.r. spectra for solutions in trifluoroacetic acid (unless otherwise stated) with Perkin-Elmer R14 or Varian HA 100 D spectrometers, mass spectra on an A.E.I. MS9 spectrometer, and e.s.r. with Hilger & Watts Microspin ESR3.

Folic acid, xanthopterin, pterin, monodeuterioacetic acid ($CH_3\ CO_2D$, > 98 atom % D), deuterium oxide (> 99.5 atom % D), and catalase were purchased from Koch-Light, ^{18}O enriched water (43.461 % enrichment) from Miles Laboratories, and deuterium gas from Matheson Gas Products. Gifts of tetrahydrobiopterin dihydrochloride and 6,7-dimethyl -5,6,7,8-tetrahydropterin from Roche Products, 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione from Dr. H.I.X. Mager (Delft University of Technology), and superoxide dismutase from Dr. A.M. Stokes (Oxford University) were received. All inorganic materials used for buffer solutions were Analytical Reagent grade.

5,6,7,8 - Tetrahydrofolic Acid. - This was prepared by the low pressure catalytic hydrogenation of folic acid over reduced platinum oxide catalyst, using a modification of the method described by Hatefi et al[39]. Since contamination by 2-mercaptoethanol (an antioxidant generally used to protect the tetrahydrofolate) might

upset the kinetic results, this was omitted in all preparations. A suspension of platinum dioxide (2g) in dry glacial acetic acid (25 cm^3) was stirred under an atmosphere of hydrogen at room temperature until consumption of gas ceased, when a suspension of folic acid (2g) in glacial acetic acid (25 cm^3) was added. After uptake of 2 mol. equiv. of hydrogen, the apparatus was flushed with nitrogen and the solution filtered under vacuum directly into a flask immersed in ice via a side arm with glass sinter incorporated. The product was freeze-dried and stored at 0^o under nitrogen in sealed glass ampoules. The compound obtained in this way was found to be 95% pure as the diacetate from its u.v. spectrum (using E 29,100[138] at λ_{max} 297 nm in 0.1M-sodium phosphate buffer, pH 7, previously de-aerated and containing 0.5% v/v of 2-mercaptoethanol). (τ) 1.7 br (amino groups, etc), 1.9 (2H,d,J 8 Hz) and 2.4 (2H, d, J 8 Hz) benzene ring, 4.85 br (1H, m, α-CH in glutamic acid of side chain), 5.55 br (1H, m, 6-H), 5.9 br (4H, 7- and 9-H_2), 7.18 and 7.4 (4H, CH_2 groups of glutamic acid), and 7.73 (6H, s, 2 x Me of diacetate).

6,7 - Dideuterio - 5,6,7,8 - tetrahydrofolic Acid.

This was prepared by the above method for THFA, using deuterium gas and replacing the glacial acetic acid by acetic (^2H) acid, $CH_3\ CO_2\ D$. (It was found that catalytic deuteriation using glacial acetic acid as solvent led to very little incorporation of deuterium in the product (n.m.r.)) The deuteriated tetrahydrofolic acid

was found to contain ca. 90% incorporation of deuterium on C(6) and ca. 50% deuterium in the methylene group at C(7) (from n.m.r. signal intensities at τ 5.55 and 5.9, respectively).

7,8 - Dihydrofolic acid - This compound was prepared for use as a t.l.c. standard in the reaction pathway studies and for preliminary kinetic studies, using the method of Futterman[193], slightly modified. Sodium hydroxide solution (0.1M) was added carefully to a suspension of folic acid (200 mg) in water (20 cm^3) until solution was achieved. Sodium ascorbate solution (50 cm^3, containing 100 mg per cm^3) was added and the solution was adjusted to pH 6 with 2M - hydrochloric acid. Sodium dithionite (2 g) was added, the solution allowed to stand at 0^oC for 15 min, and then adjusted to pH 2.8 with 2M- hydrochloric acid. The precipitate of dihydrofolic acid was separated by centrifugation, washed several times with 0.5% HCl solution and once with acetone to remove ascorbate, and dried under vacuum. The product, a white solid, homogeneous on t.l.c. in three solvents (blue fluorescence under UV, 366 nm), was stored under nitrogen at 0^oC in sealed glass ampoules. λ_{max} (pH 13) 221, 239 sh, 288, and 305 sh. nm, λ_{max} (pH 7) 227, 280 and 303 sh nm. (lit.,[17] λ_{max}(pH 13) 221, 239 sh, 285, and 306 sh nm, λ_{max} (pH 7) 227, 282, and 303 sh nm).

The carbon - 13 n.m.r. spectra in alkaline D_2O of folic acid and DHFA prepared in the above way are

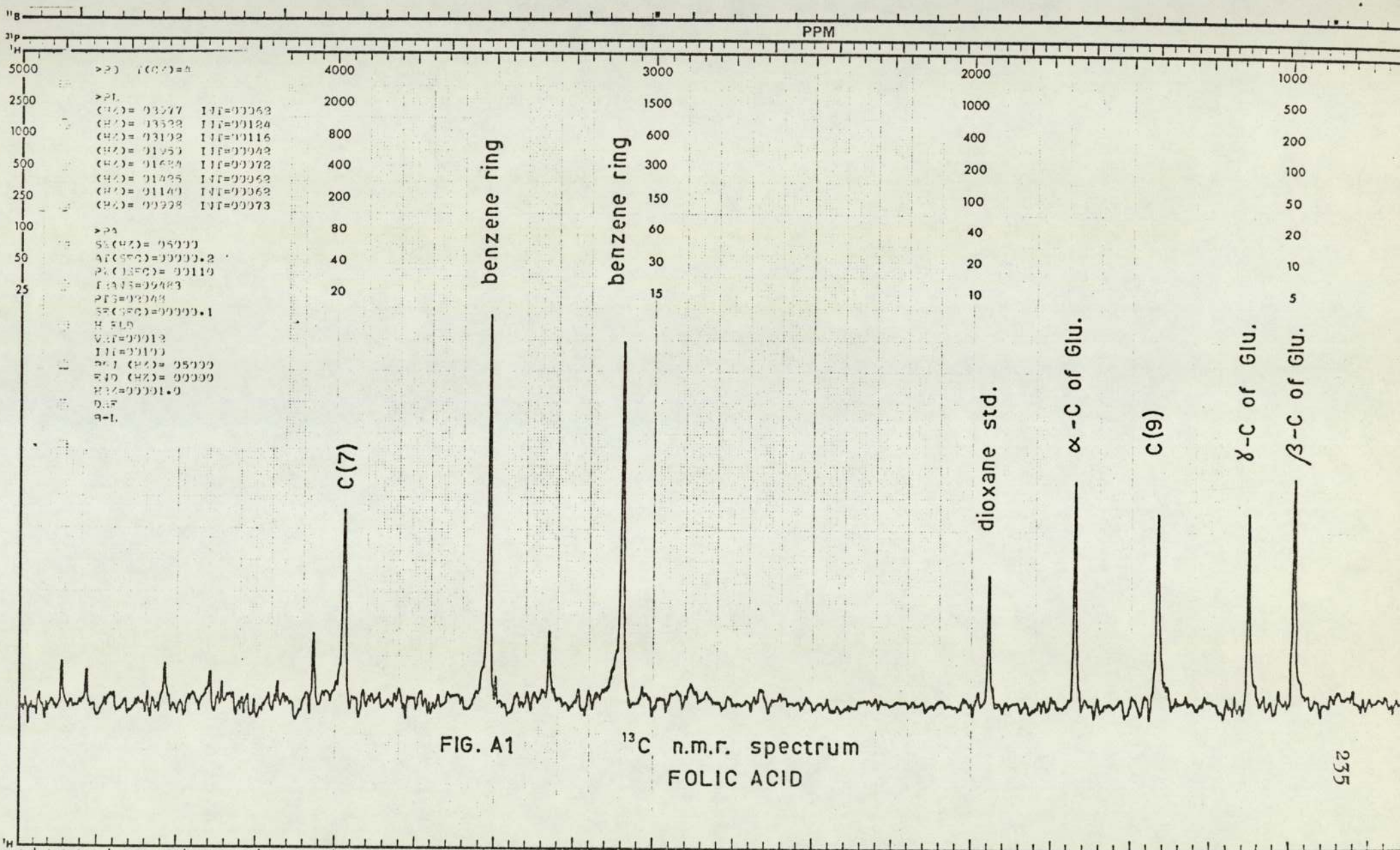


FIG. A1 ^{13}C n.m.r. spectrum
FOLIC ACID

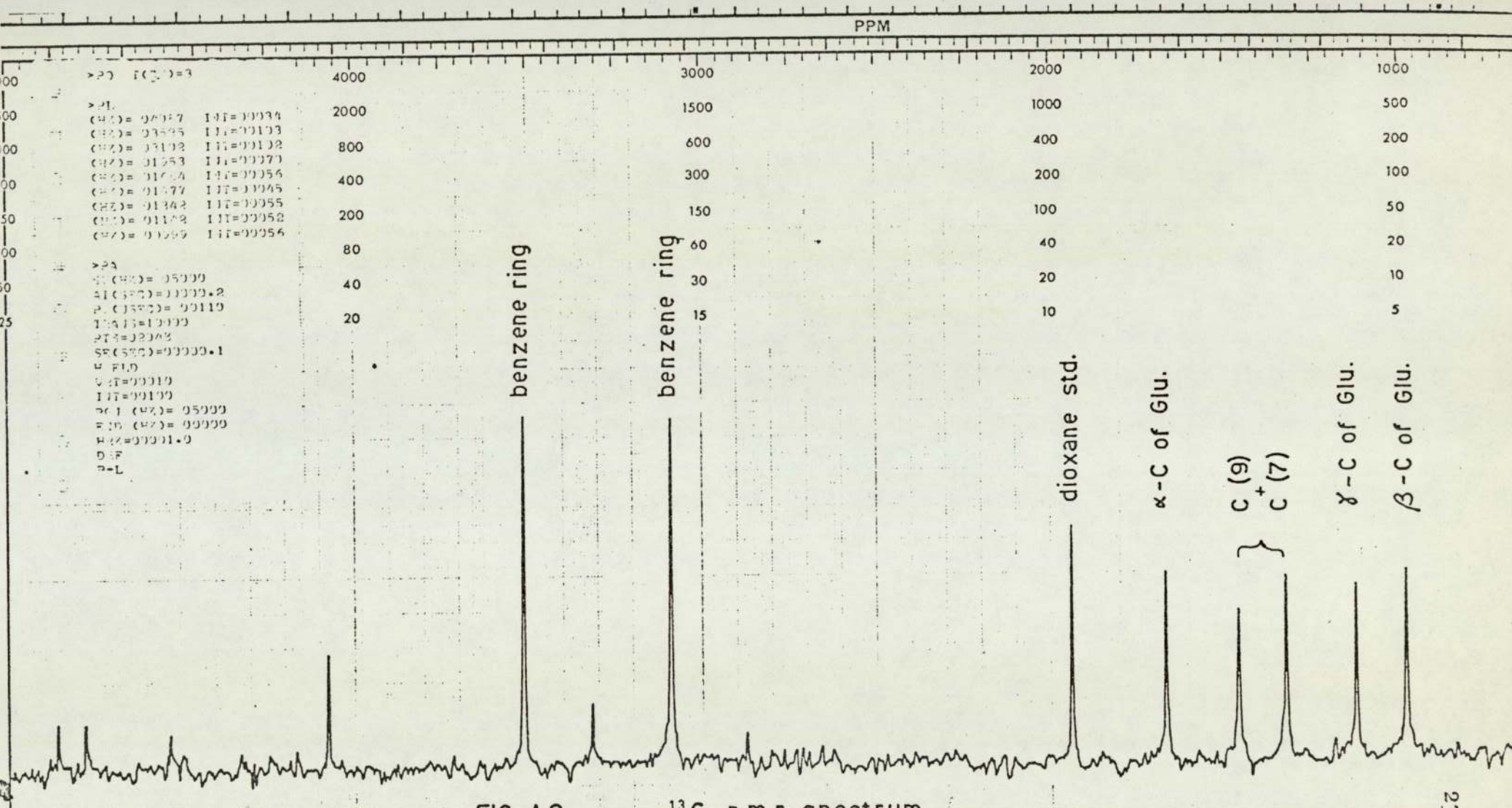


FIG. A2 ^{13}C n.m.r. spectrum
DIHYDROFOLIC ACID

included in Figs. A1 and A2 for comparison. The loss of the 7 - C resonance of folic acid at -81 p.p.m. relative to dioxane together with the appearance of one new absorption in the high field saturated carbon region at + 24 p.p.m. are consistent with the structure 7,8 - dihydrofolic acid for the product, even in the absence of absolute chemical shift assignments. (Conditions of pulse rate, etc., used in obtaining spectra did not allow quaternary carbon resonances to be recorded, hence these are absent, or weak, and the spectrum is simplified considerably). The partial chemical shift assignments in Figs. A1 and A2 are based on those given by Lyon et al.[194].

7,8 - dihydroxanthopterin. A slurry of sodium borohydride (1.14 g) in water (10 cm^3) was added to a stirred solution of xanthopterin (0.50 g) in 200 ml. of Tris buffer, pH 7.8 (Tris ≡ 2 - amino - 2 - hydroxymethylpropane - 1,3 - diol). After 20 minutes excess sodium borohydride was destroyed and the solution adjusted to pH 7-8 by the addition of 50% aq. acetic acid (6 cm^3), giving a buff precipitate. The latter was removed by centrifuging, washed several times with dilute acetic acid and dried under vacuum. Yield 0.45 g.

τ 2.1 br, 2H, (amino group), 5.62, s, 2H, 7-H_2
(consistent with reduction of xanthopterin to dihydroxanthopterin).

λ_{max} (nm) pH 13, 224, 279, 310 sh;
pH 7, 224, 275, 311;
pH 1, 279, 310.
cf. lit.[67] pH 13 223, 278, 310 sh.
pH 1 278, 315

N.m.r. of tetrahydrobiopterin. This was obtained in order to eliminate the possibility of unoxidised material giving spurious results in the characterisation of 7,8 - dihydrobiopterin and was: -

τ 1.85, 3H, amino groups; centred 5.75, 5H, 6-H, 7-H_2, plus side chain 2 x 1H; 8.45, 3H, Me.

All signals rather broad to allow resolution of similar shifts.

APPENDIX B.

Calibration of Manometric apparatus.

Method.

The assembled apparatus and connecting tubing was weighed dry and then re-weighed completely filled with water. Thus the weight of water and hence the volume of the vessel was determined, and this led to the gas volume present after introduction of a known volume of reactant solution. In order to convert change in height recorded on the manometer to moles of oxygen consumed the following formula was employed: -

$$\Delta n = \frac{V.\ \Delta h.\ \rho_1}{P.\ \rho_2.\ R\ T}$$

Where Δn = mol. O_2 consumed

V = vol. of gas in apparatus (cm^3)

Δh = change in manometric reading (cm.)

ρ_1 = density of manometer fluid

(Brodie's solution $\rho_1 = 1.033$ g. cm^{-3}).

P = standard atmosphere pressure (76 cm. Hg).

ρ_2 = density of mercury (13.6 g. cm^{-3}).

R = gas constant (82.05 cm^3. atm. $degree^{-1}.mol^{-1}$).

T = gas temperature (K).

Results.

Vessel A - this vessel was used for the majority of kinetic studies and consisted of a 250 cm^3 Pyrex glass

reaction flask. The total volume was found to be 370 cm^3.

$\therefore$ Using 100 cm^3 of reaction solution, volume of gas = 270 cm^3.

$$\therefore \quad \Delta n = \frac{270 \times 1.033}{76 \times 13.6 \times 82.05 \times 298} \Delta h$$

i.e. $$\underline{\Delta n = (1.1 \times 10^{-5}) \Delta h}$$

Naturally, this factor is only applicable at 25°C, and factors for runs at higher temperatures were re-calculated.

Vessel B

Small soda-glass vessel used for a limited number of experiments with THFA. (Chapter 3). Using 50 cm^3 reactant solution, V = 165 cm^3.

$$\therefore \text{ at } 25^\circ C \quad \Delta n = \frac{165 \times 1.033}{76 \times 13.6 \times 82.05 \times 298} \Delta h$$

i.e. $$\underline{\Delta n = (0.67 \times 10^{-5}) \Delta h}$$

It may be noted that in order to attain highest accuracy for thermobarometer corrections, the gas volume in the thermobarometer vessel was adjusted to equal the reaction vessel gas volume by addition of the appropriate volume of water to the former.

APPENDIX C.

Tabulation of all numerical initial rate data.

(1) Tetrahydrofolic acid oxidations in large pyrex vessel - solution volume = 100 cm^3.

Aqueous Solvent and pH.	THFA concn. (mM)	Gas phase % oxygen	Temperature (°C)	Additives or other modifications.	10^6 x initial rate/mol.O_2.min^{-1}
0.1M-phosphate pH 7	0.40	100	25	Blacked-out vessel - dark rates.	2.28
0.1M-phosphate pH 7	0.20	100	25	ditto	1.39
0.1M-phosphate pH 7	0.20	100	25	ditto	1.33
0.1M-phosphate pH 7	0.44	100	25	ditto	3.04
0.1M-phosphate pH 7	0.60	100	25	ditto	3.43
0.1M-phosphate pH 7	0.60	100	25	ditto	3.47
0.1M-phosphate pH 7	0.40	100	25	Laboratory illumination - light rates	3.09
0.1M-phosphate pH 7	0.40	100	25	ditto	3.09
0.1M-phosphate pH 7	0.40	100	25	ditto	3.14
0.1M-phosphate pH 7	0.40	80	25	ditto	2.70
0.1M-phosphate pH 7	0.40	40	25	ditto	2.16
0.1M-phosphate pH 7	0.40	20	25	ditto	1.76
0.1M-phosphate pH 7	0.60	20	25	ditto + 0.02M EDTA	2.0
0.1M-phosphate pH 7	0.44	20	25	ditto	1.36
0.1M-phosphate pH 7	0.30	20	25	ditto	1.0
0.1M-phosphate pH 7	0.20	20	25	ditto	0.54

(1) continued.

Aqueous Solvent and pH	THFA concn. (mM)	Gas phase % oxygen	Temperature (°C)	Additives or other modifications	10^6 x initial rate/ mol.O_2.min^{-1}
0.1M-phosphate pH 7	0.50	100	25	Lab. illumination - light rates	4.22
0.05M-phosphate pH 7	0.50	100	25	ditto	4.76
0.05M-phosphate pH 7	0.50	100	25	ditto	4.16
1.0M-ammonium acetate	0.40	100	25	ditto	2.10
0.1M-ammonium acetate	0.40	100	25	ditto	2.20
NaOH pH 6.0	0.40	100	25	ditto	1.82
NaOH pH 9.85	0.40	100	25	ditto	2.34
NaOH pH 10.3	0.40	100	25	ditto	3.30
NaOH/D_2O pH 10.2	0.40	100	25	ditto	2.36
NaOH pH 10.5	0.40	100	25	ditto	4.44
NaOH pH 10.4	0.40	100	25	ditto	3.33
NaOH pH 10.5	0.40	100	25	ditto	3.42
NaOH pH 10.8	0.40	100	25	ditto	3.88
NaOH pH 12.4	0.40	100	25	ditto	6.60
NaOH pH 13	0.40	100	25	ditto	6.14
NaOH pH 10.35	0.40	100	25	ditto	3.10

(1) continued.

Aqueous Solvent and pH.	THFA concn. (mM)	Gas phase % oxygen	Temperature (°C)	Additives or other modifications.	10^6 x initial rate/mol.O_2.min^{-1}
NaOH pH 11.1	0.40	100	25	lab. illumination - light rates.	4.7
NaOH pH 10.8	0.40	100	25	ditto	4.2
NaOH pH 7.0	0.40	100	25	ditto + 10^{-5}M - Fe^{2+}	1.77
NaOH pH 13	0.23	100	25	lab. illumination - light rates.	3.1
NaOH pH 13	0.23	60	25	ditto	2.56
NaOH pH 13	0.23	20	25	ditto	1.52
0.1M-phosphate pH 7	0.30	20	25	ditto	1.00
0.1M-phosphate pH 7	0.30	20	25	light rate; 10^{-4}M-8-hydroxyquinoline-5-sulphonic acid.	0.50
0.1M-phosphate pH 7	0.30	20	25	light rate; $3x10^{-4}$M-phenol	0.50
0.1M-phosphate pH 7	0.30	20	25	light rate; 10^{-4}M-Cu^{2+}	4.9
0.1M-phosphate pH 7	0.30	20	25	light rate; 10^{-4}M-Cu^{2+}/ $2.4x10^{-4}$M-EDTA	1.0
0.1M-phosphate pH 7	0.30	20	25	light rate; 10^{-4}M-Fe^{3+}	1.0
0.1M-phosphate pH 7	0.30	20	25	light rate; 0.8g powdered glass.	1.0
0.1M-phosphate pH 7	0.30	20	25	light rate; $1.5x10^{-4}$M CrO_3	2.0
0.1M-phosphate pH 7	0.30	20	30	light rate	1.50
NaOH pH 11.1	0.40	100	25	ditto	4.5

(1) continued.

Aqueous Solvent and pH.	THFA concn. (mM)	Gas phase % oxygen	Temperature (°C)	Additives or other modifications.	10^6 x initial rate/mol.O_2.min^{-1}
0.1M-phosphate pH 7	0.30	20	35	light rates	2.0
HCl pH 1	0.40	100	25	light rates	0.50
HCl pH 1	0.40	100	25	ditto	0.53
HCl pH 1	0.40	100	25	ditto	0.60
HCl pH 3.4	0.40	100	25	ditto	0.74
HCl pH 4.0	0.40	100	25	ditto	0.90
HCl pH 4.0	0.40	100	25	ditto	1.46
H_2O pH 5.1	0.40	100	25	ditto	1.50
H_2O pH 5.1	0.40	100	25	ditto	1.90
NaOH pH 6.0	0.40	100	25	ditto	1.82
NaOH pH 6.0	0.40	100	25	ditto	2.22
NaOH pH 7.0	0.40	100	25	ditto	2.16
NaOH pH 8.0	0.40	100	25	ditto	2.26
NaOH pH 8.0	0.40	100	25	ditto	2.38

(2) 6,7-dideuterio tetrahydrofolic acid oxidations in large pyrex vessel; solution volume = 100 cm^3

Aqueous solvent and pH	"THFA" concn. (mM)	Gas phase % oxygen	Temperature (°C)	Additives or other Modifications.	10^6 x initial rate/ $mol.O_2.min^{-1}$.
NaOH pH 9.25	0.40	100	25	light rates	2.20
NaOH pH 10.3	0.40	100	25	ditto	2.95
NaOH pH 10.5	0.40	100	25	ditto	3.25
NaOH pH 10.8	0.40	100	25	ditto	3.87
NaOH pH 11.0	0.40	100	25	ditto	4.45
NaOH pH 11.65	0.40	100	25	ditto	5.62
NaOH pH 12.8	0.40	100	25	ditto	6.10
NaOH pH 13.0	0.40	100	25	ditto	6.08

(3) Tetrahydrofolic acid oxidations in small soda glass vessel; solution volume = 50 cm^3 (N.B.)

Aqueous solvent and pH.	THFA concn. (mM)	Gas phase % oxygen	Temperature (^oC)	Additives or other modifications.	10^6 x initial rate/ mol.O_2.min^{-1} *
0.1M-phosphate pH 7	0.7	100	25	light rates	3.5
0.1M-phosphate pH 7	0.7	50	25	ditto	2.5
0.1M-phosphate pH 7	0.7	50	25	ditto	1.93
0.1M-phosphate pH 7	0.7	30	25	ditto	1.11
0.1M-phosphate pH 7	0.7	20	25	ditto	1.41
0.1M-phosphate pH 7	0.7	10	25	ditto	0.89
0.1M-phosphate pH 7	0.7	20	25	ditto	1.10
0.1M-phosphate pH 7	0.7	20	25	0.3 mM - phenol	0.60
0.1M-phosphate pH 7	0.7	20	25	0.1 mM - Cu^{2+}	5.50
0.1M-phosphate pH 7	0.7	20	25	0.1 mM - Cu^{2+} + 0.24 mM EDTA	1.0

* not adjusted for solution volume change.

(4) Tetrahydrobiopterin oxidations in large pyrex vessel; solution volume = 100 cm^3

Aqueous solvent and pH.	Tetrahydro-biopterin concn.(mM)	Gas phase % oxygen	Temperature (^oC)	Additives or other modifications	10^6 x initial rate/ mol.O_2.$min.^{-1}$
0.1M-phosphate pH 7	0.156	100	25	light rates	1.05
0.1M-phosphate pH 7	0.312	100	25	ditto	2.18
0.1M-phosphate pH 7	0.468	100	25	ditto	3.13
0.1M-phosphate pH 7	0.625	100	25	ditto	4.10
0.1M-phosphate pH 7	0.625	75	25	ditto	3.20
0.1M-phosphate pH 7	0.625	50	25	ditto	2.57
0.1M-phosphate pH 7	0.625	20	25	ditto	1.97
0.1M-phosphate pH 7	0.625	10	25	ditto	1.55
0.1M-phosphate pH 7	0.312	60	25	ditto	1.70
0.1M-phosphate pH 7	0.312	20	25	ditto	1.06
0.1M-phosphate pH 7	0.156	50	25	ditto	0.64
0.1M-phosphate pH 7	0.156	20	25	ditto	0.45
0.1M-phosphate pH 7	0.468	20	25	ditto	1.25
0.01M-phosphate	0.625	100	25	ditto	3.60
0.01M-phosphate	0.625	20	25	ditto	1.45
20 mM - EDTA	0.625	20	25	0.1 mM Cu^{2+} (EDTA)	1.50
20 mM - EDTA	0.625	20	25	0.5 mM Cu^{2+} (EDTA)	1.46
20 mM - EDTA	0.625	20	25	0.3 mM H_2O_2	1.57

(4) continued.

Aqueous solvent and pH.	Tetrahydro-biopterin concn.(mM)	Gas phase % oxygen	Temperature (°C)	Additives or other modifications	10^6 x initial rate/ mol.O_2.min.$^{-1}$
0.1M-phosphate pH 7	0.312	100	25	light rate; 0.02M-EDTA	2.13
0.1M-phosphate pH 7	0.312	20	25	light rate; 0.02M-EDTA	0.94
0.1M-phosphate pH 7	0.312	100	25	light rate; 0.002M-8-hydroxy quinoline-5-sulphonic acid.	1.22
0.1M-phosphate pH 7	0.312	20	25	ditto	0.50
0.1M-phosphate pH 7	0.312	100	25	light rate; 2 mM-phenol	1.16
0.1M-phosphate pH 7	0.625	100	25	Dark rate	3.80
0.1M-phosphate pH 7	0.625	60	25	Dark rate	2.48
0.1M-phosphate pH 7	0.625	20	25	Dark rate	1.16
0.01M-phosphate	0.625	100	25	Dark rate	3.52
0.01M-phosphate	0.625	60	25	Dark rate	2.20
0.01M-phosphate	0.625	20	25	Dark rate	1.10
0.1M-phosphate pH 7	0.312	100	25	Dark rate	1.90
0.1M-phosphate pH 7	0.312	60	25	Dark rate	1.15
0.1M-phosphate pH 7	0.312	20	25	Dark rate	0.46
0.1M-NaOH pH 13	0.312	100	25	Dark rate	5.64
0.1M-NaOH pH 13	0.312	60	25	Dark rate	3.82
0.1M-NaOH pH 13	0.312	20	25	Dark rate	1.98

(4) continued.

Aqueous solvent and pH.	Tetrahydrobiopterin concn. (mM)	Gas phase % oxygen	Temperature (°C)	Additives or other modifications	10^6 x initial rate/ mol.O_2.$min.^{-1}$
0.1M-ammonium acetate	0.625	20	25	Light rate	2.30
0.1M-ammonium acetate	0.625	100	25	ditto	4.90
0.1M-ammonium acetate	0.625	20	30	ditto	3.40
0.1M-ammonium acetate	0.625	20	35	ditto	4.80
1.25M-ammonium acetate	0.625	20	25	ditto	2.43
2.5M-ammonium acetate	0.625	20	25	ditto	4.50
2.5M-ammonium acetate	0.625	20	25	Light rate; 0.24 mM-EDTA	1.60
0.1M-ammonium acetate	0.625	20	25	Light rate; 1.0mM-EDTA	1.40
0.1M-ammonium acetate	0.625	20	30	ditto	2.0
0.1M-ammonium acetate	0.625	20	35	ditto	2.30
0.1M-ammonium acetate	0.625	20	45	ditto	4.10
0.1M-ammonium acetate/ D_2O	0.625	20	25	ditto	1.30
0.1M-phosphate pH 7	0.625	100	25	Light rate;0.17mM-p-tyrosine	4.20
0.1M-phosphate pH 7	0.625	100	25	Light rate; 0.16 mM-phenylalanine	5.60
0.1M-phosphate pH 7	0.625	100	25	Light rate; 2% ethanol	3.20
0.1M-phosphate pH 7	0.625	100	25	Light rate; 2% methanol	3.60

(4) continued.

Aqueous solvent and pH.	Tetrahydro-biopterin concn. (mM)	Gas phase % oxygen	Temperature (°C)	Additives or other modifications	10^6 x initial rate/ mol.O_2.min.$^{-1}$
0.1M-phosphate pH 7	0.625	100	25	Light rate; 2% propan-2-ol	4.90
0.1M-phosphate pH 7	0.625	20	25	Direct irradiation; Hanova UV lamp.	1.70
0.1M-phosphate pH 7	0.625	100	25	ditto	3.20
NaOH pH 13	0.312	100	25	Lab. illumination; light rate	7.57
NaOH pH 13	0.312	100	25	ditto + 0.1mM-EDTA	8.00
NaOH pH 11.5	0.312	100	25	Light rate; no additive	6.45
NaOH pH 11.4	0.312	100	25	ditto	6.5
NaOH pH 11.4	0.312	100	25	ditto	6.34
NaOH pH 11.1	0.312	100	25	ditto	6.30
NaOH pH 10.6	0.312	100	25	ditto	5.04
NaOH pH 10.4	0.312	100	25	ditto	4.20
NaOH pH 10.2	0.312	100	25	ditto	3.30
NaOH pH 9.8	0.312	100	25	ditto	2.92
NaOH pH 8.9	0.312	100	25	ditto	1.23
H_2O pH 3.4	0.312	100	25	ditto	0.68
HCl pH 1.0	0.312	100	25	ditto	0.07

APPENDIX D.

Use of Steady-State Approximation in deriving theoretical rate laws for possible autoxidation chain mechanisms.

1. Thermal oxygen initiation with termination by reaction of semiquinone and hydroperoxyl radicals.

$$AH_2 + O_2 \xrightarrow{k_1} AH. + HO_2. \qquad \text{initiation}$$

$$AH. + O_2 \xrightarrow{k_2} A + HO_2. \qquad \text{propagation}$$

$$AH_2 + HO_2. \xrightarrow{k_3} AH. + H_2O_2 \qquad \text{propagation}$$

$$AH. + HO_2. \xrightarrow{k_4} A + H_2O_2 \qquad \text{termination}$$

where AH_2 represents tetrahydropterin, AH. semiquinone radical and A dihydropterin (quinonoid).

Note: Mechanism chosen using $HO_2.$ chain carrier not AHOO. in order to illustrate actual mechanism proposed. The results of rate equation are the same in both cases.

At steady state we have: -

$$(1)\ \frac{d\,(AH.)}{dt} = 0 = k_1(AH_2)(O_2) - k_2(AH.)(O_2) + k_3(AH_2)(HO_2) - k_4(AH.)(HO_2.)$$

$$(2)\ \frac{d\,(HO_2)}{dt} = 0 = k_1(AH_2)(O_2) + k_2(AH.)(O_2) - k_3(AH_2)(HO_2) - k_4(AH.)(HO_2)$$

These are linear simultaneous equations with two unknowns, solution of which gives: -

$$(AH.) = (k_1k_3/k_2k_4)^{\frac{1}{2}}\ (AH_2)$$

But the rate of oxygen consumption is given by:

$$-\frac{d(O_2)}{dt} = k_1 (AH_2)(O_2) + k_2(AH.)(O_2)$$

neglecting the slow initiation, at steady state we obtain:

$$\text{Rate} = \frac{-d(O_2)}{dt} = (k_1 k_2 k_3 / k_4)^{\frac{1}{2}} (AH_2)(O_2)$$

This is the rate equation approximated to by the dark reaction, and it is therefore assumed that this occurs by essentially the above mechanism.

2. Thermal oxygen initiation with termination by HO_2 disproportionation

$$AH_2 + O_2 \xrightarrow{k_1} AH. + HO_2. \quad \text{initiation}$$

$$AH. + O_2 \xrightarrow{k_2} A + HO_2.$$

$$AH_2 + HO_2. \xrightarrow{k_3} AH. + H_2O_2 \quad \text{propagation}$$

$$2HO_2. \xrightarrow{k_4} H_2O_2 + O_2 \quad \text{termination}$$

At steady-state we have: -

$$(1)\ \frac{d(AH.)}{dt} = 0 = k_1(AH_2)(O_2) - k_2(AH.)(O_2) + k_3(AH_2)(HO_2)$$

$$(2)\ \frac{d(HO_2)}{dt} = 0 = k_1(AH_2)(O_2) + k_2(AH.)(O_2) - k_3(AH_2)(HO_2) - k_4(HO_2.)^2$$

Solution of simultaneous equations gives:

$$(3)\ k_2(AH.)(O_2) = k_1(AH_2)(O_2) + k_3\left\{\frac{2k_1}{k_4}\right\}^{1/2}(AH_2)^{3/2}(O_2)^{1/2}$$

But the rate of oxygen uptake is: -

$$\frac{-d(O_2)}{dt} = k_1(AH_2)(O_2) + k_2(AH.)(O_2) - k_4(HO_2)^2$$
$$= k_2(AH.)(O_2) - k_1(AH_2)(O_2)$$

Using $k_2(AH.)(O_2)$ given by equation (3) we obtain: -

$$\frac{-d(O_2)}{dt} = k_3\,(2k_1/k_4)^{1/2}\,(AH_2)^{3/2}\,(O_2)^{1/2}$$

Since the reaction is first order w.r.t. tetrahydropterin, this mechanism may be assumed inoperative.

3. Thermal oxygen initiation with termination by semiquinone disproportionation.

$$AH_2 + O_2 \xrightarrow{k_1} AH. + HO_2. \qquad \text{initiation}$$

$$AH. + O_2 \xrightarrow{k_2} A + HO_2. \qquad \text{propagation}$$

$$AH_2 + HO_2. \xrightarrow{k_3} AH. + H_2O_2 \qquad \text{propagation}$$

$$2AH. \xrightarrow{k_4} AH_2 + A \qquad \text{termination.}$$

At steady state we have: -

$$(1)\ \frac{d(AH.)}{dt} = 0 = k_1(AH_2)(O_2) - k_2(AH.)(O_2) + k_3(AH_2)(HO_2) - k_4(AH.)^2$$

$$(2)\ \frac{d(HO_2)}{dt} = 0 = k_1(AH_2)(O_2) + k_2(AH.)(O_2) - k_3(AH_2)(HO_2)$$

Solution gives:

$$(AH.) = (2\,k_1/k_4)^{1/2}\ (AH_2)^{1/2}\ (O_2)^{1/2}$$

$\therefore$ Neglecting slow initiation we obtain:

$$\frac{-d(O_2)}{dt} = k_2\ (2\,k_1/k_4)^{1/2}\ (AH_2)^{1/2}\ (O_2)^{3/2}$$

Again this does not agree with observed kinetics and may be neglected.

4. Possible photochemically assisted initiation with termination as in 1. above.

$$AH_2 + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AH_2 \ldots O_2 \ (X) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} {}^{+}AH_2 \ldots O_2^{-} \ (Y) \quad \left.\begin{array}{l}\\\\\\\\\end{array}\right\} \text{initiation}$$

$$AH_2^{+} \ldots O_2^{-} \xrightarrow{k_3} AH. + HO_2.$$

and k_2 is proposed as light-dependent.

$$AH. + O_2 \xrightarrow{k_4} A + HO_2. \quad \left.\begin{array}{l}\\\\\\\\\end{array}\right\} \text{propagation}$$

$$AH_2 + HO_2. \xrightarrow{k_5} AH. + H_2O_2$$

$$AH. + HO_2. \xrightarrow{k_6} A + H_2O_2 \qquad \text{termination.}$$

At steady state we have: -

(1) $\frac{d(X)}{dt} = 0 = k_1(AH_2)(O_2) - k_{-1}(X) - k_2(X) + k_{-2}(Y)$

(2) $\frac{d(Y)}{dt} = 0 = k_2(X) - k_{-2}(Y) - k_3(Y)$

(3) $\frac{d(AH.)}{dt} = 0 = k_3(Y) - k_4(AH.)(O_2) + k_5(AH_2)(HO_2) - k_6(AH.)(HO_2)$

(4) $\frac{d(HO_2)}{dt} = 0 = k_3(Y) + k_4(AH.)(O_2) - k_5(AH_2)(HO_2) - k_6(AH.)(HO_2)$

Four linear simultaneous equations with four unknowns; solution for (AH.) gives: -

$$(AH.) = \left[\frac{k_3 k_a}{2\,k_4} \pm \left\{ \frac{k_3^2 k_a^2}{4\,k_4^2} + \frac{k_3 k_a}{4\,k_4 k_6} \right\}^{1/2} \right] (AH_2)$$

where $k_a = k_1 k_2 \,/(\, k_{-1}\, k_{-2} + k_{-1}\, k_3 - 2k_{-2} - k_3)$

The rate of oxygen uptake is given by : -

$$\frac{-d(O_2)}{dt} = k_1\,(AH_2)(O_2) - k_{-1}\,(X) + k_4\,(AH.)(O_2)$$

but it may be shown that $(X) \propto (AH_2)(O_2)$

$$\therefore \quad -\frac{d(O_2)}{dt} = k^1\,(AH_2)(O_2) + k_4\,(AH.)(O_2)$$

Neglecting the first term on the right hand side due to initiation (usually slow) and substituting for (AH.) we obtain:

$$\frac{-d(O_2)}{dt} = \left[\frac{k_3\, k_a}{2} \pm \left\{ \frac{k_3^2 k_a^2}{4} + \frac{k_3 k_4 k_a}{4\, k_6} \right\}^{1/2} \right] (AH_2)(O_2)$$

Since k_a is expected to show a complex dependence on light intensity, the rate will also depend on this in a similar fashion.

Similar results are obtained if an analogous light-dependence is introduced into the propagation steps.

APPENDIX E.

Derivation of corrected rates and % ionisation used in pH-dependence of autoxidation rates.

(1) Effect of protonation at N(5) for THFA.

Assuming rate equation, at constant oxygen concentration, of the form: -

$$\text{Rate} = k_a \text{ (protonated THFA)} + k_b \text{(deprotonated THFA)}$$

and putting k_a (protonated THFA) = R_a

k_b (deprotonated THFA) = R_b

k_a determined from rate at pH 1

pH	10^6 x observed rate/mol.O_2.min^{-1}	% deprotonated THFA	R_a term	R_b term
1.0	0.50	0	0.50	0
3.4	0.74	3.6	0.48	.26
4.0	1.18 average	13	0.44	0.74
5.1	1.7 average	60	0.20	1.50
6.0	2.02 average	94	0.03	2.0
8.0	2.32	100	0	2.32

% deprotonated THFA estimated using $pK_a = 4.82$

Plot of R_b v. % deprotonated THFA given in Chapter 3.

(2) Effect of ionisation of 3,4-amide function.

Using rate law (at constant oxygen concentration):

Rate = k_c(neutral tetrahydropterin) + k_d(imino-enolate)

and putting:

k_c (neutral tetrahydropterin) = R_c

k_d (imino-enolate) = R_d = Observed rate - R_c

k_c estimated from extrapolated rate at ca pH 8

(a) Tetrahydrofolic acid values. % amide ionisation estimated using pKa=10.8

pH	10^6 x Observed rate/mol.O_2.min.$^{-1}$	% amide ionisation	R_c term	R_d term
9.85	2.34	7.5	1.85	0.49
10.30	3.30	18.5	1.63	1.67
10.35	3.10	20.5	1.59	1.51
10.5	3.40	26.5	1.47	1.93
10.8	3.87	41.7	1.16	2.71
11.0	4.35	53.3	0.93	3.42
11.1	4.15	56.3	0.82	3.33
12.4	6.10	96.5	0.07	6.03
13.0	6.10	100	0	6.10

(b) 6,7 dideuterio-tetrahydrofolic acid values using amide pKa = 10.8

pH	10^6 x Observed rate/mol.O_2.min.$^{-1}$	% amide ionisation	R_c term	R_d term
9.25	2.20	1.8	1.96	0.24
10.30	2.95	18.5	1.63	1.32
10.50	3.25	26.5	1.47	1.78
10.80	3.87	41.7	1.16	2.71
11.0	4.45	53.3	0.93	3.52
11.65	5.62	83.3	0.33	5.29
13.0	6.10	100	0	6.10

(c) Tetrahydrobiopterin values % amide ionisation estimated using pKa = 10.5

pH	10^6 x Observed rate/mol.O_2.min^{-1}	% amide ionisation	R_c term	R_d term
	1.0 = extrapolated value for low pH.			
8.9	1.23	3	0.97	0.26
9.8	2.92	17	0.83	2.09
10.2	3.30	35	0.65	2.65
10.4	4.20	46	0.54	3.66
10.6	5.04	56	0.44	4.60
11.1	6.34	92.5	0.08	6.25
13	8.00	100	0	8.00
13	7.57	100	0	7.57

For the above data, plots of R_d v. % amide ionisation are made in the text.

APPENDIX F.

Spectrophotometric estimation of pKa values for ionisation of 3,4-amide group.

Method.

On increasing the solution pH in the region 9 to 13, reduced pterins show a shift of the absorption maximum centred around 290-300 nm, and this is due to ionisation of the 3,4-amide group. Since the extinction coefficient is only slightly affected by this pH change (see, for example, Blakley[18]) it follows that the fractional shift in λ_{max} is approximately proportional to the degree of ionisation. Consequently, a reasonable estimate of dissociation constant may be obtained from a plot of λ_{max} vs. pH, since the pKa value is equal to the pH corresponding to 50% dissociation, i.e., the mid-point of the plot. The pKa values for THFA, tetrahydrobiopterin and DHFA were determined as follows.

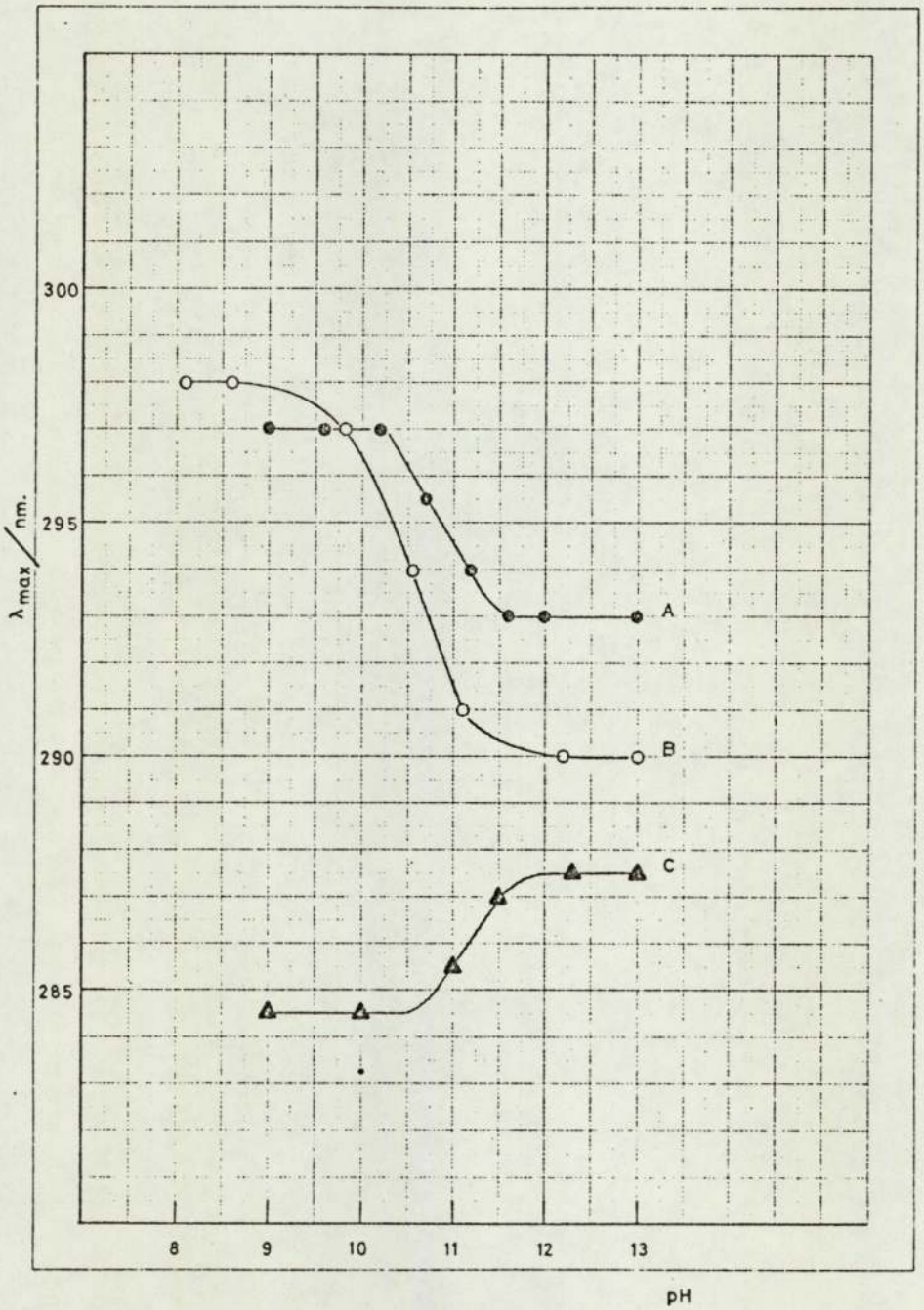
A solution of the compound (ca. 2 mg) in water (100 cm^3), adjusted to the desired pH with 0.1M-NaOH, previously de-aerated with a stream of nitrogen gas and containing 0.1% 2-mercaptoethanol as antioxidant, was prepared and its UV spectrum immediately recorded against a solvent blank. The λ_{max} was noted and the pH determined ($\pm$ 0.1 pH units). A plot of λ_{max} vs. pH was constructed and the point of maximum slope estimated, the corresponding pH being taken as the pKa value.

Results.

The appropriate plots are shown in Fig. F.1, and the pKa values indicated therein.

FIG. F.1

pH - Dependence of λ_{max} for reduced pterins.



A) Tetrahydrofolic acid pK_a 10.8

B) Tetrahydrobiopterin pK_a 10.5

C) Dihydrofolic acid pK_a 11.2

APPENDIX G.

L C A O coefficients for all atoms from Huckel calculations on tetrahydropterin molecular system using h = 1.0 and k = 0.90 for N(5). Other heteroatom parameters as in Chapter 6.

Molecular orbital energy level, units of β relative to α-value	L C A O (Huckel) coefficients for atom at column head.									
	N(1)	C(2)	N(3)	C(4)	C(4a)	N(5)	N(8)	C(8a)	C(2)-Amino Nitrogen	Amide carbonyl oxygen
+2.8942	+0.1586	+0.3025	+0.5318	+0.4389	+0.2477	+0.1177	+0.1234	+0.1720	+0.2170	+0.4908
+2.3080	-0.2312	-0.3853	-0.2274	+0.2016	+0.0381	+0.0262	-0.1699	-0.1372	-0.4769	+0.6546
+2.2274	+0.1424	-0.1766	-0.2645	-0.0158	+0.2988	+0.2191	+0.6656	+0.4841	-0.2428	-0.0695
+1.6597	+0.0868	+0.0836	-0.4541	-0.1561	-0.2637	-0.3598	+0.2643	+0.0422	+0.5234	+0.4587
+1.5161	-0.0006	-0.0067	+0.4073	+0.0133	-0.3597	-0.6273	+0.3692	+0.0059	-0.4169	-0.0274
+0.9240	+0.7703	+0.1714	-0.1603	-0.0791	+0.0136	-0.1612	-0.4111	+0.2368	-0.2976	+0.0735
+0.2953	+0.1764	+0.2325	+0.1205	-0.3776	-0.4535	+0.5792	+0.2304	-0.2776	-0.1930	+0.2215
-0.8397	-0.3200	-0.0564	+0.2793	-0.5971	+0.0118	-0.0058	-0.2532	+0.5923	+0.0241	+0.2105
-1.0673	+0.3327	-0.7583	+0.2478	+0.1221	-0.3383	+0.1473	-0.0415	+0.1064	+0.2954	-0.0398
-1.9176	-0.2364	+0.2454	-0.2096	+0.4709	-0.5732	+0.1768	-0.1373	+0.4691	-0.0718	-0.1202

APPENDIX H.

The synthesis of 2,3- and 2,5-dihydroxyphenylalanines.

2,3- and 2,5- dihydroxyphenylalanines were synthesised as t.l.c. standards for the investigations in Chapter Eight, using the Erlenmeyer azlactone method described by Lambooy[251] and starting from 2,3- and 2,5- dimethoxybenzaldehydes, respectively. The method will be described for the 2,3- isomer, the other being identical but using the appropriate starting material. Spectral data, etc., are given for intermediates and final products after the description. M. pts. were determined on a Reichter hot stage and are uncorrected. U.V. spectra were recorded with Perkin-Elmer 137UV or Unican SP700 spectrophotometers, n.m.r. with Perkin Elmer R14 or Varian HA100D spectrometers, mass spectra on an A.E.I. MS9 spectrometer, and i.r. on a Perkin-Elmer 157G spectrometer. Starting materials were purchased from Phase Separations, Koch-Light and B.D.H. Ltd.

4-(2,3-Dimethoxybenzylidene)-2-phenyl-5-oxazolone.

2,3-Dimethoxybenzaldehyde was purified by recrystallisation from n-hexane. The purified compound (16.6g, 0.1 mole), N-benzoylglycine (18.3g, 0.102 mole), freshly fused sodium acetate (8.1g. 0.099 mole) and acetic anhydride (100 cm^3) were heated on the steam-bath for 45 mins., the acetic anhydride removed on the rotary evaporator, and the product permitted to stand for several hours. Water (125 cm^3) was added, the product suspended and allowed to stand overnight, and filtered and washed

thoroughly on the filter with water. The precipitate was re-suspended in ether (75 cm^3), filtered and washed with ether (40 cm^3). The product was recrystallised from benzene.

2,3 - Dihydroxyphenylalanine.

The azlactone prepared above (6.18 g., 0.02 mole), glacial acetic acid (37 cm^3), hydriodic acid (sp. gr. 1.7, 30 cm^3) and red phosphorus (1.9 g.) were heated under reflux and a stream of hydrogen for $2\frac{1}{2}$ hours. The hot mixture was filtered through asbestos and evaporated to dryness (rotary evaporator). The residue was treated with water (100 cm^3) and evaporated as before. It was then suspended in water at 50^o (100 cm^3) and extracted three times with 50 cm^3 portions of ether. The water solution was concentrated to 50 cm^3, and a layer of n-hexane added followed by an excess of ammonia solution. The mixture was taken to dryness (rotary evaporator) and the residue suspended in cold water (50 cm^3), filtered and recrystallised from water containing a little sulphur dioxide. It was then further recrystallised to constant m. pt. from water containing a little decolorising charcoal.

Characterisation, etc.

Azlactones.

4-(2,3-Dimethoxybenzylidene)-2-phenyl-5-oxazolone.

Yield: 16 g. (53%) m.pt. 169-169.5°, Lit.[251] 168-171°

λ_{max} ($\log_{10}$ E), EtOH 225 (4.25), 259 (4.11), 366 (4.61) 387 sh (4.44) nm.

ν_{max} (KBr) cm^{-1} 3075, 3055, 3010, 2980, 2935, 2900, 2870, 2835 (w) CH, 1800 (s.), 1755 (m.) 1650 (s.), C = O, C = N and C = C 1600, 1593, 1575 C = C (Ar).

τ ($CDCl_3$) 1.5 -3.0, complex aromatic + olefinic, total 9H, 6.10, s, 3H, and 6.15, s, 3H, 2 x methoxyl.

MS M^+ 309.100421 ($C_{18}H_{15}NO_4$ gives 309.100100)

m/e (%) 309 (9.9) 106 (8.8) 105 (100) 78 (3.3) 77 (35) 51 (7.7)

$\underline{m}^*$ 56.6 (105 ⟶ 77)

Analysis: C 69.70, H 4.75, N 4.38%

calculated $C_{18}H_{15}NO_4$ C 69.90, H 4.85, N 4.53%

4-(2,5-Dimethoxybenzylidene)-2-phenyl-5-oxazolone.

Yield 16.5 g (54%) m.pt. 170-171° literature values not available.

λ_{max} ($\log_{10}E$), EtOH 227 (4.04) 263 (3.87) 353 (4.09) 427 (4.11) nm.

ν_{max} (KBr) cm^{-1} 3050, 3000, 2960, 2910, 2835 (w) CH str. 1785 (s) 1765 (m), 1645 (m), C = O, C = N and C = C 1600, 1580, 1555 (w) C = C (Ar).

τ ($CDCl_3$) 1.5 - 3.12, complex aromatic and olefinic, total 9H, 6.18, s, 3H, and 6.20, s, 3H, 2 x methoxyl.

MS M^+ 309.098596 ($C_{18}H_{15}NO_4$ gives 301.100100) large ppm error due to inability to resolve instrument owing to very small intensity of standard peak compared with 309.

m/e (%) 309 (15.8) 119 (14.5) 105 (100) 91 (10.5) 78 (15.8) 77 (39.5) 59 (16) 58 (35.6) 51 (16)

$\underline{m}^*$ 56.5 (105 ⟶ 77)

Analysis C 69.80, H 4.80, N4.61 %

calculated $C_{18}H_{15}NO_4$ C69.90, H4.85 N 4.53%

Dihydroxyphenylalanines.

2,3-Dihydroxyphenylalanine.

Yield 1.90 g. (44%) m. pt. 268 - 270° (d.)
lit. 280° (d)[251] and 265° [252]

λ_{max} (log E), pH 1 2.79 (3.36) nm.

ν_{max} (KBr) cm^{-1} 3500 - 2200 CO_2H , 1660 C = O

τ (trifluoroacetic acid) 2.5 br, 3H, amino (protonated);
3.1, 4 peaks, 3H, aromatic;
5.23 br, 1H, α - H of alanine subs.;
6.4 m, 2H, β-H_2 of alanine.

MS M^+ 197.068673 ($C_9H_{11}NO_4$ gives 197.068802)

m/e (%)				
197 (5.7)	179 (2.9)	177 (5.7)		
164 (20)	151 (100)	136 (20)	134 (23)	
124 (26)	123 (34)	122 (34)	110 (40)	
95 (11)	94 (17)	79 (17)	78 (43)	
77 (31)	53 (14)	52 (23)	51 (37)	
50 (20)				

Analysis C 54.61 H 5.67 N 6.93 %
calc. $C_9H_{11}NO_4$ C 54.8 H 5.58 N 7.1%

2,5 - Dihydroxyphenylalanine.

Yield 1.6 g. (37%) m. pt. 257.5 - 258.5° (d.)

lit.[251] 257 - 258°

λ_{max} (log E) pH 1 205 (3.89), 218 sh.(3.73)
294 (3.56) nm.

ν_{max} (KBr) cm^{-1} 3545 N - H 3500 - 2300 CO_2H
1630 C = O

τ (trifluoroacetic acid) 2.45 br, 3H, amino (protonated)
2.90, 1H, and 3.08, 2H, aromatic
5.25 br, 1H, and 6.45, m, 2H, α - H
and β - H_2 of alanine subs.

MS Compound appears to lose elements of water on heating to give M^+ 179.058694
($C_9H_9NO_3$ gives 179.058238)

Analysis C 50.50 H 5.99 N 6.48 %
Calc. $C_9H_{11}NO_4.H_2O$ C50.22, H 6.09, N 6.51

cf. Lambooy reports this compound as monohydrate[253].

REFERENCES.

1. R. Purrmann. Ann. Chem. (1940) 544, 182.

2. R. Purrmann. Ann. Chem. (1940) 546, 98.

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

4. D. J. Brown and N. W. Jacobsen. J.Chem.Soc. (1961), 4413.

5. D. J. Brown and S. F. Mason. J.Chem.Soc. (1956) 3443.

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

7. W. Pfleiderer. Chem. Ber. (1957) 90, 2582.

8. W. Pfleiderer. Chem. Ber. (1957) 90, 2617.

9. W. Pfleiderer. Chem. Ber. (1957) 90, 2604.

10. W. Pfleiderer. Chem. Ber. (1957) 90, 2624.

11. W. Pfleiderer. Chem. Ber. (1957) 90, 2631.

12. W. Pfleiderer. Chem. Ber. (1958) 91, 1671.

13. G. Nubel and W. Pfleiderer. Chem. Ber.(1962) 95,1605.

14. E. Lippert and H. Prigge. Z.Elektrochem(1960) 64,662.

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

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

17. J. C. Rabinowitz in "The Enzymes" Vol. 2, 2nd Edition, ed. P. D. Boyer, H. Lardy and K. Myrback.
Academic Press, New York (1960). p.185.

18. R. L. Blakley "The Biochemistry of Folic Acid and Related Pteridines". Frontiers of Biology Series Vol. 13, ed. A. Neuberger and E. L. Tatum.
North-Holland (1969).

19. E. L. Patterson, H. P. Broquist, A. M. Albrecht, M. H. von Saltza and E. L. R. Stokstad.
J. Am. Chem. Soc. (1955) 77, 3167.

20. E. L. Patterson, M. H. von Saltza and E. L. R. Stokstad.
J. Am. Chem. Soc. (1956) 78, 5871.

21. E. L. Patterson, R. Milstrey and E. L. R. Stokstad.
J. Am. Chem. Soc. (1956) 78, 5868.

22. S. Kaufman. Biochim. Biophys. Acta (1957) 23, 445.

23. S. Kaufman. J. Biol. Chem. (1957) 226, 511.

24. S. Kaufman. J. Biol. Chem. (1958) 230, 931.

25. S. Kaufman. Biochim. Biophys. Acta (1958) 27, 428.

26. S. Kaufman. J. Biol. Chem. (1959) 234, 2677.

27. S. Kaufman and B. Levenberg. J. Biol. Chem. (1959). 234, 2683.

28. S. Kaufman, W. F. Bridgers, F. Eisenberg and S. Friedman. Biochem. Biophys. Res. Commun. (1962) 9, 497.

29. S. Kaufman. Proc. Nat. Acad. Sci. U.S. (1963) 50, 1085.

30. S. Kaufman. J. Biol. Chem. (1967) 242, 3934.

31. J. Renson, F. Goodwin, H. Weissbach and S. Udenfriend. Biochem. Biophys. Res. Commun. (1961) 6, 20.

32. T. Noguchi, M. Noshino and R. Kido. Biochem. J. (1973) 131, 375.

33. S. G. Bhat, M. Ramanarayanan and C. S. Vaidyanathan. Biochem. Biophys. Res. Commun. (1973) 52, 834.

34. P. F. T. Vaughan and V. S. Butt. Biochem. J. (1972) 127, 641.

35. G. W. Kidder and L. L. Nolan. Biochem. Biophys. Res. Commun. (1973) 53, 929.

36. H. R. Mahler and E. H. Cordes. "Basic Biological Chemistry" Harper International Edition (1969) p. 439.

37. E. L. R. Stokstad and J. Koch. Physiol. Rev. (1967) 47, 83.

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

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

40. K. Uyeda and J. C. Rabinowitz. Anal. Biochem. (1963) 6, 100.

41. C. K. Mathews and F. M. Huennekens. J. Biol. Chem. (1960), 235, 3304.

42. R. L. Blakley. Biochem. J. (1957) 65, 331.

43. A. Miller and H. Waelsch. J.Biol.Chem.(1957) 228,397.

44. M. May, T. J. Bardos, F. L. Barger, M. Lansford, J. M. Ravel, G. L. Sutherland and W. Shive. J. Am. Chem. Soc. (1951) 73, 3067.

45. M. Silverman and J. M. Noronha. Biochem. Biophys. Res. Commun. (1961) 4, 180.

46. K. G. Scrimgeour and K. S. Vitols. Biochemistry (1966) 5, 1438.

47. M. Friedkin, E. J. Crawford and D. Misra. Fed. Proc. (1962) 21, 176.

48. K. Smith, K. G. Scrimgeour and F. M. Huennekens. Biochem. Biophys. Res. Commun. (1963) 11, 388.

49. C. K. Mathews and F. M. Huennekens. J. Biol. Chem. (1963) 238, 4005.

50. S. F. Zakrzewski. Fed. Proc. (1963) 22, 231.

51. E. J. Pastore, M. Friedkin and O. Jardetzky. J. Am. Chem. Soc. (1963) 85, 3058.

52. S. F. Zakrzewski. J. Biol. Chem. (1966) 241, 2962.

53. A. Albert. Nature (1956) 178, 1072.

54. E. L. Wittle, B. L. O'Dell, J. M. Vandenbilt and J. J. Pfiffner. J.Am.Chem.Soc. (1947) 69, 1786.

55. R. L. Dion and T. L. Loo. J.Org.Chem.(1961) 26, 1857.

56. S. F. Zakrzewski and C. A. Nichol. Fed. Proc. (1956) 15, 390.

57. D. Chippel and K. G. Scrimgeour. Can. J. Biochem.(1970) 48, 999.

58. R. Stocks-Wilson, Ph.D. Thesis. University of Aston in Birmingham (1971).

59. J. M. Whitely, J. Drais, J. Kirchner and F. M. Huennekens. Arch. Biochem Biophys. (1968). 126, 955.

60. D. R. Seeger, D. B. Cosulich, J. M. Smith, Jr., and M. E. Hultquist. J.Am.Chem.Soc.(1949), 71, 1753.

61. A. Albert, D. J. Browne and G. Cheeseman. J. Chem. Soc. (1952), 4219.

62. A. Albert and S. Matsuura. J. Chem. Soc. (1962), 2162.

63. P. R. Brook and G. R. Ramage. J.Chem.Soc. (1957), 1.

64. M. Viscontini and H. R. Weilenmann. Helv.Chim.Acta (1958) 41, 2170.

65. S. Kaufman, J. Biol. Chem. (1964) 239, 332.

66. E. C. Taylor and W. R. Sherman. J. Am. Chem. Soc. (1959) 81, 2464.

67. A. Stuart, D. W. West and H. C. S. Wood. J. Chem. Soc. (1964), 4769.

68. J. A. Blair and A. J. Pearson. Tetrahedron Letts. (1973), 203.

69. H. Rembold, H. Metzger, P. Sudershan and W. Gutensohn. Biochim. Biophys. Acta. (1969) 184, 386.

70. H. Rembold, H. Metzger, and W. Gutensohn. Biochim. Biophys. Acta (1971) 230, 117.

71. M. Viscontini and M. Argentini. Liebigs Ann. Chem. (1971) 745, 109.

72. M. Viscontini and M. Cogoli-Greuter. Helv. Chim. Acta. (1971), 54, 1125.

73. M. Viscontini and T. Okada. Helv. Chim. Acta (1969) 52, 306.

74. A. W. Schreker and F. M. Huennekens. Biochem. Pharmacol. (1964) 13, 731.

75. J. R. Bertino, J. P. Perkins and D. G. Johns.
Biochemistry (1965) 4, 839.

76. R. L. Blakley. Biochem. J. (1960) 74, 71.

77. B. V. Ramasastri and R. L. Blakley. J. Biol. Chem.
(1964) 239, 106.

78. J. B. Wittenberg, J. M. Noronha and M. Silverman.
Biochem. J. (1962) 85, 9.

79. S. Kaufman. J. Biol. Chem. (1961) 236, 804.

80. D. J. Vonderschmitt and K. G. Scrimgeour.
Biochem. Biophys. Res. Commun. (1967) 28, 302.

81. M. C. Archer and K. G. Scrimgeour.
Can. J. Biochem. (1970) 48, 526.

82. M. C. Archer and K. G. Scrimgeour.
Can. J. Biochem. (1970) 48, 278.

83. M. C. Archer, D. J. Vonderschmitt and K. G. Scrimgeour.
Can. J. Biochem. (1972) 50, 1174.

84. W. Jaenicke and K. Kretzschmar.
Experientia Suppl. (1972) 18, 375.

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

86. A. Ehrenberg, P. Hemmerich, F. Muller, T. Okada and M. Viscontini. Helv.Chim.Acta (1967) 50, 411.

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

88. M. Viscontini and H. R. Weilenmann. Helv. Chim. Acta. (1959) 42, 1854.

89. E. G. E. Hawkins. "Organic Peroxides" E. and F.F. Spon Ltd., London (1961).

90. F. M. Huennekens. Biochemistry (1963) 2, 151.

91. M. Viscontini and A. Bobst. Helv.Chim.Acta (1965) 48, 816.

92. M. Viscontini and T. Okada. Helv. Chim. Acta (1967) 50, 1492.

93. M. Viscontini and T. Okada. Helv. Chim. Acta (1967) 50, 1845.

94. M. Viscontini and M. Argentini. Helv. Chim. Acta (1971) 54, 2287.

95. H.I.X. Mager and W. Berends. Rec. Trav. Chim. (1965) 84, 1329.

96. H.I.X. Mager and W. Berends. Biochim. Biophys. Acta (1966) 118, 440.

97. H.I.X. Mager and W. Berends. Rec. Trav. Chim. (1972) 91, 1137.

98. H.I.X. Mager, R. Addink and W. Berends. Rec. Trav. Chim. (1967) 86, 833.

99. H.I.X. Mager and W. Berends. Rec. Trav. Chim. (1972) 91, 611.

100. H.I.X. Mager and W. Berends. Rec. Trav. Chim. (1972) 91, 630.

101. S. Kaufman. Ann. Rev. Biochem. (1967) 36, 171.

102. W. F. Coulson, M. J. Powers and J. B. Jepson. Biochim. Biophys. Acta (1970) 222, 606.

103. W. B. Watt. J. Biol. Chem. (1972) 247, 1445.

104. B. L. Hillcoat, P. F. Nixon and R. L. Blakley. Anal. Biochem. (1967) 21, 178.

105. J. M. Whitely and F. M. Huennekens. Biochemistry (1967) 6, 2620.

106. B. P. Tong, Roche Products Ltd., Personal Communication.

107. G. K. Cain, M. F. Mallette and E. C. Taylor, Jr. J. Am. Chem. Soc. (1946) 68, 1996.

108. R. M. Johnson and I. W. Siddiqi "The Determination of Organic Peroxides" Pergamon Press, Monographs in Organic Functional Group Analysis Vol. 4 (1970) Chapter 11.

109. B. J. R. Nicolaus. J. Chromatog. (1960) 4, 384.

110. W. F. Linke and A. Seidell "Solubilities of Inorganic and Metal Organic Compounds" Vol. 2, 4th Ed. Am. Chem. Soc. (1965).

111. P. George. Trans. Faraday Soc. (1946) 42, 210.

112. M. Ardon. "Oxygen" Benjamin (1965).

113. J. A. Blair and A. J. Pearson. J. Chem. Soc. Perkin II (1974), 80.

114. A. J. Pearson. Chem. & Ind. (1974), 233.

115. W. A. Waters "The Chemistry of Free Radicals" Clarendon Press (1946) p. 232 and following.

116. J. L. Bolland and P. ten Have. Trans. Faraday Soc. (1947) 43, 201.

117. I. T. Brownlie and K. U. Ingold. Can. J. Chem. (1966) 44, 861.

118. J. A. LuValle and A. J. Weissberger. J. Am. Chem. Soc. (1949) 69, 1821.

119. F. G. R. Gimblett "Introduction to the Kinetics of Chemical Chain Reactions" McGraw-Hill (1970).

120. W. A. Waters. "Mechanisms of Oxidation of Organic Compounds" Methuen (1964) P p. 40.

121. L. Bateman. Quart. Rev. (1954) 8, 147.

122. T. Yano, T. Suetaka and T. Umekara Nippon Kagaku Kaishi (1972), 2194.

123. D. L. Allara. J. Org. Chem. (1972) 37, 2448.

124. P. K. Adolf and G. A. Hamilton. J. Am. Chem. Soc. (1971) 93, 3420.

125. M. M. T. Kahn and A. E. Martell. J. Am. Chem. Soc. (1967) 89, 4176.

126. M. M. T. Kahn and A. E. Martell. J. Am. Chem. Soc. (1967) 89, 7104.

127. A. Weissberger, J. E. LuValle and D. S. Thomas, Jr. J. Am. Chem. Soc. (1943) 65, 1934.

128. A. Weissberger and J. E. LuValle. J. Am. Chem. Soc. (1944) 66, 700.

129. A. E. Martell. "Stability Constants of Metal-ion Complexes". Chem. Soc. Special publication No.17 (1964).

130. H. Taube "Oxygen" Little, Brown and Co., Boston, Mass. (1965).

131. J. L. Bolland. Quart. Rev. (1949) 3, 1.

132. G. A. Russell. J. Chem. Educ. (1959) 36, 111.

133. A. A. Miller and F. R. Mayo. J. Am. Chem. Soc. (1956) 78, 1017.

134. A. A. Frost and R. G. Pearson. "Kinetics and Mechanism" 2nd Edition, Wiley International (1961) p. 249.

135. N. Kulevsky, P. J. Sneeringer and V. I. Stenberg. J. Org. Chem. (1972) 37, 438.

136. J. A. LuValle and A. J. Weissberger. J. Am. Chem. Soc. (1949) 69, 1567.

137. J. A. LuValle and A. J. Weissberger. J. Am. Chem. Soc. (1949) 69, 1576.

138. R. G. Kallen and W. P. Jencks. J. Biol. Chem. (1966) 241, 5845.

139. R. S. Davidson. Quart. Rev. (1967) 21, 249.

140. K. J. Laidler "Chemical Kinetics" 2nd Ed. McGraw-Hill (1965).

141. U.S. Department of Commerce National Bureau of Standards. Circular 514 (1951).

142. M. Stewart and C. H. Nicholls. Australian J. Chem. (1972) 25, 2139.

143. R. F. Bartholomew and R. S. Davidson. J. Chem. Soc. C (1971), 2342.

144. E. G. E. Hawkins. J. Chem. Soc. Perkin I (1972), 13.

145. A. J. Robb, unpublished data, University of Aston in Birmingham.

146. W. E. Doering and J. D. Chanley. J. Am. Chem. Soc. (1946) 68, 586.

147. W. E. Doering and R. M. Haines. J. Am. Chem. Soc. (1954) 76, 482.

148. E. J. Bailey, J. Elks and D. H. R. Barton. Proc. Chem. Soc. (1960), 214.

149. H. R. Gersmann and A. F. Bickel. J. Chem.Soc.(1959), 2711.

150. M. S. Karasch and B. S. Joshi.
J. Org. Chem. (1957) 22, 1439.

151. A. Nishinaga and T. Matsuura. Chem. Commun. (1973),9.

152. J. O. Hawthorne, K. A. Schowalter, A. W. Simpson, M. H. Wilt, and M. S. Morgan in "Oxidation of Organic Compounds", Vol. 1., Advances in Chemistry Series No. 75, ed. R. F. Gould.
Am. Chem. Soc. (1967) p. 203.

153. K. Ingold. Acc. Chem. Res. (1969) 2, 1.

154. C. Walling and G. M. El-Taliavi.
J. Am. Chem. Soc. (1973) 95, 844.

155. J. Weiss. Nature (1934) 133, 648.

156. J. H. Baxendale and S. Lewin. Trans. Faraday Soc. (1946) 42, 126.

157. J. L. Bolland and H. R. Cooper. Proc. Roy. Soc. A (1954) 225, 405.

158. M. G. Evans, N. S. Hush and N. Uri.
Quart. Rev. (1952) 6, 186.

159. D. H. Reid. Chem. & Ind. (1956), 1504.

160. D. A. Blackadder and C. Hinshelwood.
J. Chem. Soc. (1957), 2898.

161. J. A. Howard and K. U. Ingold. Can. J. Chem.
(1967) 45, 785.

162. R. O. C. Norman and P. R. West.
J. Chem. Soc. B. (1969), 389.

163. J. F. Corbett. J. Soc. Cosmet. Chem. (1972)
23, 683.

164. B. H. J. Bielski and P. C. Chan.
Arch. Biochem. Biophys. (1973) 159, 873.

165. H. Gutfreund. Biochem. J. (1960) 74, 17P.

166. E. Saito and B. H. J. Bielski.
J. Am. Chem. Soc. (1961) 83, 4467.

167. Q. H. Gibson and J. W. Hastings.
Biochem. J. (1962) 83, 368.

168. V. Massey, F. Muller, R. Feldberg, M. Schuman,
P. A. Sullivan, L. G. Howell, S. G. Mayhew,
R. G. Matthews and G. P. Foust.
J. Biol. Chem. (1969) 244, 3999.

169. D. Ballou, G. Palmer and V. Massey.
Biochem. Biophys. Res. Commun.(1969) 36, 891.

170. P. F. Knowles, J. F. Gibson, F. M. Pick and R. C. Bray. Biochem. J. (1969) 111, 53.

171. V. Massey, G. Palmer and D. Ballou in "Flavins and Flavoproteins" Proc. 3rd Int. Symposium, ed. H. Kamin, University Park Press, Baltimore (1971) p. 349.

172. C. F. Koelsch. J. Am. Chem. Soc. (1957) 79, 4439.

173. G. M. Coppinger. J. Am. Chem. Soc. (1957) 79, 501.

174. J. M. McCord and I. Fridovich. J. Biol. Chem. (1968) 243, 5753.

175. J. M. McCord and I. Fridovich. J. Biol. Chem. (1969) 244, 6049.

176. I. Fridovich. Acc. Chem. Res. (1972) 5, 321.

177. H. P. Misra and I. Fridovich. J. Biol. Chem. (1972) 247, 3170.

178. S. Kaufman and D. B. Fisher. J. Biol. Chem. (1973) 248, 4300.

179. D. Behar, G. Czapski, J. Rabani, L. M. Dorfman and H. A. Schwarz. J. Phys. Chem. (1970) 74 , 3209.

180. P. Hemmerich, V. Massey and G. Weber.
Nature (1967) 213, 728.

181. G. R. Gapski, J. M. Whitely and F. M. Huennekens.
Biochemistry (1971) 10, 2930.

182. J. A. Blair and A. J. Pearson. Tetrahedron Letts.
(1973), 1681.

183. H.I.X. Mager and W. Berends. Tetrahedron Letts.
(1973), 4051.

184. B. Pullman and A. Pullman "Quantum Biochemistry"
Interscience (1963) Chapter 8.

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

186. B. Neely. Mol. Pharmacol. (1967) 3, 108.

187. A. Bobst. Helv. Chim. Acta (1967) 50, 1480.

188. A. Streitwieser "Molecular Orbital Theory for
Organic Chemists" Wiley (1961).

189. A. Wheway, Unpublished data, University of Aston
in Birmingham.

190. J. R. Bolton, A. Carrington and J. dos Santos-Veiga. Mol. Physics (1962) 5, 465.

191. P. George in "Oxidases and Related Redox Systems" Vol. 1., ed. T.E. King, H.S. Mason and M. Morrison (1964) p. 3.

192. K. Scully. B.Sc. Project Report, University of Aston in Birmingham. (1973).

193. S. Futterman in "Methods in Enzymology" Vol. 6, ed. S. P. Colowick and N. O. Kaplan. Academic Press, New York (1963) p. 801.

194. J. A. Lyon, P. D. Ellis and R. B. Dunlap. Biochemistry (1973) 12, 2425.

195. H. Y. Neujahr and A. Gaal. Eur. J. Biochem (1973) 35, 386.

196. D. B. Fisher, R. Kirkwood and S. Kaufman. J. Biol. Chem. (1972) 247, 5161.

197. H. Rembold and M. Osanai. Hoppe-Seyler's Z. Physiol. Chem. (1971) 352, 1359.

198. J. E. Ayling, G. R. Boehm, S. C. Textor and R. A. Pirson. Biochemistry (1973) 12, 2045.

199. M. Ikeda, M. Levitt and S. Udenfriend.
Biochem. Biophys. Res. Commun. (1965) 18, 482.

200. R. J. Taylor, Jr., C.S. Stubbs, Jr., and
L. Ellenbogen. Biochem. Pharmacol. (1968) 17, 1779.

201. R. J. Taylor, Jr., C.S.Stubbs, Jr., and
L. Ellenbogen. Biochem. Pharmacol. (1969) 18, 587.

202. M. Ikeda, L.A. Fahien and S. Udenfriend.
J. Biol. Chem. (1966) 241, 4452.

203. C. J. Sih. Science (1969) 163, 1297.

204. G. Guroff, C. A. Reifsnyder and J. W. Daly.
Biochem. Biophys. Res. Commun. (1966) 24, 720.

205. G. Guroff, M. Levitt, J. W. Daly and S. Udenfriend.
Biochem. Biophys. Res. Commun. (1966) 25, 253.

206. F. Haber and J. Weiss. Proc. Roy. Soc. A (1934) 147,
332.

207. J. H. Baxendale, M. G. Evans and G. S. Park.
Trans. Faraday Soc. (1946) 42, 155.

208. J. H. Mertz and W. A. Waters. J. Chem.Soc.(1949) S,15.

209. W. T. Dixon and R. O. C. Norman. Nature (1962) 196,891.

210. J. R. Lindsay Smith and R. O. C. Norman.
J. Chem. Soc. (1963), 2897.

211. L. N. Dorfman, I. A. Taub and R. E. Buhler.
J. Chem. Phys. (1962) 36, 3051.

212. D. R. Augood, D. H. Hey, A. Nechvatal and G. H. Williams. Nature (1959) 167, 725.

213. R. O. C. Norman and G. K. Radda.
Proc. Chem. Soc. (1962) 138.

214. C. Nofre, A. Cier, C. Michon-Saucet and J. Parnet.
Compt. Rend. (1960) 251, 811.

215. C. Nofre, J. P. Charrier and A. Cier.
Bull. Soc. Chim. Biol. (1963) 45, 913.

216. R. L. Wilson. Int. J. Radiat. Biol.(1972) 21, 401.

217. D. M. Jerina, J. W. Daly, W. Landis, B. Witkop and S. Udenfriend. J.Am.Chem.Soc. (1967) 89, 3347.

218. B. B. Brodie, J. Axelrod, P. A. Shore and S. Udenfriend. J. Biol. Chem. (1954) 208, 741.

219. H. S. Mason and I. Onoprienko. Fed. Proc. (1956) 15, 310.

220. H. S. Mason in "Advances in Enzymology" Vol. 19. ed. F.F. Nord, Interscience, New York, N.Y., (1957) p. 79.

221. R. Breslow and L. N. Lukens. J. Biol. Chem. (1960) 235, 292.

222. R. R. Grinstead. J. Am. Chem. Soc. (1960) 82, 3472.

223. G. A. Hamilton, R. J. Workman and L. Woo. J. Am. Chem. Soc. (1964) 86, 3390.

224. G. A. Hamilton. J. Am. Chem. Soc. (1964) 86, 3391.

225. R. O. C. Norman and J. R. Lindsay Smith in "Oxidases and Related Redox Systems" Vol. 1. ed. T.E. King, H. S. Mason and M. Morrison, John Wiley & Sons (1964) p. 131.

226. J. L. Hoard, G. S. Smith and M. Lind in "Advances in the Chemistry of Coordination Compounds" ed. S. Kirschner, The Macmillan Co., N.Y., (1961) p. 296.

227. J. L. Hoard, M. Lind and J. V. Silverton. J. Am. Chem. Soc. (1961) 83, 2770

228. J. L. Hoard, C. H. L. Kennard and G. S. Smith. Inorg. Chem. (1961) 2, 1317.

229. F. Schmidt and W. Klinger.Z.Physiol. Chem. (1958) 310, 31.

230. C. E. Dalgliesh. Biochem. J. (1954) 58, xlv

231. C. E. Dalgliesh. Arch. Biochem. Biophys. (1955) 58, 214.

232. A. Bobst and M. Viscontini. Helv. Chim. Acta (1966) 49, 884.

233. L. I. Woolf, A. Jakubovic and E. Chan-Henry. Biochem. J. (1971) 125, 569.

234. G. Guroff, J.W. Daly, D. M. Jerina, J. Renson, B. Witkop, and S. Udenfriend. Science (1967) 157, 1524.

235. J. W. Daly, G. Guroff, D. M. Jerina, S. Udenfriend, and B. Witkop in "Oxidation of Organic Compounds" Vol. 3, Advances in Chemistry Series No. 77, ed. R. F. Gould, Am. Chem. Soc. (1968) p. 279.

236. J. W. Daly, D. M. Jerina and B. Witkop. Experientia (1972) 28, 1129.

237. D. M. Jerina. Chem. Tech. (1973), 120.

238. D. M. Jerina, J. W. Daly and B. Witkop. Biochemistry (1971) 10, 366.

239. D. M. Jerina, D. R. Boyd and J. W. Daly.
Tetrahedron Letts. (1970),457.

240. H. Hart. Acc. Chem. Res. (1971) 4, 337.

241. D. M. Jerina, J. W. Daly and B. Witkop.
J. Am. Chem. Soc. (1967) 89, 5488.

242. D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend.
Arch. Biochem. Biophys. (1968) 128, 176.

243. D. M. Jerina, J. W. Daly and B. Witkop.
J. Am. Chem. Soc. (1968) 90, 6523.

244. D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend.
J. Am. Chem. Soc. (1968) 90, 6525.

245. D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend.
Biochemistry (1970) 9, 147.

246. N. Kaubisch, J. W. Daly and D. M. Jerina.
Biochemistry (1972) 11, 3080.

247. D. R. Boyd, J. W. Daly and D. M. Jerina.
Biochemistry (1972) 11, 1961.

248. E. A. Fehnel J. Am. Chem. Soc.(1972) 94, 3961.

249. G. J. Kasperek and T. C. Bruice.
J. Am. Chem. Soc. (1972) 94, 198.

250. G. J. Kasperek, T. C. Bruice, H. Yagi, N. Kaubisch and D. M. Jerina.
J. Am. Chem. Soc. (1972) 94, 7876.

251. J. P. Lambooy. J. Am. Chem. Soc. (1954) 76, 133.

252. G. R. Clemo and F. K. Duxbury.
J. Chem. Soc. (1950), 1795.

253. J. P. Lambooy. J. Am. Chem. Soc. (1949) 71, 3758.

254. A. von Henglein, W. Karmann, W. Roebke and G. Beck.
Makromolekulare Chem. (1966) 92, 105.

255. L. M. Dorfman and G. E. Adams. Nat. Stand. Ref. Data Ser., Nat. Bur. Stand.(1973) No. 46, p.1.

256. M. B. Dearden, C.R.E. Jefcoate and J.R. Lindsay Smith in "Oxidation of Organic Compounds" Vol. 3, Advances in Chemistry Series No. 77, ed. R. F. Gould, Am. Chem. Soc. (1968) p. 260.

257. B. M. Hoffman, D. L. Diemente and F. Basolo.
J. Am. Chem. Soc. (1970) 92, 61.

258. J. Ellis, J. M. Pratt and M. Green.
J. Chem. Soc. Chem. Commun. (1973), 781.

259. J. Klevan, J. Peone and S. K. Madan.
J. Chem. Educ. (1973) 50, 670.

Tetrahedron Letters No. 3, pp 203 - 204, 1973. Pergamon Press. Printed in Great Britain.

A KINETIC STUDY OF THE AUTOXIDATION OF TETRAHYDROBIOPTERIN

J.A. Blair and A.J. Pearson

Department of Chemistry, University of Aston in Birmingham, Birmingham B4 7ET.

(Received in UK 5 December 1972; accepted for publication 14 December 1972)

The kinetics of autoxidation of tetrahydrobiopterin (1) in aqueous solution have been studied manometrically at 25°C using the initial rates method. The reaction has a 1:1 stoichiometry and is first order in (1) and oxygen (rate constant = 1.0 ± 0.1 l $mol^{-1}sec^{-1}$). Retardation by phenols and the absence of any effect of light suggests a free-radical chain reaction. A linear dependence of rate on per cent ionisation of the 3,4-amide group (pK = 10.5, estimated spectrophotometrically) is found on varying the solution pH. (Fig. 1).

Hydrogen peroxide has been reported in solutions of autoxidised tetrahydropterins[1,2], and e.s.r. studies[3,4,5] have shown the presence of free-radicals during oxidation.

This data is consistent with the mechanism shown, involving formation of transient hydroperoxides having the structure shown (IV) (atom 4a is the only carbon atom having unpaired electron density in radical (III)). Rapid loss of H_2O_2 and subsequent rearrangement of the quinonoid from (V) gives the observed first product, 7,8-dihydrobiopterin (VI) indicated by T.L.C. and characterised spectroscopically: U.V. : λ_{max}(nm.) pH 1, 267, 350; pH 7, 240, 280, 330; pH 13, 280, 330, cf. ref. 6. N.m.r : 2 - proton singlet τ 4.9, characteristic of 7,8-dihydropterins, CH_3 group at τ8.7, doublet J = 8 Hz, two CH groups at τ5.5 and τ6.1). This is then oxidised more slowly to give the final product, biopterin (VII)[1,7]. Present work in this laboratory indicates the same mechanism for other tetrahydropterins.

Tetrahydropterins are cofactors in enzymatic hydroxylation[6] of phenylalanine to tyrosine, a process in which hydroperoxides may have a significant role.

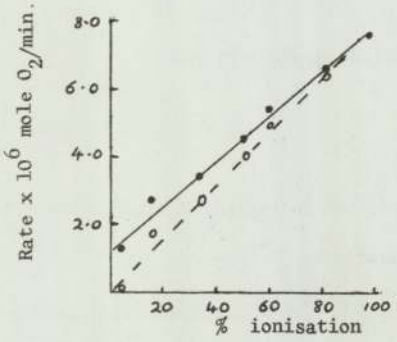
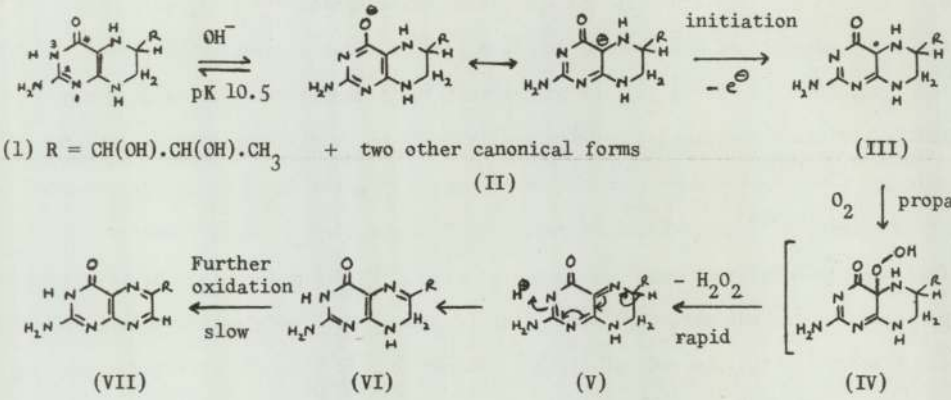


Fig 1

—— (a) 0.3 mM tetrahydrobiopterin solutions under pure O_2. pH varied using NaOH solutions of different concentrations. Intercept due to slow reaction of non-ionised compound.

- - - (b) Rates corrected for reaction of residual non-ionised compound.

Mechanism:



References:

1. R. Stocks-Wilson, Ph.D. Thesis, (1971) University of Aston in Birmingham.
2. H.I.X. Mager and W. Berends, Rec. Trav. Chem. 84, 1329 (1965).
3. P. Doyle, Unpublished data, University of Aston in Birmingham.
4. A. Bobst, Helv. Chim. Acta., 50, 2222 (1967).
5. A. Ehrenberg, P. Hemmerich, F. Müller, T. Okada and M. Viscontini, Helv. Chim. Acta., 50, 411 (1967).
6. R.L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines" North-Holland (1969).
7. H. Rembold, H. Metzger and W. Gutensohn, Biochim. Biophys. Acta, 230, 117 (1971).

Tetrahedron Letters No. 19, pp 1681 - 1682, 1973. Pergamon Press. Printed in Great Britain.

THE AUTOXIDATIVE RING CONTRACTION OF BLOCKED TETRAHYDROPTERIDINES: ORIGIN OF INTRODUCED OXYGEN FUNCTION.

J.A. Blair and A.J. Pearson

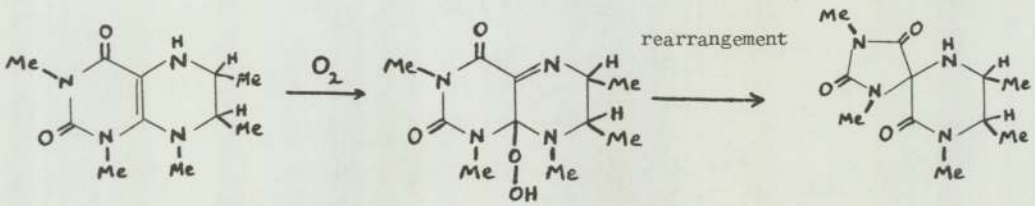
Department of Chemistry, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET.

(Received in UK 15 March 1973; accepted for publication 28 March 1973)

It has been established[1] that 1,3,6,7,8 - pentamethyl - 5,6,7,8 - tetrahydropteri - 2,4 - dione (I) undergoes a contraction of the pyrimidine ring, when autoxidised in water, to give 4,1',3' - trimethyl - 3 - oxopiperazine - 2 - spiro - 5' - hydantoin (III). This has been presented[1] as evidence for the intermediacy of transient 8a - hydroperoxides(II) in the autoxidation (scheme 1), a postulate which is not in agreement with kinetic data which we have reported[2].

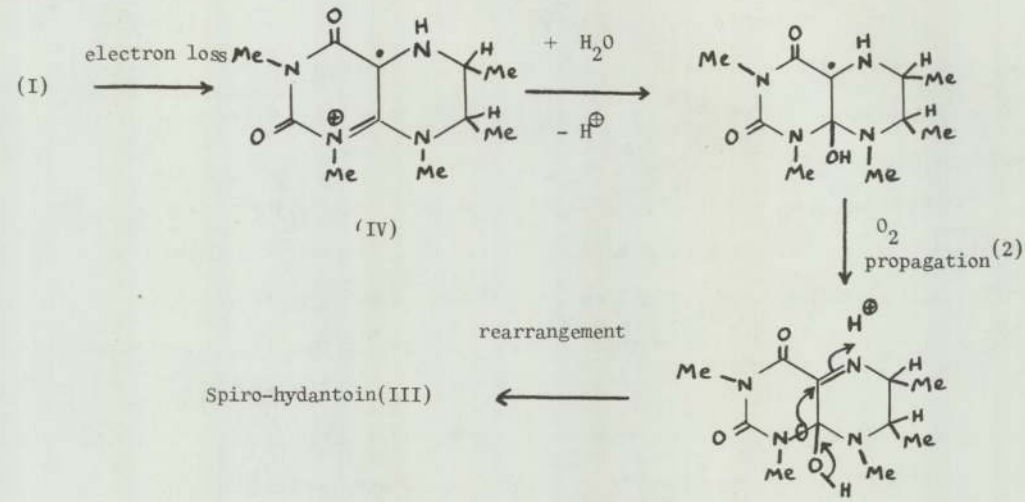
We have now shown conclusively that the new oxygen function introduced during the reaction is derived from the water used as solvent. The blocked pteridine (10 mg.) was dissolved in pyridine/water (10:1, 2 cm^3) and shaken under oxygen for two days, and the solvent was then removed under vacuum. The mass spectrum of the product corresponded to a mixture of spiro - hydantoin[1] (M^+ , $^m/_e$ = 254) and starting material (M^+ , $^m/_e$ = 238). The experiment was repeated using oxygen - 18 enriched water (43.46% enrichment), when the resulting mass spectrum showed that no isotopic exchange between the starting material amide groups and water had occurred (M^+ at $^m/_e$ = 238 obtained , but no peaks at $^m/_e$ 240 or 242). However, the spiro - hydantoin spectrum showed the incorporation of ^{18}O (M : M + 2 ratio 1.6 ; theoretical ratio, from water = ~~1.53~~ 1·3). Treatment of the labelled product with $H_2{}^{16}O$ did not lead to loss of label (shown by persistence of M + 2 at $^m/_e$ = 256). This demonstrates that the label is introduced by addition of water and not through exchange and this is not consistent with the mechanism proposed by Mager and Berends[1]. It is probable that the addition occurs at the radical - cation stage (IV) and not at the quinonoid stage, since no 7,8 - dihydropteridine is observed in the products[1], as would be expected from rearrangement of the quinonoid form (see, for example, reference 2). A reasonable mechanism is presented in scheme 2, not requiring the formation of 8a - hydroperoxides, and in which molecular oxygen is reduced to hydrogen peroxide.

Scheme 1 (Mager & Berends[1]):-



(I) (II) (III)

Scheme 2:-



Acknowledgements

Thanks are due to Prof. H.I.X. Mager for a generous gift of the blocked pteridine (I), and to Mr. M. Horton for mass spectra.

References.

(1) H.I.X. Mager and W. Berends, Rec. Trav. Chim. 91, 1137, (1972)

(2) J.A. Blair and A.J. Pearson, Tetrahedron Lett., No. 3, 203, (1973).

Kinetics and Mechanism of the Autoxidation of the 2-Amino-4-hydroxy-5,6,7,8-tetrahydropteridines

By **John A. Blair** and **Anthony J. Pearson,*** Department of Chemistry, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET

Reprinted from

JOURNAL
OF
THE CHEMICAL SOCIETY

PERKIN TRANSACTIONS II

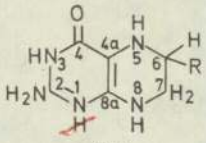
1974

Kinetics and Mechanism of the Autoxidation of the 2-Amino-4-hydroxy-5,6,7,8-tetrahydropteridines

By **John A. Blair** and **Anthony J. Pearson,*** Department of Chemistry, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET

The autoxidation of tetrahydrofolic acid and tetrahydrobiopterin, 6-substituted derivatives of 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridines (tetrahydropterins), has been investigated by product analysis and kinetic methods. The overall reaction is first order in tetrahydropterin and approximately first order in oxygen, with an activation energy of 58 kJ mol^{-1}. There is a linear dependence of rate on the percentage ionisation of the 3,4-amide group. Kinetic data are in accord with a free radical chain reaction in which the chain carrier is the hydroperoxyl radical (HO_2·), *not* alkylperoxyl radical, and propagation is accompanied by formation of a transient quinonoid dihydropterin intermediate, rearrangement of which results in the formation of the products described. Oxygen-18 labelling experiments show that the autoxidative ring contraction of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione does not provide evidence for the intermediacy of 8a-hydroperoxides during the reaction.

TETRAHYDROBIOPTERIN (1b) has been shown [1] to be the cofactor required during the enzymatic hydroxylation of phenylalanine to tyrosine, in which molecular oxygen is introduced as the hydroxy-group in tyrosine.[2] No



(1)

a; R = CH_2·NH–C$_6$H$_4$–CO·NH·CH(CO_2H)·$[CH_2]_2$·CO_2H

b; R = CH(OH)·CH(OH)·CH_3

detailed mechanism for the activation of molecular oxygen has been established, though it has been suggested [3-5] that transient hydroperoxide intermediates formed in autoxidation of the cofactor may be the source of the hydroxy-group. Although evidence for the intermediacy of quinonoid dihydropterins during oxidation has been presented,[6] the steps leading to this species have not been elucidated.

It has also been suggested [3,7] that autoxidation occurs by a chain reaction involving hydroperoxide formation at C(6) or C(7), a reasonable proposal considering that it is the reduced pyrazine ring of the molecule which appears to undergo oxidation.

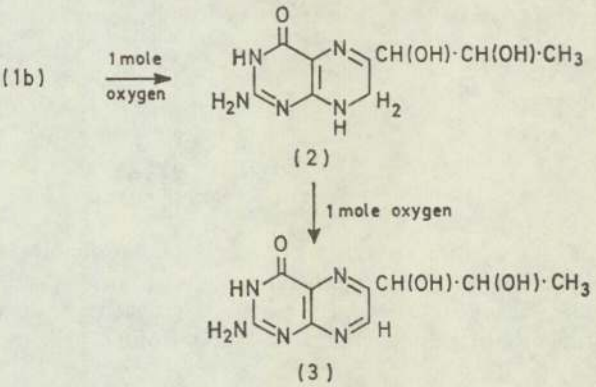
In an attempt to resolve the present controversy regarding the existence or non-existence of hydroperoxides in these autoxidations, the present study was undertaken, using tetrahydrobiopterin and tetrahydrofolic acid (1a) as model compounds.

RESULTS AND DISCUSSION

Products.—It has been shown previously [4,8] that the reaction pathway for tetrahydrobiopterin autoxidation at ambient temperatures involves formation of 7,8-dihydrobiopterin (2) followed by its subsequent oxidation to biopterin (3) (Scheme 1).

Tetrahydrofolic acid is known [9] to be oxidised to folic acid (5), xanthopterin (10), and pterin (8); 7,8-dihydrofolic acid (4), 7,8-dihydropterin (6), and dihydroxanthopterin (9) are recognised as intermediate compounds which are further autoxidised to the final products.†

The pH dependence of products from tetrahydrofolic acid autoxidation was investigated by Stocks-Wilson,[3] who determined the ratio of pterin : xanthopterin in the final products over a range of pH, by isolation on preparative paper chromatography. The results are shown in Table 1, and it will be noted that this ratio decreases drastically as the solution pH increases.



(1b) → (2) → (3)

SCHEME 1 Reaction pathway for autoxidation of tetrahydrobiopterin at 25°

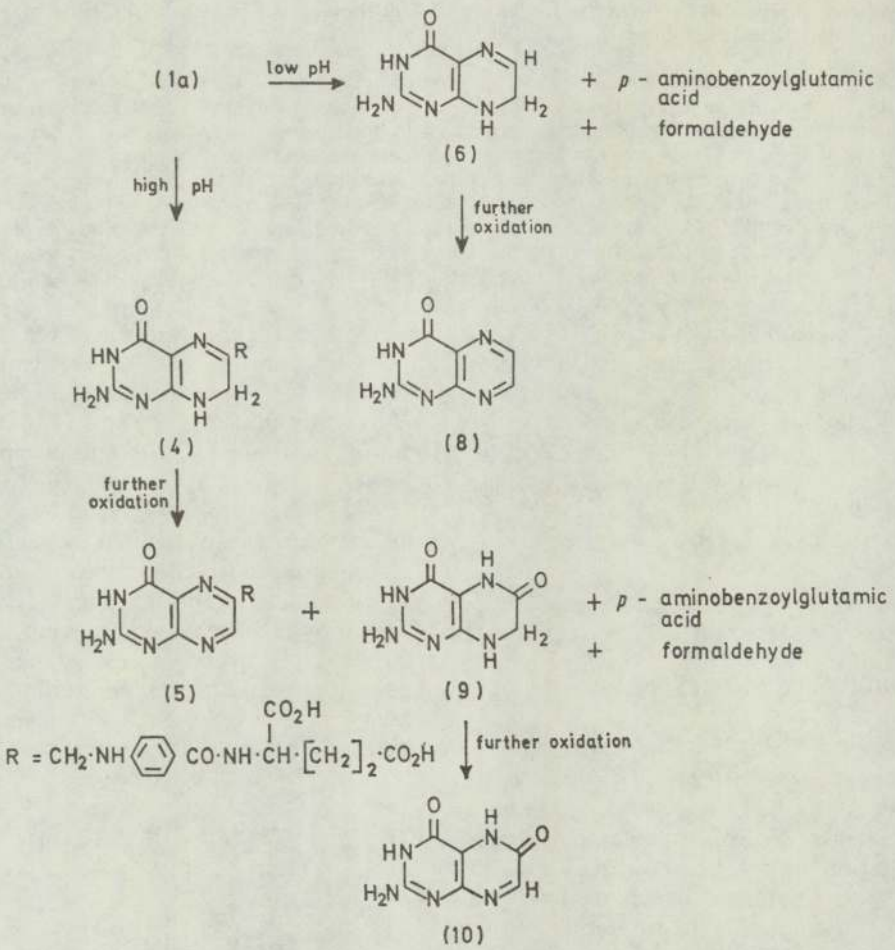
The reaction pathway has been further elucidated by determining the products at different times, using t.l.c., the results being shown in Table 2. It is clear that, at low pH, pterin or dihydropterin (inseparable on t.l.c.)

† Recent experiments show that, at 40°, autoxidation of tetrahydrobiopterin yields a more complex mixture of products among which appear to be xanthopterin and pterin (detectable on t.l.c.), so that it is not dissimilar to tetrahydrofolic acid regarding oxidation products.

[1] S. Kaufman, *J. Biol. Chem.*, 1959, **234**, 2683; *Proc. Nat. Acad. Sci., U.S.A.*, 1963, **50**, 1085.
[2] S. Kaufman, W. F. Bridgers, F. Eisenberg, and S. Friedman, *Biochem. Biophys. Res. Comm.*, 1962, **9**, 497.
[3] R. Stocks-Wilson, Ph.D. Thesis, University of Aston, 1971.
[4] J. A. Blair and A. J. Pearson, *Tetrahedron Letters*, 1973, 203.
[5] H. I. X. Mager and W. Berends, (*a*) *Rec. Trav. chim.*, 1965, **84**, 1329; (*b*) *Biochim. Biophys. Acta*, 1966, **118**, 440; (*c*) *Rec. Trav. chim.*, 1967, **86**, 833; (*d*) *ibid.*, 1972, **91**, 1137.
[6] (*a*) M. C. Archer and K. G. Scrimgeour, *Canad. J. Biochem.*, 1970, **48**, 278; (*b*) S. Kaufman, *J. Biol. Chem.*, 1961, **236**, 804; 1964, **239**, 332.
[7] E. G. E. Hawkins, ' Organic Peroxides,' Spon, London, 1961, p. 401.
[8] H. Rembold, H. Metzger, and W. Gutensohn, *Biochim. Biophys. Acta*, 1971, **230**, 117.
[9] D. Chippel and K. G. Scrimgeour, *Canad. J. Biochem.*, 1970, **48**, 999.

arise directly from oxidative cleavage of the tetrahydrofolic acid. At higher pH, formation of xanthopterin is preceded by production of dihydrofolic acid, and is a peroxide, some kinetic studies were undertaken which go some way towards elucidating the detailed mechanism.

Kinetic Studies.—Oxygen uptake curves were analysed



SCHEME 2 Reactions pathway for autoxidation of tetrahydrofolic acid at 23°

known oxidation product of the latter, probably *via* dihydroxanthopterin.[9] Therefore the pterin : xanthopterin ratios recorded also reflect the relative rates of formation of dihydropterin and dihydrofolic acid, and the overall reaction pathway can be written as in Scheme 2.

TABLE 1

pH-Dependence of pterin : xanthopterin ratio in oxidation products of tetrahydrofolic acid at ambient temperatures

Buffer and pH	Pterin : xanthopterin ratio
Dilute acetic acid; pH 3	12·64 : 1·00
0·1M-Ammonium acetate; pH 7	1·00 : 1·90
NaOH–$NaHCO_3$; pH 10	1·00 : 17·38

Since most 'normal' autoxidations proceed *via* the formation of hydroperoxide intermediates, attempts were made to detect these on t.l.c. at low temperatures. However, the only peroxide detectable was hydrogen peroxide (see Experimental section). Since it can be argued that such intermediates from tetrahydropterins are very unstable, undergoing easy loss of hydrogen by the method of initial rates (following any induction period), thus overcoming difficulties due to oxidation o

TABLE 2

T.l.c. investigation of reaction pathway during tetrahydrofolic acid autoxidation

Oxidation medium and pH	Sampling time	Observable products (by comparison with known standards)
Dilute AcOH; pH 3	1·5 h	Pterin or dihydropterin (not distinguishable)
	39 h	Pterin or dihydropterin
0·1M-Phosphate; pH 7	40 min	Dihydrofolic acid (major); pterin (strong spot)
	24 h	Suspected 6-formyl-7,8-dihydropterin (minor, from dihydrofolic acid oxidation); pterin (strong spot)
NaOH; pH 11	40 min	Dihydrofolic acid (major); dihydroxanthopterin (faint); pterin (faint)
	24 h	Xanthopterin (major); pterin (faint)
NaOH; pH 13	35 min	Dihydrofolic acid
	24 h	Xanthopterin

initial products, and interference from secondary reactions with the hydrogen peroxide generated. The manometric technique was employed rather than more accurate spectrophotometric methods, owing to the fact that spectra are affected greatly by the solution pH. In fact, there is very little apparent oxidation of tetrahydrofolic acid at pH 13 if examined spectrophotometrically, due to the almost identical spectra of products and starting material. The results below show that, in fact, oxidation is more rapid at higher pH.

In 0·1M-phosphate buffer at pH 7 the reaction is first order in tetrahydropterin (Figure 1) and approximately first order in oxygen concentration (both light and dark rates) although a small zero-order dependence on

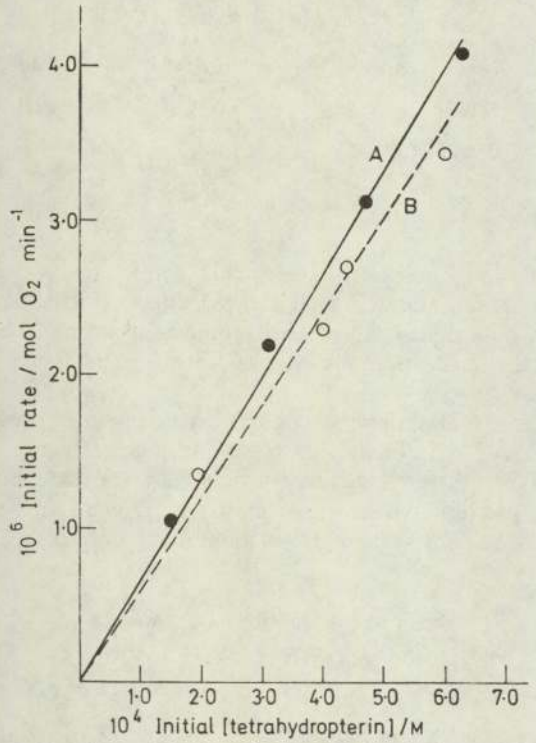


FIGURE 1 First-order plots for tetrahydropterin autoxidation (phosphate buffer; pH 7; pure oxygen; 25°): A, tetrahydrobiopterin (light rates); B, tetrahydrofolic acid (dark rates)

oxygen was also obtained (Figure 2), this possibly being due to initiation by trace impurities. Under these conditions the overall rate law is of the form (1) where

$$\text{Rate} = k_a[H_4\text{Pter.}](1 + k_b[O_2]) \qquad (1)$$

H_4Pter. represents tetrahydropterin. Values for k_a and k_b have not been estimated.

The apparent activation energy (not corrected for oxygen solubility changes) for both compounds is 58 kJ mol^{-1} (Figure 3).

The reaction rate is retarded by a factor of *ca.* 2 by phenols, which are known chain-breaking inhibitors, is catalysed by small concentrations of copper ions, and shows a very small reduction in rate when light is excluded (Table 3). The changes in rate in various buffer solutions are probably due to copper impurities present, as shown by the data for ammonium acetate solutions (Table 3); atomic absorption analysis of a

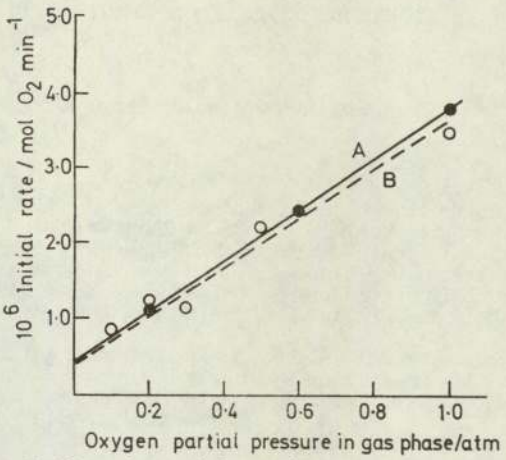


FIGURE 2 Dependence of O_2 uptake rate on oxygen concentration (0·1M-phosphate buffer; pH 7; 25°): A, 0·625mM-tetrahydrobiopterin dark rates; B, 0·7mM-tetrahydrofolic acid light rates

sample of 2·5M-ammonium acetate solution showed the presence of $1{\cdot}4 \times 10^{-6}$ g-atom l^{-1} copper, whereas 0·1M-phosphate and 0·1M-sodium hydroxide contain only $0{\cdot}8 \times 10^{-6}$ and $0{\cdot}6 \times 10^{-6}$ g-atom l^{-1} respectively. EDTA was found to eliminate the catalysis.

Protonation of N(5) has been shown to lead to a decrease in rate, due to decrease in electron density in the pteridine ring by removal of the N(5) lone pair from

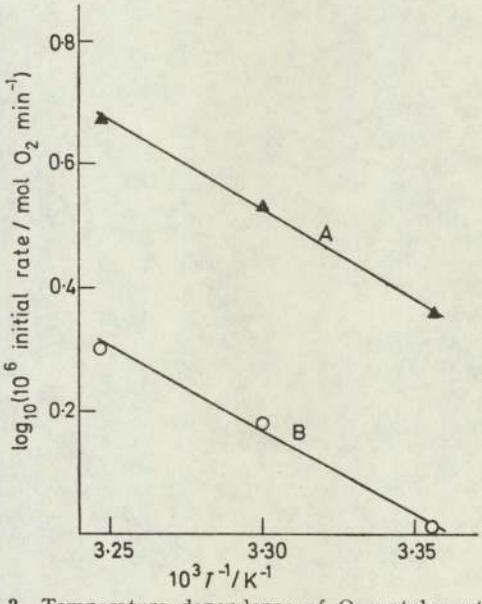


FIGURE 3 Temperature dependence of O_2 uptake rates (uncorrected). 'Arrhenius' plots for oxidations under air: A, 0·625mM-terahydrobiopterin in 0·1M-ammonium acetate buffer; B, 0·7mM-tetrahydrofolic acid in 0·1M-phosphate buffer

conjugation. We have observed [4] a large increase in rate of oxygen uptake on increasing the solution pH from 9 to 13, the region in which ionisation of the 3,4-

amide group occurs.[10] Using pK_a values for this group (tetrahydrobiopterin 10·5; tetrahydrofolic acid 10·8), estimated spectrophotometrically, and assuming a rate equation (at constant oxygen concentration) of the form (2) the first term on the right-hand side due to un-ionised compound can be estimated at each pH, and

$$\text{Rate} = k_c[\text{non-ionised } H_4\text{Pter.}] + k_d[\text{Imino-enolate anion}] \quad (2)$$

the overall rate corrected by subtraction of this. A plot of corrected rate against percent ionisation of the amide group gives an excellent straight line (Figure 4), showing that equation (2) is correct. Furthermore, the plot for 6,7-dideuteriated tetrahydrofolic acid coincides with that for the non-deuteriated compound, showing that homolysis of neither of these C–H bonds occurs during the reaction, and also that the pH effect is attributable to no heterolysis other than amide ionisation. Therefore, the suggestions by Stocks-Wilson [3] and Hawkins [7] are not correct.

TABLE 3

Additive effects on autoxidation rates of tetrahydrofolic acid and tetrahydrobiopterin at 25°

Reactant	Buffer solution	Gas phase	Additive or other modification	10^6 Initial rate (mol O_2 min^{-1})
Tetrahydrofolic acid (0·7 mM)	0·1M-Phosphate; pH 7	Air	None	1·10
Tetrahydrofolic acid (0·7 mM)	0·1M-Phosphate; pH 7	Air	3×10^{-4}M-phenol	0·60
Tetrahydrofolic acid (0·7mM)	0·1M-Phosphate; pH 7	Air	10^{-4}M-Cu^{2+}	5·50
Tetrahydrofolic acid (0·7 mM)	0·1M-Phosphate; pH 7	Air	10^{-4}M-Cu^{2+} + 0·24mM-EDTA	1·0
Tetrahydrobiopterin (0·31mM)	0·1M-Phosphate; pH 7	Pure oxygen	None	2·18
Tetrahydrobiopterin (0·31mM)	0·1M-Phosphate; pH 7	Pure oxygen	0·02M-EDTA	2·13
Tetrahydrobiopterin (0·31mM)	0·1M-Phosphate; pH 7	Pure oxygen	0·002M-8-Hydroxyquinoline-5-sulphonic acid	1·22
Tetrahydrobiopterin (0·31mM)	0·1M-Phosphate; pH 7	Pure oxygen	0·002M-Phenol	1·16
Tetrahydrobiopterin (0·31mM)	0·1M-Phosphate; pH 7	Pure oxygen	Light excluded	2·0
Tetrahydrobiopterin (0·625mM)	0·1M-Ammonium acetate	Air	None	2·30
Tetrahydrobiopterin (0·625mM)	2·5M-Ammonium acetate	Air	None	4·50
Tetrahydrobiopterin (0·625mM)	2·5M-Ammonium acetate	Air	0·24mM-EDTA	1·60
Tetrahydrobiopterin (0·31mM)	0·1M-Sodium hydroxide	Pure oxygen	None	7·5
Tetrahydrobiopterin (0·31mM)	0·1M-Sodium hydroxide	Pure oxygen	0·24mM-EDTA	8·0

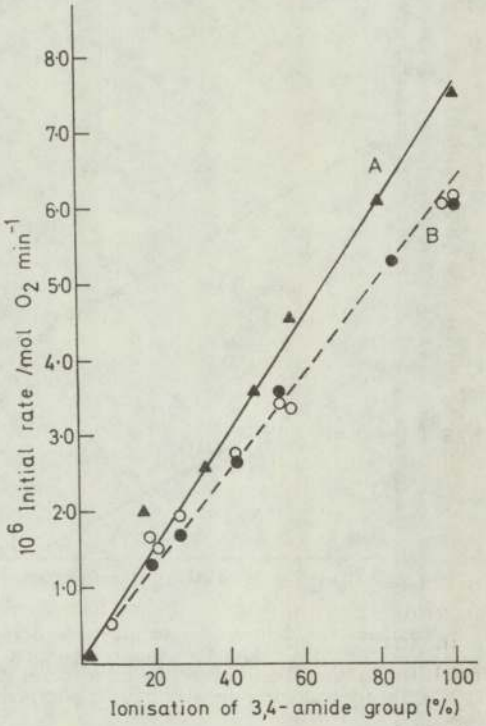


FIGURE 4 Dependence of corrected rates on ionisation of the 3,4-amide group under pure oxygen at 25°: A, 0·31mM-tetrahydrobiopterin (pK_a 10·5); B, 0·4mM-tetrahydrofolic acid (○) and 0·4mM-6,7-dideuteriated tetrahydrofolic acid (●) (pK_a 10·8)

These results, particularly the involvement of ground-state oxygen, indicate that the oxidation is a free radical chain reaction. The observation that the rate is the same (within experimental error) in D_2O as in water (Table 4) shows there is no homolysis of the N(5)–H bond involved, so the reaction occurs by electron removal, followed by rapid proton loss from N(3) to give the free radical (11). This conclusion is supported by the linear relationship between rate and imino-enolate concentration. A parallel is indicated between the canonical forms for this anion and the free radical as shown in Scheme 3, further stabilisation being gained by conjugation with the N(5) lone pair. The structure

TABLE 4

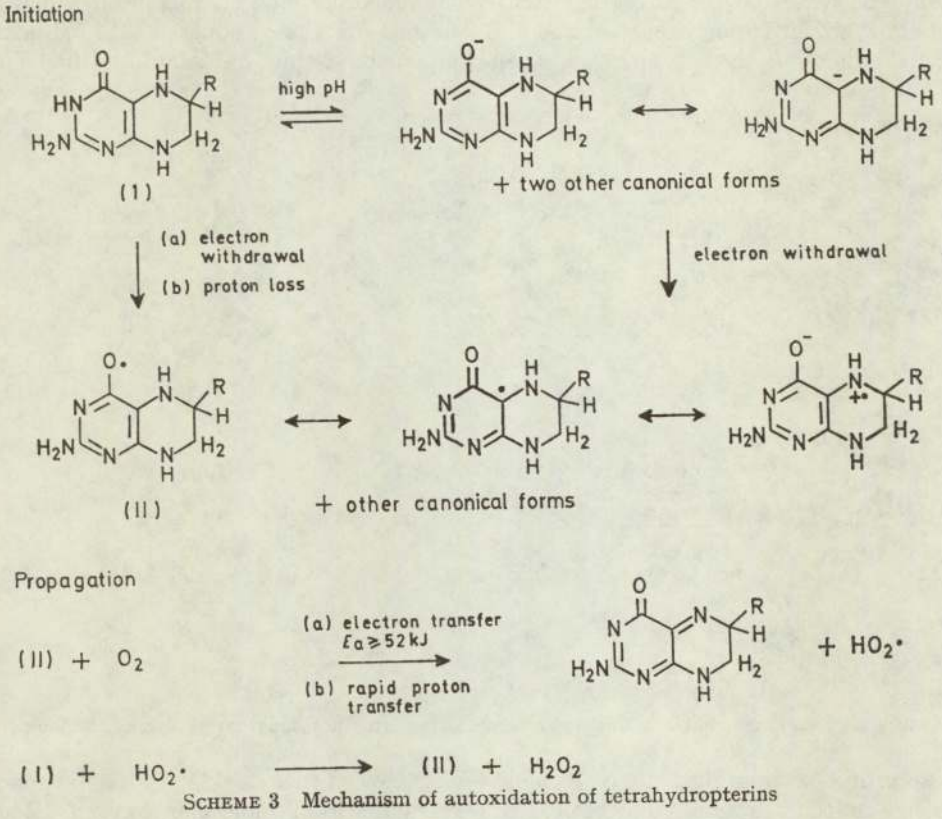
Free-radical kinetic characteristics

Buffer	Gas phase	Steady-state O.D. at 550 nm	10^6 Steady-state O_2 uptake rate (mol min^{-1})	$k_2(O_2)$ (See text)	Temp. (°C)
0·1M-Ammonium acetate	Pure oxygen	0·290			25
0·1M-Ammonium acetate	Air	0·300	0·40	1·33	25
0·1M-Ammonium acetate	Air	0·245	0·57	2·33	30
0·1M-Ammonium acetate	Air	0·220	0·70	3·18	35
0·1M-Ammonium acetate	Air	0·155	1·25	8·06	45
0·1M-Ammonium acetate–D20	Air	0·275	0·35	1·27	25

[10] R. G. Kallen and W. P. Jencks, *J. Biol. Chem.*, 1966, **241**, 5845.

hown for (11) is in complete agreement with e.s.r. ata [11] and with HMO calculations of unpaired spin ensities which we have made (Table 5).

During propagation (11) reacts with oxygen to give he chain carrier, and there are three possible ways this N(5) to give the quinonoid form (12) and a hydroperoxyl radical (HO_2·) as chain carrier. (3) An electron is transferred from (11) to oxygen, followed by a rapid proton transfer to give the quinonoid form (12) and the hydroperoxyl radical.



SCHEME 3 Mechanism of autoxidation of tetrahydropterins

could occur. (1) Oxygen adds to the angular C(4a) atom (the only carbon with unpaired electron density) in a rapid diffusion-controlled reaction to give an organic peroxyl radical chain carrier, as we have previously suggested.[4] (2) Hydrogen is abstracted from

TABLE 5

HMO calculations of spin densities in semiquinone radicals from tetrahydropterins

Atom	Unpaired electron density
N(1)	0·0470
C(2)	0·0569
N(2′)	0·0461
N(3)	0·0176
C(4)	0·1517
Amide oxygen	0·0578
C(4a)	0·2714
N(5)	0·2162
N(8)	0·0595
C(8a)	0·0747

Using heteroatom parameters (heteroatom X)

$\alpha_x = \alpha_c + h_x \beta_{cc}$
$\beta_{cx} = k_x \beta_{cc}$

	h	k
N(5), N(3), N(8), N(2′)	1·50	1·00
Amide oxygen	2·00	1·00
N(1)	~~0·50~~ 0·56	0·80

On consideration of the general autoxidation process (Scheme 4), where X is denoted as the chain carrier

$AH_2 + O_2 \xrightarrow{k_1} AH\cdot + HO_2\cdot$ initiation
$AH\cdot + O_2 \xrightarrow{k_2} X$
$AH_2 + X \xrightarrow{k_3} AH\cdot + XH$ propagation
$AH\cdot + X \xrightarrow{k_4}$ non-radical products termination

SCHEME 4

and neglecting the slow initiation reaction and using the steady-state approximation, the rate of oxygen uptake is given by equation (3) in agreement with the observed

$$\text{Rate} = (k_2 k_3 / k_4)^{\frac{1}{2}} [AH_2][O_2] \quad (3)$$

kinetics if the impurity initiation is omitted, showing that the correct termination has been chosen.

More important, the rate is given by equation (4), whatever the initiation or termination chosen.

$$\text{Rate} = k_2[AH\cdot][O_2] \quad (4)$$

$$\therefore \; k_2[O_2] = \text{Rate}/[AH\cdot] \quad (5)$$

[11] A. Bobst, *Helv. Chim. Acta*, 1968, **51**, 607.

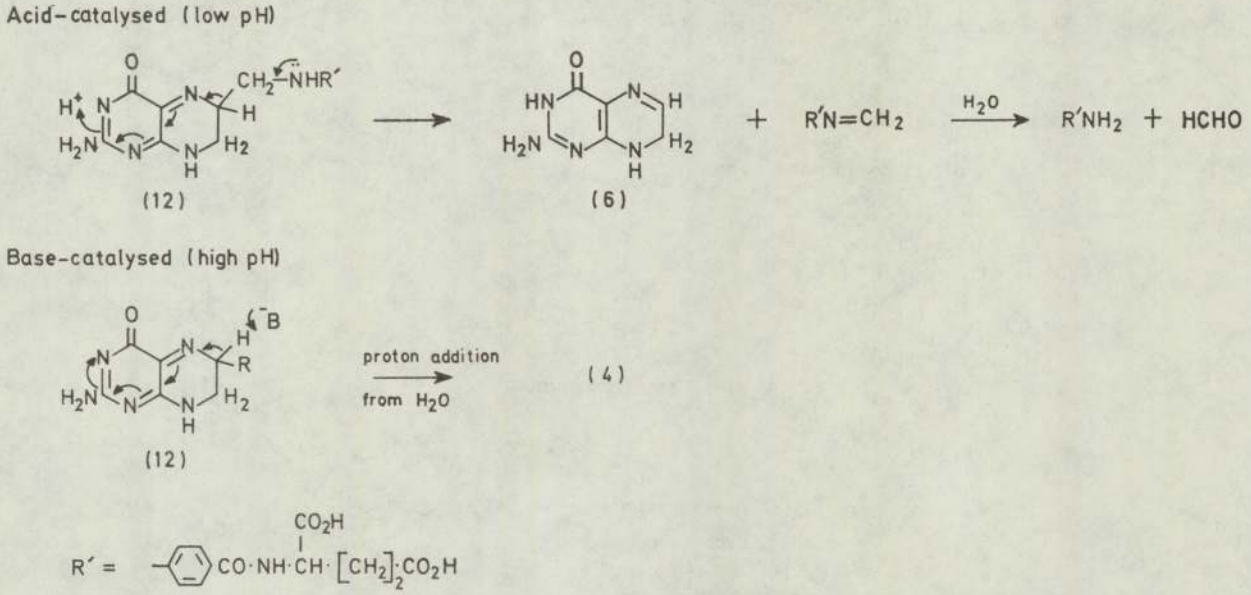
Spectrophotometric observation of the steady-state concentration of free radicals formed during tetrahydrobiopterin autoxidation ($\lambda_{max.}$ 550 nm *), and the corresponding overall rate, over a range of temperatures, shows that the product $k_2[O_2]$ increases considerably as temperature is raised (Table 4). Since oxygen concentration actually decreases with increasing temperature, k_2 must show an activation energy of at least 52 kJ mol^{-1}. Furthermore, k_2 is not changed when D_2O is used as solvent, showing that no N–H bond cleavage is involved in the reaction. Therefore, the propagation reaction must be that described in (3) above, and *not* addition of oxygen to the free radical (a zero activation energy process).

This conclusion is in agreement with the observation [13] that the free radicals formed using other oxidants are not scavenged by oxygen, therefore precluding oxygenated intermediates.

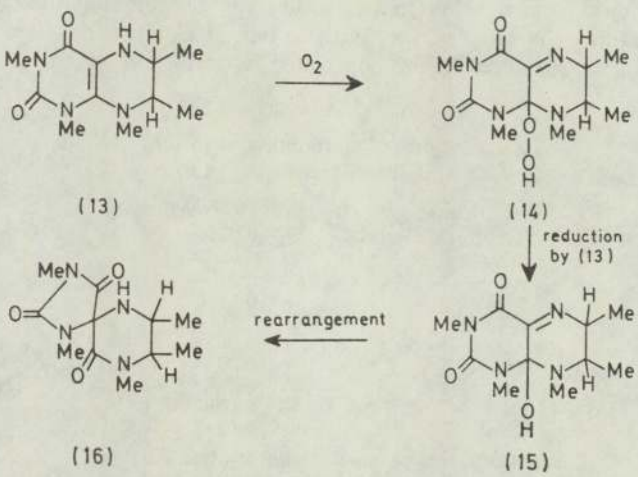
Thus, autoxidation must be as shown in Scheme 3, a possible mechanism recognised [14,15] for other compounds but for which no definite evidence has been described.

The observed products and their pH-dependence can be explained by considering the possible rearrangements of the quinonoid intermediate shown in Scheme 5. Apparently, loss of side chain during tetrahydrobiopterin oxidation demands higher activation energy than with tetrahydrofolic acid, thereby explaining the differences in products at room temperature.

Incorporation of ^{18}O *during the Autoxidative Ring Contraction of* 1,3,6,7,8-*Pentamethyl*-5,6,7,8-*tetrahydropteri-2,4-dione.*—The autoxidative ring contraction of this blocked tetrahydropteridine (13) has been proposed [5d] as evidence for the intermediacy of 8a-hydroperoxides (14) during the reaction, as shown in Scheme 6. We have earlier reported [16] that autoxidation of this compound in pyridine–^{18}O-labelled water (10 : 1) results in a high incorporation of label into the new oxygen function formed in the product 1′,3′,4,5,6-pentamethyl-3-oxopiperazine-2-spiro-5′-hydantoin (16). These experiments also showed that *ca.* 12% of the oxygen introduced does originate from molecular oxygen. This apparent incorporation of molecular oxygen has now been shown to arise *via* a concerted reaction of (13) with the hydrogen peroxide formed during the oxidation, using conditions



SCHEME 5 Rearrangement of quinonoid intermediate during autoxidation of tetrahydrofolic acid



SCHEME 6 Autoxidative ring contraction of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione (13) (from ref. 5*d*)

* Bobst [12] has reported that red colouration produced during oxidation is due to the free radical. This is supported by the independence of steady-state concentration on oxygen partial pressure (Table 4).

[12] A. Bobst, *Helv. Chim. Acta*, 1967, **50**, 2222.
[13] M. C. Archer, K. G. Scrimgeour, and D. J. Vonderschmitt, *Canad. J. Biochem.*, 1972, **50**, 1174.
[14] G. A. Russell, *J. Chem. Educ.*, 1959, **36**, 111.
[15] J. F. Corbett, *J. Soc. Cosmetic Chemists*, 1972, **23**, 683.
[16] J. A. Blair and A. J. Pearson, *Tetrahedron Letters*, 1973, 1681.

designed to test this hypothesis (see Experimental section). As can be seen from Table 6, the inclusion of

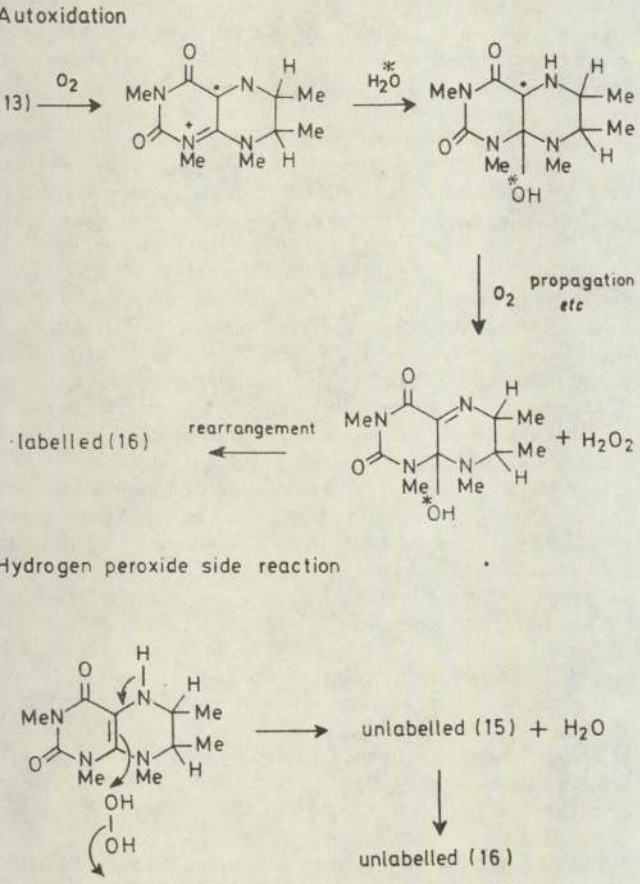
TABLE 6

Incorporation of ^{18}O during autoxidative ring contraction of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione using ^{18}O-labelled water (43·46% enriched)

Reaction conditions	Ratio of $(M + 2) : M$ peaks in spirohydantoin mass spectrum	Incorporation of oxygen from water (%)
4 Days; unlabelled oxygen gas, unlabelled water	0·019	
4 Days; unlabelled oxygen gas, labelled water	0·535*	80
4 Days; unlabelled oxygen gas, labelled water + excess of catalase	0·766*	100
5 h; unlabelled H_2O_2, labelled water, anaerobic	0·073*	1·57

* After correction for natural $(M + 2)$ peak (first result in Table).

catalase (an enzyme which decomposes hydrogen peroxide) in the autoxidation mixture results in the



SCHEME 7 Autoxidative ring contraction of blocked tetrahydropteridine, alternative mechanisms

formation of (16) in which there is 100% incorporation of water into the 3-oxo-group produced. Furthermore, unlabelled spirohydantoin is observed to be formed using unlabelled hydrogen peroxide in ^{18}O-labelled water under anaerobic conditions. In all cases, no exchange was observed between carbonyl groups of starting material and the water used.

Thus, during the autoxidation there is a consecutive side reaction with hydrogen peroxide generated by reduction of the molecular oxygen, as shown in Scheme 7. All these results can be explained without the intervention of 8a-hydroperoxides, and Scheme 7 is consistent with the mechanism proposed in the preceding discussion.

EXPERIMENTAL

U.v. and visible spectra were recorded with a Perkin-Elmer 137 u.v. spectrophotometer, n.m.r. spectra for solutions in trifluoroacetic acid with Perkin-Elmer R14 or Varian HA 100D spectrometers, and mass spectra on an A.E.I. MS9 spectrometer. Folic acid, xanthopterin, pterin, monodeuterioacetic acid (≥98 atom % D), and catalase were purchased from Koch–Light, ^{18}O enriched water (43·461% enrichment) from Miles Laboratories, and deuterium gas from Matheson Gas Products. Tetrahydrobiopterin dihydrochloride and 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione were gifts from Roche Products and Dr. H. I. X. Mager, respectively.

5,6,7,8-*Tetrahydrofolic Acid*.—This was prepared by the low pressure catalytic hydrogenation of folic acid over reduced platinum oxide catalyst, using a modification of the method described by Hatefi *et al.*[17] Since contamination by 2-mercaptoethanol (an antioxidant generally used to protect the tetrahydrofolate) might upset the kinetic results, this was omitted in all preparations. A suspension of platinum oxide (2 g) in dry glacial acetic acid (25 cm³) was stirred under an atmosphere of hydrogen at room temperature until consumption of gas ceased, when a suspension of folic acid (2 g) in glacial acetic acid (25 cm³) was added. After uptake of 2 mol. equiv. of hydrogen, the apparatus was flushed with nitrogen and the solution filtered under vacuum directly into a flask immersed in ice *via* a side arm with glass sinter incorporated. The product was freeze-dried and stored at 0° under nitrogen in sealed glass ampoules. The compound obtained in this way was found to be 95% pure as the diacetate from its u.v. spectrum (using ε 29,100 [18] at $\lambda_{max.}$ 297 nm in 0·1M-phosphate buffer; pH 7; previously de-aerated and containing 0·5% v/v of 2-mercaptoethanol), τ 1·7br (amino groups, *etc.*), 1·9 (2H, d, *J* 8 Hz) and 2·4 (2H, d, *J* 8 Hz, benzene ring), 4·85br (1H, m, α-CH in glutamic acid of side chain), 5·55br (1H, m, 6-H), 5·9br (4H, 7- and 9-H_2), 7·18 and 7·4 (4H, CH_2 groups of glutamic acid), and 7·73 (6H, s, 2 × Me of diacetate).

6,7-*Dideuterio*-5,6,7,8-*tetrahydrofolic Acid*.—This was prepared by the same method as for tetrahydrofolic acid, using deuterium gas and replacing the glacial acetic acid by acetic [^2H]acid. [It was found that catalytic deuteriation using glacial acetic acid as solvent led to very little incorporation of deuterium in the product (n.m.r.).] The deuteriated tetrahydrofolic acid was found to contain *ca.* 90% incorporation of deuterium on C(6) and *ca.* 50%

[17] Y. Hatefi, P. T. Talbert, M. J. Osborn, and F. M. Huennekens, *Biochem. Prep.*, 1960, **7**, 89.

[18] R. G. Kallen and W. P. Jencks, *J. Biol. Chem.*, 1966, **241**, 5845.

deuterium in the methylene group at C(7) (from the n.m.r. signal intensities at τ 5·55 and 5·9, respectively).

7,8-Dihydrofolic Acid.—This compound was prepared for use as a t.l.c. standard in the reaction pathway studies, using the method of Futterman,[19] slightly modified. Sodium hydroxide solution (0·1M) was added carefully to a suspension of folic acid (200 mg) in water (20 cm³) until solution was achieved. Sodium ascorbate solution (50 cm³, containing 100 mg per cm³) was added and the solution was adjusted to pH 6 with 2M-hydrochloric acid. Sodium dithionite (2 g) was added, the solution allowed to stand at 0° for 15 min, and then adjusted to pH 2·8 with 2M-hydrochloric acid. The precipitate of dihydrofolic acid was separated by centrifugation, washed several times with 0·5% HCl solution and once with acetone to remove ascorbate, and dried under vacuum. The product, a white solid, homogeneous on t.l.c. in three solvents (blue fluorescence under u.v., 366 nm), was stored under nitrogen at 0° in sealed glass ampoules, $\lambda_{max.}$ (pH 13) 221, 239sh, 288, and 305sh nm, $\lambda_{max.}$ (pH 7) 227, 280, and 303sh nm [lit.,[20] $\lambda_{max.}$ (pH 13) 221, 239sh, 285, and 306sh nm, $\lambda_{max.}$ (pH 7) 227, 282, and 303sh nm]. T.l.c. data are given in Table 7.

Reaction Pathway of Tetrahydrofolic Acid Autoxidation.—Solutions of tetrahydrofolic acid (25 mg) in the appropriate buffer (25 cm³; dilute acetic acid for pH 3 oxidations; 0·1M-phosphate buffer, pH 7; sodium hydroxide adjusted to pH 11; 0·1M-sodium hydroxide, pH 13) were shaken

TABLE 7

R_F Values for oxidation products of tetrahydrofolic acid

Compound and appearance under u.v. light	R_F Values in solvent system indicated: 0·1M-Phosphate buffer	n-Propanol–1% ammonia (2 : 1)	n-Butanol–acetic acid–water (4 : 1 : 5)
Tetrahydrofolic acid (absorbing spot)	0·78	0·35	0·43
Dihydrofolic acid (blue fluorescence)	0·22	0·12	0·43
Xanthopterin (green fluorescence)	0·49	0·25	0·42
Pterin (blue fluorescence)	0·52	0·38	0·46

under air to achieve oxidation, 2 µl samples withdrawn at the times shown in Table 2, and run together with appropriate standards (dihydrofolic acid, pterin, xanthopterin) using cellulose MN300 layer (0·1 mm) in the solvent systems shown in Table 7. The developed chromatograms were viewed under u.v. (366 nm) and the observed fluorescent products compared with the standards.

Isolation of 7,8-Dihydrobiopterin.—A solution of tetrahydrobiopterin (50 mg) in water (50 cm³) was stirred under oxygen at 25° until 0·8 mol. equiv. of oxygen had been absorbed (measured manometrically). This ensures that any hydrogen peroxide formed during autoxidation does not lead to over-oxidation of the dihydrobiopterin. The product was freeze-dried, and since it appeared as a single fluorescent spot on t.l.c. in three solvent systems no attempt was made at further purification. The spectral properties of the compound were consistent with 7,8-dihydrobiopterin, $\lambda_{max.}$ (pH 1) 267 and 350 nm, $\lambda_{max.}$ (pH 7) 240, 280, and 330 nm, $\lambda_{max.}$ (pH 13) 280 and 330 nm (*cf.* ref. 21), τ 1·5br (amino-groups), 4·6 [2H, s, 7-H_2, characteristic of 7,8-dihydropterins (ref. 22, p. 98)], 5·3 and 5·7 (1H, each, poorly resolved, CH groups of side chain), and 8·55 (3H, d, *J* 8 Hz, methyl group of side chain).

Attempted Detection of Peroxide Intermediates.—Tetrahydrobiopterin is soluble in a mixture of ethanol and aqueous 0·1M-sodium hydroxide (4 : 1). Solutions of 2 mg tetrahydrobiopterin per cm³ in this medium were oxidised with constant stirring at −78° (dry ice–acetone) to allow accumulation of peroxides, when a yellow solution was produced. This was spotted onto previously chilled (0°) silica gel t.l.c. plates alongside standard of hydrogen peroxide dissolved in the solvent used [silica gel plates previously eluted with methanol–concentrated hydrochloric acid (10 : 1) to remove any iron present, and re-activated at 110° for 35 min]. The chromatograms were run in three solvent systems (below) at 0° and developed by spraying with a 1% aqueous solution of *NN*-dimethyl-*p*-phenylenediamine and warming, when pink spots appeared where peroxides were present. The only peroxide detectable in the oxidation mixture had identical R_F values with those of the hydrogen peroxide standard [R_F values: 0·82 in water–ethanol–chloroform (20 : 17 : 2); 0·56 in propanol–1% ammonia (2 : 1); 0·27 in carbon tetrachloride–acetone (2 : 1)].

Kinetic Studies.—These were done using a simple manometric technique. The apparatus consisted of a 250 cm³ glass reaction flask connected to a conventional Warburg water manometer, and having an inlet for oxygen which could be closed by means of a three-way glass tap. The flask, containing 100 cm³ of aqueous solvent, was immersed in a thermostatted bath (temperature variation not more than ±0·01°) and was purged with a stream of oxygen for 20 min with rapid magnetic stirring. The weighed tetrahydropterins were introduced into a side arm in an open glass ampoule and suspended above the solvent, the gas supply shut off, and thermal equilibration awaited. Thus, the sample was kept dry and under these conditions did not oxidise. The ampoule was then allowed to fall into the rapidly stirred solvent and the pressure change after adjustment to constant volume was noted at intervals of 1 min. Correction for variation in atmospheric pressure and bath temperature was made using an identical blank reaction vessel as thermobarometer. Calibration of the apparatus was achieved by determining the weight of water required to completely fill it, hence calculation of the volume of gas contained in the system and conversion of pressure change to moles of oxygen could be made. Reproducibility of results using different reaction vessels showed that calibration was accurate to within ±5%; runs done under identical conditions showed rates to be accurate to within ±10%. Variation of oxygen partial pressures was achieved by mixing known volumes of oxygen and nitrogen and introducing the mixture into a vacuum de-gassed reactor.

The pH dependence was studied by incorporating into the solvent the quantity of sodium hydroxide required to give the desired solution pH, and then measuring the pH immediately after oxidation.

Determination of pK_a *Values.*—This was done by plotting the $\lambda_{max.}$ at *ca.* 300 nm against pH of de-aerated solutions

[19] S. Futterman, 'Methods in Enzymology,' eds. S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1963, vol. 6, p. 801.

[20] J. C. Rabinowitz, 'The Enzymes,' eds. P. D. Boyer, H. Lardy, and K. Myrback, Academic Press, New York, 1960, vol. 6, 2nd edn., p. 185.

[21] R. L. Blakley, *Nature*, 1960, **188**, 231.

[22] R. L. Blakley, 'The Biochemistry of Folic Acid and Related Pteridines,' North Holland Research Monographs, 'Frontiers of Biology,' North Holland, 1969, vol. 13, p. 69.

f tetrahydropterin containing 0·1% 2-mercaptoethanol, sing appropriate blanks containing 2-mercaptoethanol s reference. Sigmoid curves were obtained between H 9 and 13 and the pH corresponding to maximum lope was taken as the pK_a of the 3,4-amide group (*cf.* ef. 11).

Kinetic Characteristics of Free Radical.—Tetrahydro-iopterin (25 mg) was stirred rapidly in 0·1M-ammonium cetate buffer solution (25 cm^3), pH 6·8, containing 10^{-4}M-DTA (to remove effects of copper impurities present in mmonium acetate), and using a continuous stream of air bove the solution. The optical density at 550 nm was etermined at 1 min intervals, and the steady-state value oted, together with the time for attainment of steady-tate conditions. The overall rate at this time was also etermined for solutions of 20 mg tetrahydrobiopterin in 00 ml buffer (since absolute values of k_2 were not required his was found convenient), and the data shown in Table 5 or different temperatures were computed.

Autoxidative Ring Contraction of 1,3,6,7,8-*Pentamethyl-*,6,7,8-*tetrahydropteri*-2,4-*dione in* ^{18}O-*Labelled Water.*—In hese experiments the blocked pteridine (3 mg) was shaken n labelled water (0·5 cm^3; 43·46% enriched) under the ollowing conditions: (1) under unlabelled oxygen gas for our days at room temperature; (2) as in (1) but including catalase (4 mg) in the reaction mixture; and (3) under argon for 5 h, using water previously de-oxygenated with a stream of dry argon, and in the presence of 2×10^{-5}M-unlabelled hydrogen peroxide (20 μl of 3% H_2O_2).

After the times indicated the products were dried *in vacuo*, and the catalase in (2) was removed by extraction of the spirohydantoin with chloroform (0·5 cm^3). Mass spectra of the dried products were recorded without further purification, and after subtraction of the starting material spectrum showed the presence of 1′,3′,4,5,6-pentamethyl-3-oxopiperazine-2-spiro-5′-hydantoin. The incorporation of ^{18}O label, and the percentage introduction of water as the 3-oxo-group, were calculated from the ratio of the *M* and *M* + 2 peaks as shown in Table 6 (Results section).

1,3,6,7,8-Pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione had *m/e* 238 (100%), 223 (54), 209 (24), 195 (27), 181 (19), 166 (18), 140 (60), 138 (19), 123 (16), 109 (14), 97 (30), 95 (35), 83 (38), and 68 (57), *m** 170·5 (223 ⟶ 195) (loss of CO from *M* − 15 peak). The unlabelled spirohydantoin (*cf.* ref. 5*d*) had *m/e* 254 (100%), 239 (25), 225 (2), 196 (4), 169 (17), 154 (15), 142 (85), 141 (25), 127 (44), and 86 (15).

We thank the S.R.C. for a studentship (to A. J. P.) and the University of Aston for financial support.

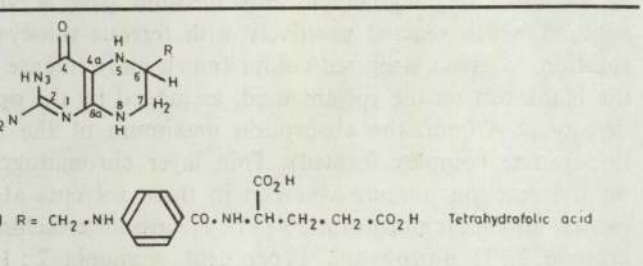
[3/1569 *Received*, 25*th July*, 1973]

Kinetics and mechanism of the autoxidation of tetrahydropterins

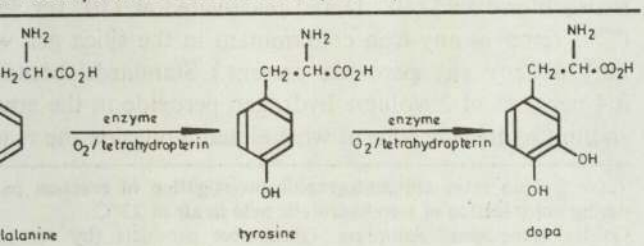
ilbert Morgan Medal Award Address

thony J Pearson

ahydrobiopterin (1b) (Fig 1) has been shown[1] to be the ıral cofactor required for the enzymatic hydroxylation henylalanine to tyrosine and of tyrosine to dopa, a process lving incorporation of molecular oxygen into the sub- te molecule. Other tetrahydropterins also may sub- ıte for the coenzyme (Fig 2).



1 Tetrahydrofolic acid and tetrahydrobiopterin



2 Enzymic conversion of phenylalanine and tyrosine

Io detailed mechanism of the incorporation of molecular gen has been worked out to date, although it has been tulated[2–6] that hydroperoxide intermediates in the dation of the cofactor are responsible. The present study made to determine whether any reactive intermediates e formed during the autoxidation of the tetrahydropterin actor, which might serve as a species capable of hydroxyl- g aromatic compounds.

Therefore, a survey of the reaction was carried out by kinetic methods and by product analysis. The present paper describes the work carried out between December 1971 and March 1973, using tetrahydrofolic acid (THFA) and tetrahydrobiopterin as model pterin compounds.

Pearson, Department of Chemistry, University of on in Birmingham, Gosta Green, Birmingham 7ET, delivered this paper to the Birmingham Section he SCI in Birmingham on 31 May 1973

Materials

Tetrahydrofolic acid (Ia) was prepared by the catalytic hydrogenation of folic acid in glacial acetic acid using pre-reduced platinum catalyst (O'Dell *et al*[7]). Tetrahydrobiopterin was a gift from Roche Products Ltd.

Products of oxidation

Final products of the oxidation including their pH dependence have been reported previously for THFA.[2,8] The effect of pH is shown in Table 1.

The main final products of THFA autoxidation are pterin (IV) or 7,8-dihydropterin, xanthopterin (V), 7,8-dihydrofolic acid (VI) (DHFA) and folic acid (VII), in amounts depending on reaction conditions (Table 1). Since formation of the first two compounds involves cleavage of the C(6) – C(9) bond any proposed mechanism must account for this and for the increase in xanthopterin yield as pH is increased.

Table 1 **pH dependence of pterin: xanthopterin ratio in oxidation products of tetrahydrofolic acid (determined after three days oxidation under air)[2]**

pH of oxidation	Pterin : xanthopterin ratio
3	12·64 : 1·00
7	1·00 : 1·90
10	1·00 : 17·38

The first isolatable product from tetrahydrobiopterin autoxidation was obtained as follows. Tetrahydrobiopterin (50mg) was stirred in distilled water (100cm³) under an atmosphere of pure oxygen at 25°C until 80 per cent of the stoichiometric amount of oxygen had been taken up (measured manometrically). The product was lyophilised and, since it appeared to be homogeneous on t.l.c. examination (two solvents: 0·1 M-phosphate buffer, pH 7; n-propanol/1 per cent ammonia solution 2 : 1), it was not further

purified. Ultraviolet spectral examination and n.m.r. were consistent with the structure of 7,8-dihydrobiopterin (II). (Ultraviolet: $\lambda_{max.}$ (nm) pH 1, 267, 350; pH 7, 240, 280, 330; pH 13, 280, 330 cf. ref. 9. N.m.r.: 2-proton singlet τ4·4, characteristic of 7,8-dihydropterins,[9] Me group at τ8·7, doublet J = 8Hz, two CH groups at τ5·5 and τ6·1.)

The final product of autoxidation, biopterin[2,10] (III) arises from a slow oxidation of 7,8-dihydrobiopterin.

Reaction pathway in THFA oxidation

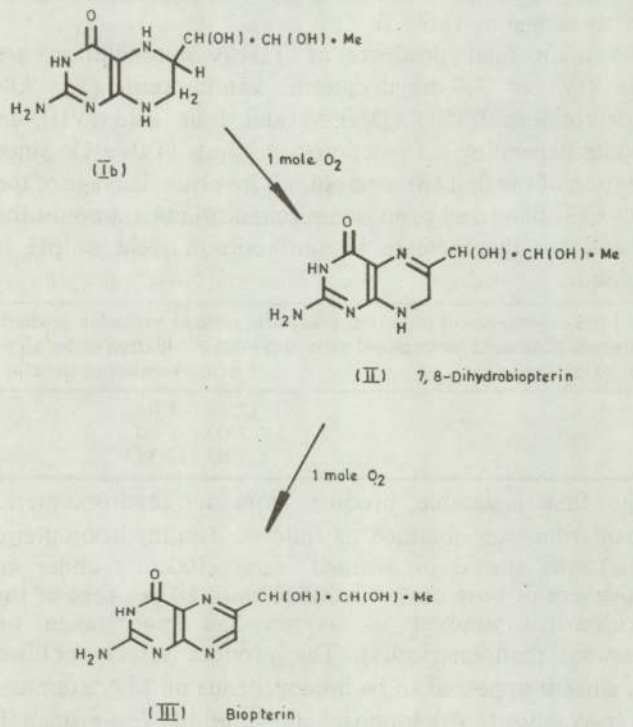
The oxidation of THFA was followed using t.l.c. in three solvent systems to identify products formed after various time intervals, by comparison with known standards (viewed under ultraviolet light for detection). Solutions of 1mg/cm³ of THFA in dilute acetic acid (pH 3), 0·1M phosphate buffer (pH 7) or sodium hydroxide solution (pH 11 and pH 13) were shaken under air and samples run on t.l.c. at the times shown (Table 2). The products are also shown in Table 2.

From these results it is clear that the pterin or 7,8-dihydropterin obtained at low pH arises directly from the THFA. Xanthopterin is only observed subsequently to the formation of DHFA and is a known oxidation product of the latter.[8] Although no folic acid was observed on t.l.c. (a weakly absorbing compound under ultraviolet light), it is a known oxidation product of both DHFA and THFA.

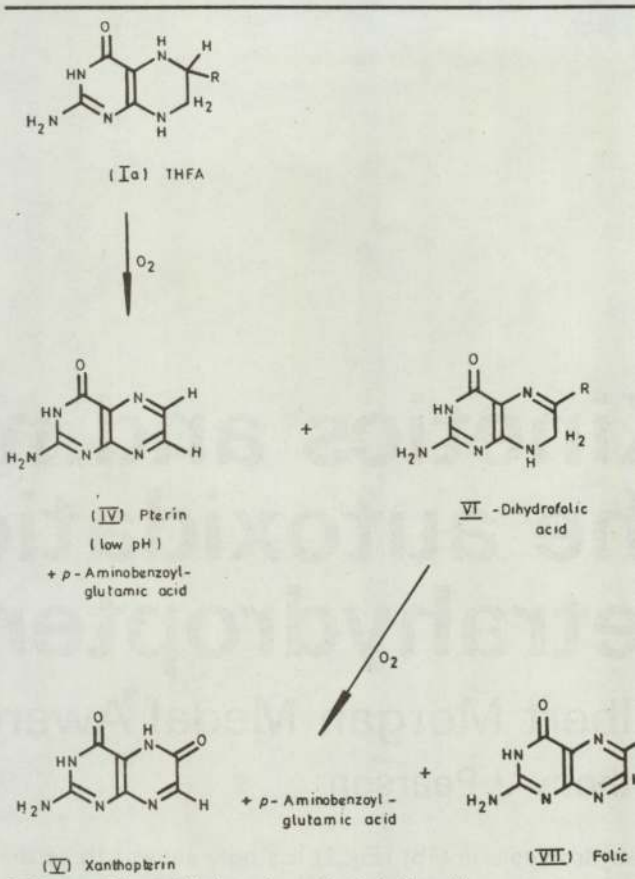
Therefore, the basic oxidation reaction pathways for tetrahydrobiopterin and THFA may be written as in Schemes 1 and 2.

Attempted detection of organic peroxides

Since most normal autoxidations proceed via hydroperoxides (ROOH), attempts were made to try and detect these compounds qualitatively. At ambient temperature only slight positive tests were obtained by adding ferrous thiocyanate solution, the most sensitive test reagent, to solutions of oxidised tetrahydropterin. These were not strong enough to allow detection by t.l.c. and thus discriminate between hydrogen peroxide and organic peroxides.



Scheme 1 Autoxidation of tetrahydrobiopterin



Scheme 2 Autoxidation of tetrahydrofolic acid

Tetrahydrobiopterin was found to be soluble in a mi of ethanol and aqueous 0·1M sodium hydroxide sol (4 : 1) at −78°C (using dry ice/acetone bath). Oxida of solutions of 2mg/cm³ in this medium gave a y solution which reacted positively with ferrous thiocya solution, to give a deep red colour (much more intense the blank test on the solvent used, as judged by the o density at 470nm, the absorption maximum of the thiocyanate complex formed). Thin layer chromatogr on the reaction mixture was run in three solvents at (water, ethanol, chloroform 20 : 17 : 2; carbon tetrachl acetone 2 : 1; n-propanol, 1 per cent ammonia 2 : silica gel previously eluted with methanol/concent hydrochloric acid (10 : 1) and reactivated at 110° for 3 (This removes any iron contaminant in the silica gel, v may destroy any peroxide present.) Standards, conta 0·4 per cent of 20volume hydrogen peroxide in the eth sodium hydroxide solvent were eluted alongside the rea

Table 2 **Thin layer chromatographic investigation of reaction pa during autoxidation of tetrahydrofolic acid in air at 23°C**

Oxidation medium and pH	Sampling time	Observable products (by comp with known standards)
Dilute AcOH pH3	15h	Pterin or dihydropterin (not guishable)
	39h	Pterin or dihydropterin
0·1M phosphate pH 7	40min	Dihydrofolic acid (major); (strong spot)
	24h	Suspected 6-formyl-7,8-dihydro (minor) – from DHFA oxida pterin (strong spot)
NaOH pH 11	40min	Dihydrofolic acid (major); dih xanthopterin (faint); pterin (fa
	24h	Xanthopterin (major); pterin (fai
NaOH pH 13	35min	Dihydrofolic acid
	24h	Xanthopterin

Solvent systems (only fluorescent compounds noted) were
(1) 0·1 M-phosphate buffer, pH 7
(2) n-propanol/1 per cent ammonia 2 : 1
(3) n-butanol/acetic acid/water 4 : 1 : 5 (organic phase)

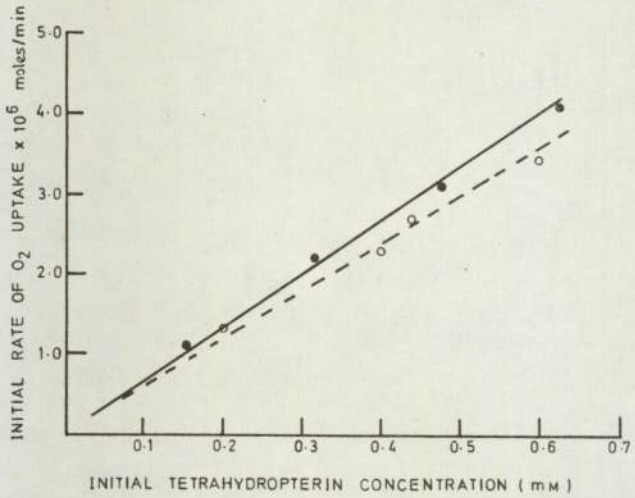


Fig 3 Plots of rate vs concentration for autoxidation of THFA and tetrahydrobiopterin in 0·1M – phosphate buffer at 25°C under pure oxygen (100 ml of solution)
● tetrahydrobiopterin ○ tetrahydrofolic acid (THFA)

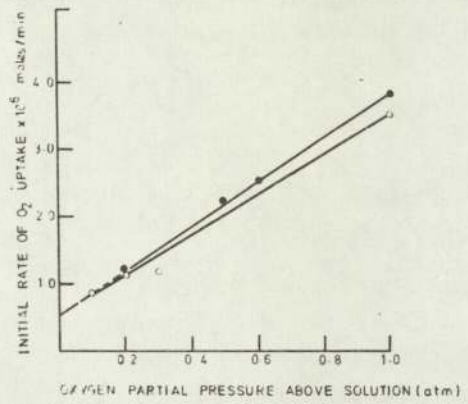


Fig 4 Oxygen concentration dependence of autoxidation rates in 0·1M – phosphate at 25°C
● tetrahydrobiopterin (20 mg/100 ml) ○ THFA (0·7 mM)

mixture. Chromatograms were sprayed with a 1 per cent solution of *N,N*-dimethyl-*p*-phenylenediamine in water, when pink spots appeared having identical R_f values to the hydrogen peroxide standards.

Hence it appears that hydrogen peroxide is the only peroxide formed. In support of this, it was observed that vacuum removal of solvent at −78°C left a solid which gave a positive peroxide test, but when the solution was carefully neutralised with dilute hydrochloric acid prior to solvent removal, the solid no longer reacted positively. Thus the residue obtained in the first case contained sodium peroxide and neutralisation caused its decomposition to hydrogen peroxide which was lost on evaporation of solvent.

Kinetic studies

The kinetics of autoxidation of THFA and tetrahydrobiopterin were followed by measurement of oxygen consumption at constant volume using a simple manometric technique. The results were analysed by determining the initial rates (following the short 'induction' period) from the plots of oxygen uptake vs time.

The following results were found. (1) The reaction is first order in tetrahydropterin compound (Fig 3). (2) There is a combination of first – and zero – order dependence on oxygen partial pressure above the reaction solution (Fig 4). The zero-order effect may be due to initiation by trace impurities. (3) The reaction is retarded by a factor of ≃2 by phenols and various other known inhibitors (Table 3). (4) Some transition metals catalyse the reaction (the greatest effect is caused by copper compounds). This catalysis is removed by inclusion of EDTA in the solution. (5) A decrease in rate is observed on decreasing the pH of the solution from 6 to 3, due to protonation of N(5).[11] (6) Rates are only very slightly reduced (within experimental error) on exclusion of light. The reaction therefore involves ground state oxygen (Table 3). (7) Tetrahydrofolic acid deuterated at C(6) and C(7) reacts at the same rate as the non-deuterated compound. Cleavage of these C–H bonds is therefore not involved in any rate-determining steps (see Fig 5). (8) The reaction rate is approximately the same in D_2O as in H_2O (within experimental error). Therefore no N–H bond homolysis is involved in a rate determining step. (9) There is an increase in rate on increasing the pH in the region pH 9 to pH 13. This is paralleled by the ionisation of the 3,4-amide group in the molecules (pK_a's determined spectrophotometrically are THFA 10·8 and tetrahydrobiopterin 10·5). At constant oxygen pressure, assuming the relationship:

Rate = k[non-ionised tetrahydropterin] + k′[enolate anion]

a linear relationship between rate and per cent ionisation is obtained (Fig 5). (10) The overall activation energy for oxygen uptake is around 14kcal/mole for both compounds (Fig 6).

These results indicate that the reaction is a free-radical chain process. Since homolysis of C–H and N–H bonds in the pteridine ring does not appear to be involved, the formation of radicals occurs primarily by electron abstraction followed by proton loss. This conclusion is supported by the observation (9) above, when an increase in electron density in the pyrimidine ring causes an increase in rate due to increased ease of electron removal.

The problem now arises as to what the remaining steps are for the propagation of the reaction. The free radical (VIII) formed by electron abstraction and loss of proton (see Scheme 3) may react with oxygen in three possible ways: (i) addition to the free radical (VIII) at carbon atom 4a

Table 3 **Effect of some additives on autoxidation rates of THFA and tetrahydrobiopterin at 25°C in 0·1 M phosphate buffer at pH 7 (sample of results only)**

Reactant and concn.	Oxygen partial pressure (atm)	Additive or other modifications	Initial rate of O_2 uptake × 10^6 moles O_2/min for 100cm³ of solution
Tetrahydrobiopterin (10mg/100cm³)	1·0	None	2·18
	1·0	0·02M-EDTA	2·13
	1·0	8-hydroxyquinoline-5-sulphonic acid (0·002M)	1·22
	1·0	Phenol (0·002M)	1·16
	1·0	Dark reaction vessel	2·0
THFA (0·7 mM)	0·2	None	1·10
	0·2	3×10^{-4} M-phenol	0·60
	0·2	10^{-4} M-Cu^{2+}	5·50

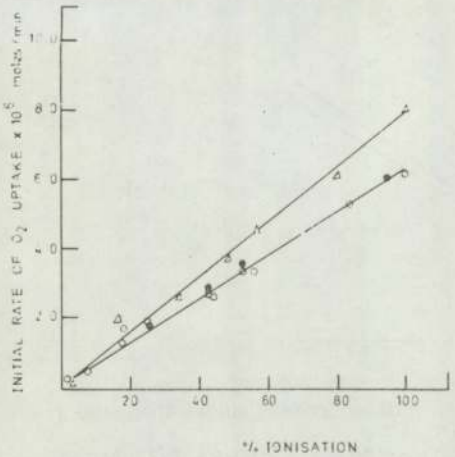


Fig 5 Plot of (observed rate-rate due to residual non-ionised compound) vs per cent ionisation of the 3, 4-amide group under pure oxygen at 25°C (per cent ionisation and residual non-ionised compound concentration estimated using pK = 10·8 for THFA and pK = 10·5 for tetrahydrobiopterin)
○ THFA (0·4 mM) ● THFA deuterated at C (6) and C (7) (0·4 mM) △ tetrahydrobiopterin (10 mg/100 ml)

to give a (4a)-alkyl peroxy radical which may then act as the chain carrier;[13] (ii) further electron abstraction to give an N(5)-protonated quinonoid dihydro compound (IX) which rapidly loses proton to give the quinonoid intermediate reported by Kaufman[14] and several other workers;[11,12] (iii) homolysis of the N(5)–H bond to give the quinonoid form (X).

In all cases it is clear that the quinonoid dihydro compound is an obligatory intermediate between removal of electrons from the pyrimidine ring of tetrahydro compounds and the formation of 7,8-dihydro compounds (and other products resulting from rearrangement of the quinonoid form).

Fortunately, it has been possible to observe the free radical (VIII) and to show that the reaction with oxygen is probably that in process (ii) above (next section). The reactions are presented in Scheme 3 below.

Kinetic characteristics of the free-radical intermediate

When solutions of tetrahydrobiopterin (25mg) in 25cm³ of 0·1 M-ammonium acetate solution, containing 10^{-4}M EDTA (to eliminate transition metal effects), were oxidised with rapid stirring, a wine red colour was produced ($\lambda_{max.}$ 550nm). Similar effects were obtained with tetrahydrofolic acid (4mg/cm³), in glacial acetic acid, but clean spectra could not be obtained in ammonium acetate buffer. This coloration has been attributed[15,16] to the formation of the free radical (VIII), a conclusion supported by the observation that the steady-state concentration of free radical (measured as optical density at 550nm) is independent of oxygen partial pressure above the solution (Table 4).

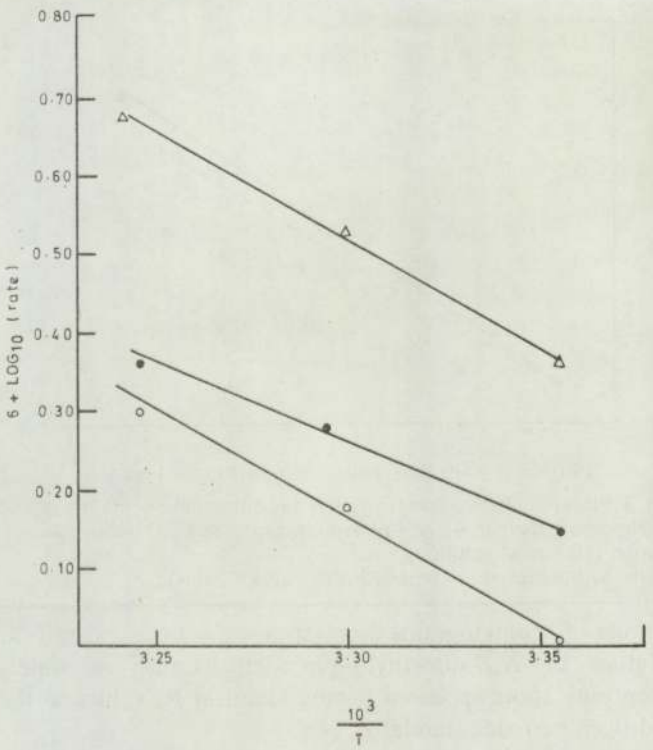


Fig 6 Temperature dependence of initial rates of autoxidation under air. Plots of $\log_{10}$ (rate) vs 1/T
△ 20 mg of tetrahydrobiopterin/100 ml of ammonium acetate buffer. E_a = 14 kcal/mole
● the same with 10^{-4}M – EDTA added. E_a = 10 kcal/mole
○ THFA in 0·1M – phosphate buffer E_a = 14 kcal/mole

Other possible intermediates (peroxy radicals or quinonoid form X) would show an increase of steady-state concentration with oxygen pressure increase.

Considering the general autoxidation scheme below:

$$\text{(1)}\ AH_2 \xrightarrow[O_2]{k_1} AH\cdot \text{ (initiation)}$$

$$\text{(2)}\ AH\cdot + O_2 \xrightarrow{k_2} \text{chain carrier}$$

$$\text{(3)}\ AH_2 + \text{chain carrier} \xrightarrow{k_3} AH\cdot$$

(4) AH· + chain carrier ⟶ non-radical products (termination)

applying steady state assumption to free radicals we obtain:

$$\text{Rate} = k_2(AH\cdot)(O_2) = k_{overall}(AH_2)(O_2)$$

or, at constant oxygen pressure:

$$\text{Rate} = k_2'(AH\cdot) = k'_{overall}(AH_2)$$

where $k_2' = k_2(O_2)$.

(This allows for the partial zero-order dependence of rate on O_2 pressure probably due to other iniation reactions).

Therefore it is possible to determine certain characteristic for reaction (2) above, since

Table 4 **Characteristics of free radicals during tetrahydrobiopterin autoxidation**

Buffer solution	O_2 partial pressure (atm)	Steady-state optical density at 550nm (O.D.$_{ss}$)	O_2 uptake rate* at steady-state × 10^6 moles. min^{-1} (R)	$k_2' = \frac{R}{O.D._{ss}}$ (arbitrary units)	Temperature
0·1M ammonium acetate	0·2	0·300	0·40	1·33	25°C
	1·0	0·290			25°C
	0·2	0·245	0·57	2·32	30°C
	0·2	0·220	0·70	3·18	35°C
	0·2	0·155	1·25	8·1	45°C
0·1M-ammonium acetate in D_2O	0·2	0·275	0·35	1·27	25°C

* Using 20mg of tetrahydrobiopterin in 100cm³

$$= \frac{\text{Overall rate}}{(AH\cdot)} \text{ (at steady-state)}$$

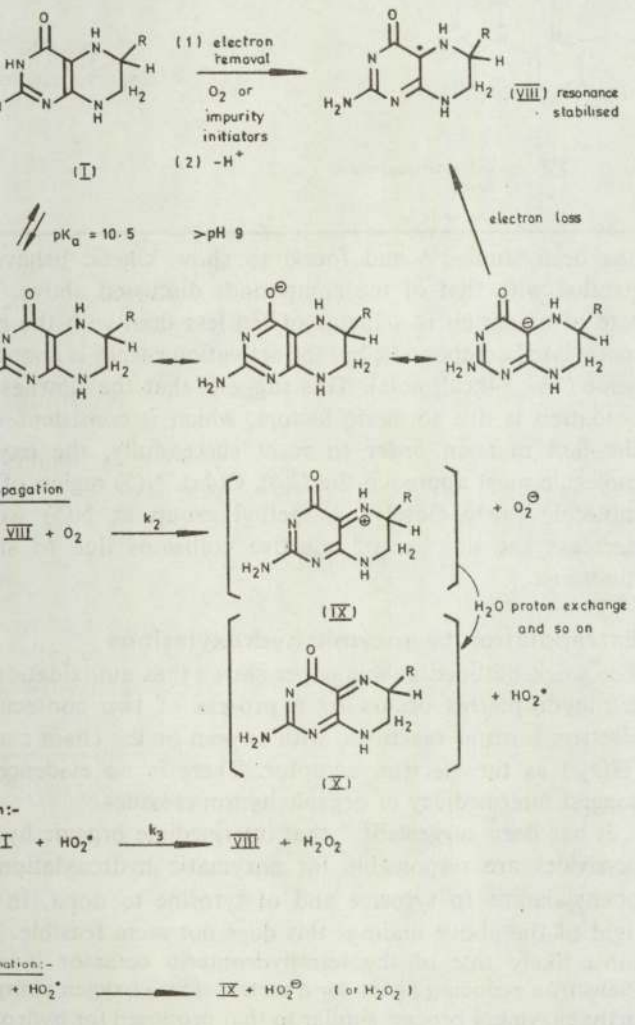
sing this and the data included for tetrahydrobiopterin able 4 we can see that k_2' is not affected by carrying out reaction in D_2O. Therefore homolysis of the N(5)–H l is not involved in step (2). (NH groups easily exchange ogen for deuterium in D_2O.)

mperature does have an effect on k_2' and the activation gy is 13 ± 2kcal/mole (from Table 4). Similar values obtained when EDTA is excluded from the reaction um, and the oxidation of THFA in glacial acetic acid gives a value of around 13kcal/mole for this activation gy.

ne rather high activation energy for reaction (2) indicates the process involved is most likely removal of electron the radical (VIII) to give a protonated quinonoid mediate. This has been recognised as a possible process her areas[17] but no definite evidence for it has appeared e literature.

n addition of oxygen to the free radical to give an nic peroxy radical chain carrier is expected to be a sion-controlled zero- (or low) activation energy process. a hypothesis is not consistent with the data presented is section.

ne mechanism proposed in Scheme 3 is consistent with ne data presented here including the reduction of oxygen ydrogen peroxide. An explanation of the final products s from a consideration of the possible acid- and base-



ame 3 Mechanism of autoxidation of tetrahydropterins

catalysed rearrangements of the quinonoid intermediate (Scheme 4) and subsequent further oxidations.

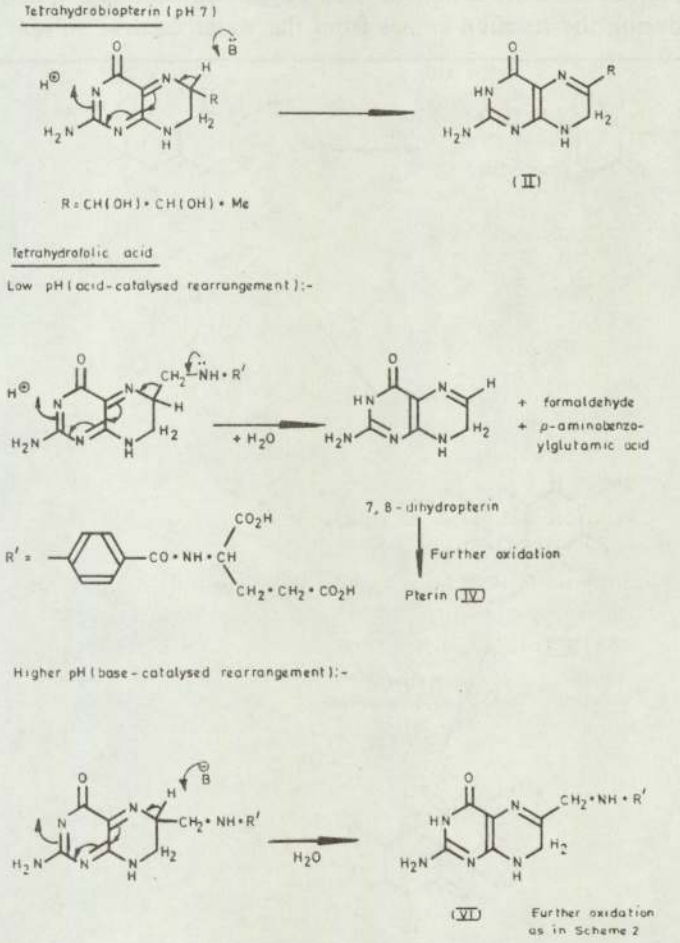
Molecular orbital calculations and rationalisation with e.s.r. data

Several workers in this field have presented e.s.r. data showing a considerable localisation of unpaired electron density on N(5) in the intermediate 'semiquinone' radical (VIII) (references 15, 16, 18). At first sight this does not appear compatible with the structure written for VIII in Scheme 3.

Some simple HMO calculations were done for the tetrahydropterin system in order to evaluate unpaired spin density distribution in the radical. Care must be exercised in using these methods as the results obtained are sensitive to the hetero atom parameters (h and k) chosen for N(5). However, in all cases a high spin density was found for N(5) and also for C(4) and C(4a). and this is consistent with both the e.s.r. data and the proposals based on the evidence presented in this paper. Typical values are found in Table 5. Furthermore, the observation that dihydrofolic acid is oxidised much more slowly than THFA is consistent with the removal of electron from a highest occupied molecular orbital, which lies at lower energy in the 7,8-dihydropterin system ($E = \alpha + 0{\cdot}5164\beta$) than in the tetrahydro system ($E = \alpha + 0{\cdot}3543\beta$).

A critique of the mechanism proposed by Mager and Berends[3–6]

Using model compounds, Mager and Berends have presented an autoxidation scheme involving formation of



Scheme 4 Rearrangement of quinonoid intermediate and subsequent oxidation at 25°C

Table 5 **HMO calculations of spin density in radicals derived from tetrahydropterin**

Using[19] $\alpha_x = \alpha_c + h_x\beta_{cc}$
$\beta_{cx} = k_x\beta_{cc}$
for heteroatom X

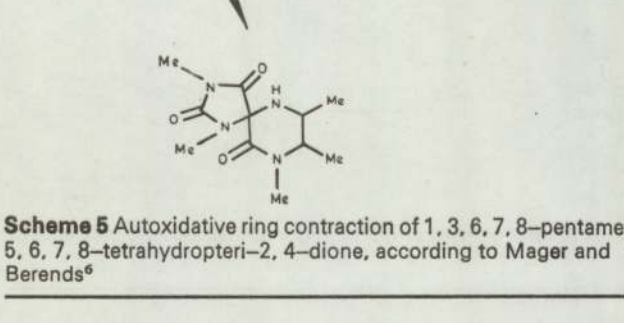
Parameters:

Atom	h	k
N(5), N(3), N(8), N(2′)	1·50	1·00
C(4) Amide oxygen	2·00	1·00
N(1)	0·50	0·80

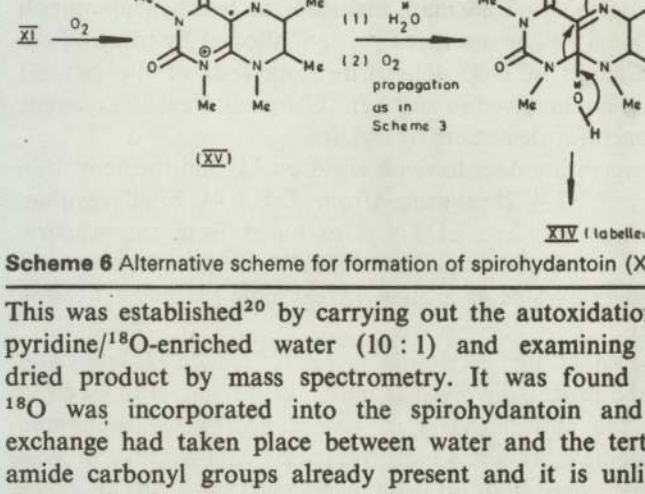
Atom	Spin density
N(1)	0·0470
C(2)	0·0579
N(2′)	0·0461
N(3)	0·0176
C(4)	0·1517
Amide oxygen	0·0578
C(4a)	0·2714
N(5)	0·2162
N(8)	0·0595
C(8a)	0·0747

transient 8a-hydroperoxide intermediates (XIII). The formation of 4,1′,3′-trimethyl-3-oxopiperazine-2-spiro-5′-hydantoin (XIV) during the autoxidation of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione (XI) is quoted as evidence for this intermediate, as depicted in Scheme 5. The hydroperoxide would be derived from addition of molecular oxygen to the free radical (XII) formed by electron removal at C(8a). However, the evidence presented above, both theoretical and kinetic, indicates that any addition of oxygen would occur at C(4a), since this is the only carbon atom having a high unpaired electron density.

In view of the fact that organic peroxy radicals are not formed during the autoxidation, another explanation of this autoxidative ring contraction must be sought. The alternative possibility is that the new oxygen function introduced during the reaction comes from the water used as solvent.



Scheme 5 Autoxidative ring contraction of 1, 3, 6, 7, 8–pentamethyl–5, 6, 7, 8–tetrahydropteri–2, 4–dione, according to Mager and Berends[6]



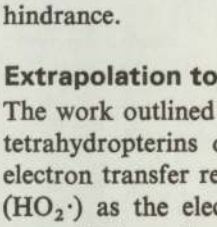
Scheme 6 Alternative scheme for formation of spirohydantoin (X

This was established[20] by carrying out the autoxidation pyridine/^{18}O-enriched water (10 : 1) and examining dried product by mass spectrometry. It was found ^{18}O was incorporated into the spirohydantoin and exchange had taken place between water and the tert amide carbonyl groups already present and it is unli that exchange would occur at the hydroperoxide s (XIII). Furthermore, treatment of the oxidation pro with unlabelled water did not lead to loss of label, so that ^{18}O was not incorporated via an exchange with solv but by an addition of water, probably at the radical ca stage (XV) as outlined in Scheme 6.

Therefore, this ring contraction cannot be cited as evid for the formation of 8a-hydroperoxide intermediates.

Oxidation of 5-methyl compounds

The autoxidation of 5-methyltetrahydrofolic acid (X



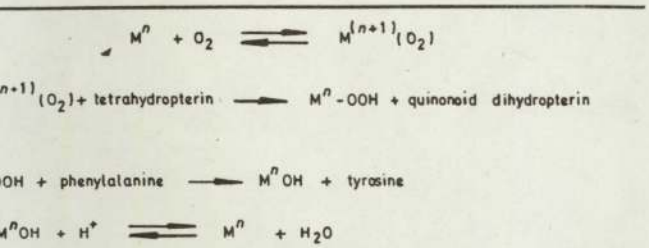
(XVI) 5-Methyltetrahydrofolic acid
R as in Ia

has been studied[21] and found to show kinetic behav parallel with that of the compounds discussed above. rate of oxidation is a factor of ten less than with the r methylated compounds, but the activation energy is abou same (13 – 14kcal/mole). This suggests that the slownes oxidation is due to steric factors, which is consistent the fact that, in order to react successfully, the oxy molecule must approach the C(4), C(4a), N(5) region of molecule fairly closely A methyl group at N(5) w decrease the number of effective collisions due to s hindrance.

Extrapolation to enzymic hydroxylations

The work outlined in this paper shows that autoxidatio tetrahydropterins occurs by a process of two consecu electron transfer reactions, with oxygen or the chain ca ($HO_2\cdot$) as the electron acceptor. There is no evidence suggest intermediacy of organic hydroperoxides.

It has been suggested[2–6] that intermediate organic hy peroxides are responsible for enzymatic hydroxylation phenylalanine to tyrosine and of tyrosine to dopa. In light of the above findings this does not seem feasible. most likely role of the tetrahydropterin cofactor is a 2-electron reducing agent for a metal – ion – oxygen com in the enzyme, a process similar to that proposed for hydro ation of steroids.[22] This also agrees with the 'ping-p mechanism suggested for the reaction as a result of enz



heme 7 Postulated mechanism of phenylalanine hydroxylation
represents a metal ion in its *n*th oxidation state, e.g. Fe^{2+})

etics studies,[23] since it requires consecutive steps involving uction of enzyme – oxygen complex by the cofactor and n hydroxylation of the substrate. A possible mechanism hown in Scheme 7.

Thanks are due to Roche Products for generous gifts of rahydrobiopterin, Professor H. I. X. Mager for a gift ,3,6,7,8-pentamethyl-5,6,7,8-tetrahydropteri-2,4-dione and Dr J. A. Blair for encouragement and helpful discussion oughout this work. Financial support was provided by University of Aston and the S.R.C.

erences

a) Kaufman, S., *J. Biol. Chem.*, 1959, **234,** 2683; (b) *Idem*, *Proc. atn. Acad. Sci.*, USA, 1963, **50,** 1085

[2] Stocks-Wilson, R., Ph.D. Thesis, 1971, University of Aston in Birmingham
[3] Mager, H. I. X. & Berends, W., *Recl. Trav. chim., Pays Bas*, 1965, **84,** 1329
[4] *Idem*, *Biochim. biophys. Acta*, 1966, **118,** 440
[5] *Idem*, *Recl. Trav. chim., Pays Bas*, 1969, **86,** 833
[6] *Idem*, *ibid*, 1972, **91,** 1137
[7] O'Dell, B. L., Vandenbilt, J. M., Bloom, E. S. & Pfiffner, J. J., *J. Am. Chem. Soc.*, 1947, **69,** 250
[8] Chippel, D. & Scrimgeour, K. G., *Can. J. Biochem.*, 1970, **48,** 999
[9] Blakley, R. L., 'The biochemistry of folic acid and related pteridines', 1969, p69, *North-Holland*
[10] Rembold, H., Metzger, H. & Gutensohn, W., *Biochim. biophys. Acta*, 1971, **230,** 117
[11] Archer, M. C., Vonderschmidt, D. J. & Scrimgeour, K. G., *Can. J. Biochem*, 1972, **50,** 1174
[12] Archer, M. C. & Scrimgeour, K. G., *ibid*, 1970, **48,** 278
[13] Blair, J. A. & Pearson, A. J., *Tetrahedron Lett.*, 1973 no 3, 203
[14] (a) Kaufman, S., *J. biol. Chem.*, 1961, **236,** 804; (b) *Idem*, *ibid*, 1964, **239,** 332
[15] Bobst, A., *Helv. chim. Acta*, 1967, **50,** 2222
[16] Ehrenberg, A. & Hemmerich, P., 'Biological oxidations', T. P. Singer (Ed), p250, 1968, *Wiley Interscience*
[17] Norman, R. O. C. & West, P. R., *J. chem. Soc. B*, 1969, 389
[18] Bobst, A., *Helv. chim. Acta*, 1968, **51,** 607
[19] Steitwieser, A., 'Molecular orbital theory for organic chemists', 1961, *Wiley*
[20] Blair, J. A. & Pearson, A. J., *Tetrahedron Lett.*, 1973, no 19, 1681
[21] Robb, A. J., Unpublished data, University of Aston in Birmingham
[22] Sih, C. J., *Science, Washington*, 1969, **163,** 1297
[23] Ikeda, M., Fahien, L. A. & Udenfriend, S., *J. biol. Chem.*, 1966, **241,** 4452