Kinetics and Mechanism of the Autoxidation of the 2-Amino-4-hydroxy-5,6,7,8-tetrahydropteridines

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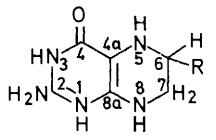
The autoxidation of tetrahydrofolic acid and tetrahydrobiopterin, 6-substituted derivatives of 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridines (tetrahydropterins), has been investigated by product analysis and kinetic methods. The overall reaction is first order in tetrahydropterin and approximately first 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 ($HO_2\cdot$), *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 = $CH_2\cdot NH$ $CO\cdot NH\cdot CH(CO_2H)\cdot[CH_2]_2\cdot CO_2H$

b; R = $CH(OH)\cdot CH(OH)\cdot CH_3$

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.

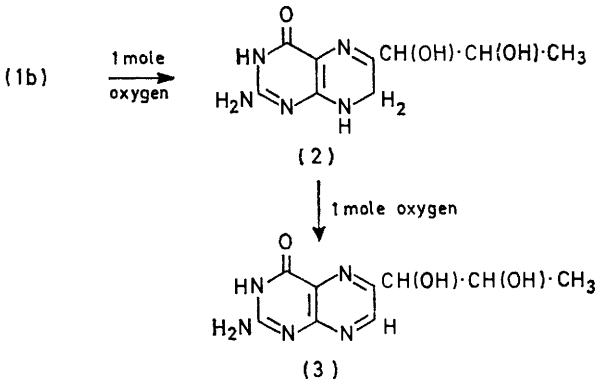
In an attempt to resolve the present controversy regarding the existence or non-existence of hydroperoxides in these autoxidations, the present study was undertaken, using tetrahydrobiopterin and tetrahydrofolic acid (1a) as model compounds.

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.



SCHEME 1 Reaction pathway for autoxidation of tetrahydrobiopterin at 25°

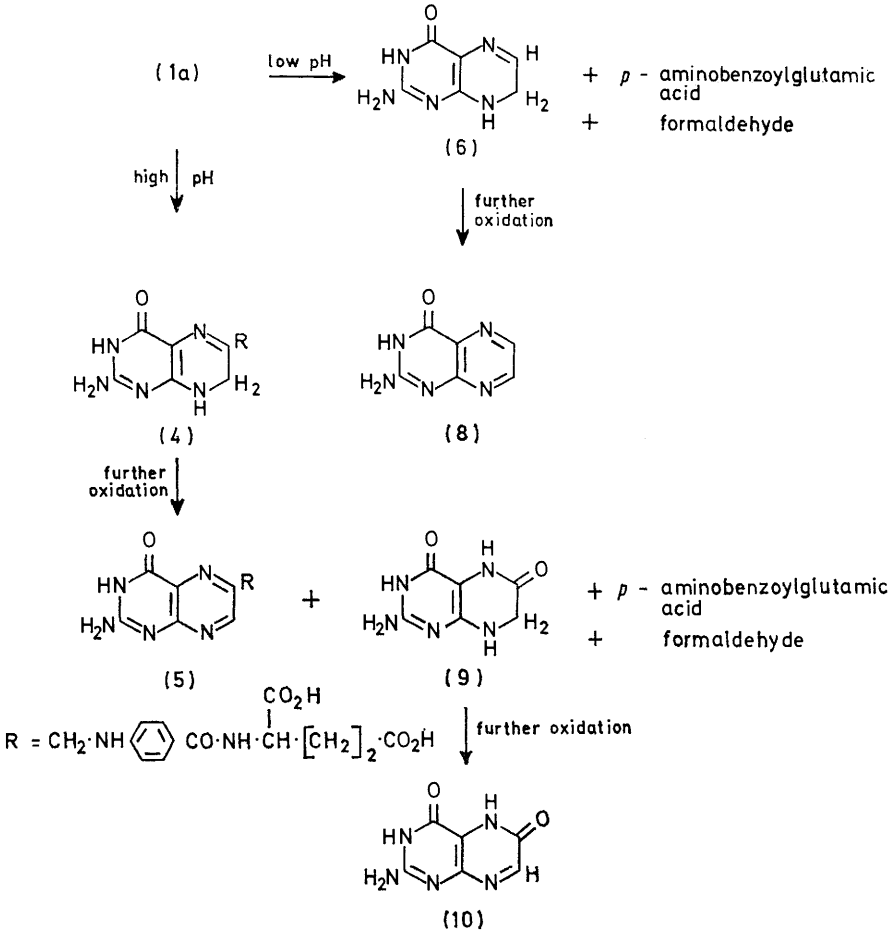
The reaction pathway has been further elucidated by determining the products at different times, using t.l.c., the results being shown in Table 2. It is clear that, at low pH, pterin or dihydropterin (inseparable on t.l.c.)

† 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 of

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

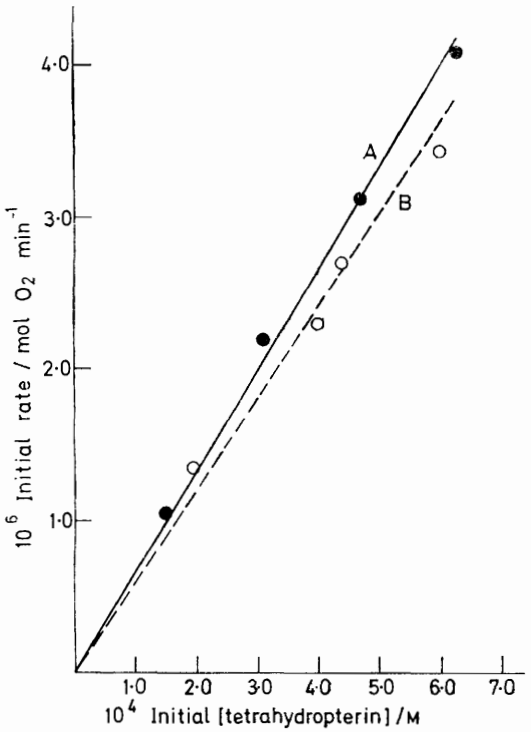


FIGURE 1 First-order plots for tetrahydropterin autoxidation (phosphate buffer; pH 7; pure oxygen; 25°): A, tetrahydrobiopterin (light rates); B, tetrahydrofolic acid (dark rates)

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

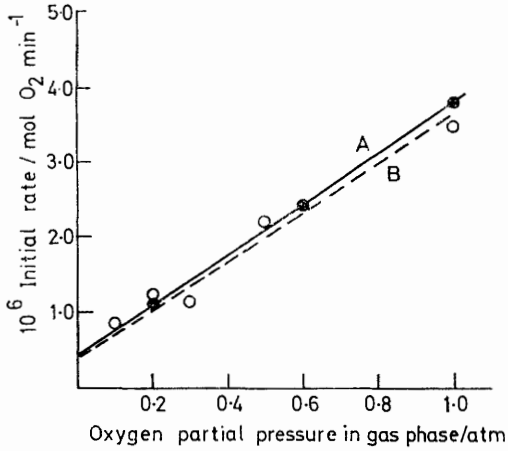


FIGURE 2 Dependence of O_2 uptake rate on oxygen concentration (0·1M-phosphate buffer; pH 7; 25°): A, 0·625mM-tetrahydrobiopterin dark rates; B, 0·7mM-tetrahydrofolic acid light rates

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

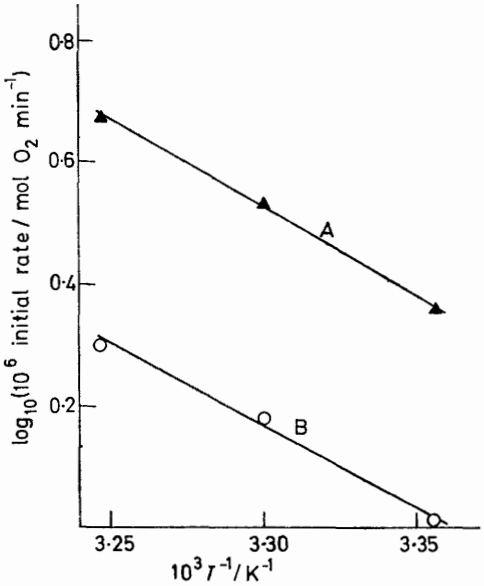


FIGURE 3 Temperature dependence of O_2 uptake rates (uncorrected). 'Arrhenius' plots for oxidations under air: A, 0·625mM-terahydrobiopterin in 0·1M-ammonium acetate buffer; B, 0·7mM-tetrahydrofolic acid in 0·1M-phosphate buffer

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

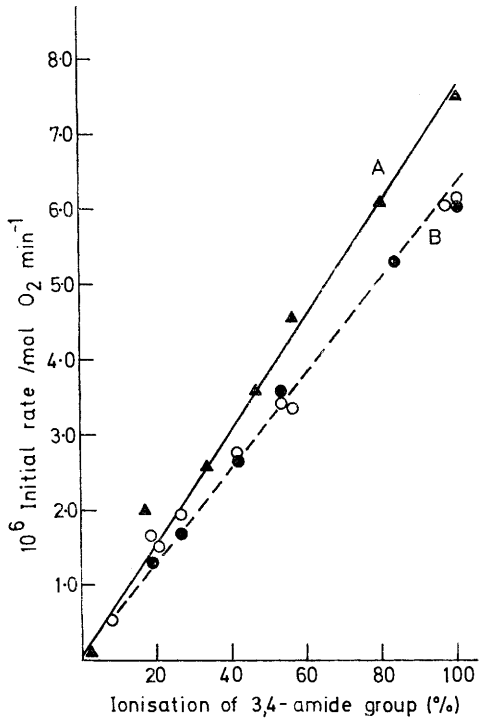


FIGURE 4 Dependence of corrected rates on ionisation of the 3,4-amide group under pure oxygen at 25°: A, 0·31mM-tetrahydrobiopterin (pK_a 10·5); B, 0·4mM-tetrahydrofolic acid (○) and 0·4mM-6,7-dideuteriated tetrahydrofolic acid (●) (pK_a 10·8)

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

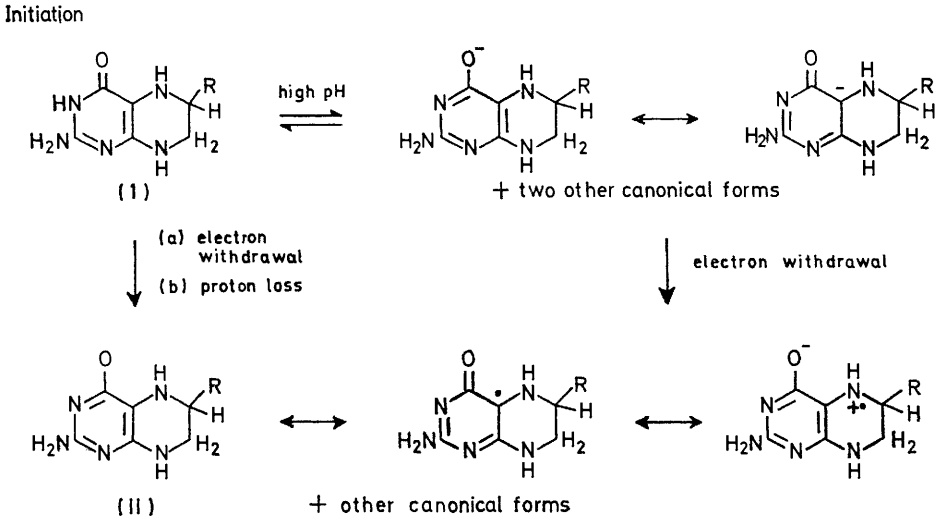
Free-radical kinetic characteristics

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.

shown for (11) is in complete agreement with e.s.r. data [11] and with HMO calculations of unpaired spin densities which we have made (Table 5).

During propagation (11) reacts with oxygen to give the chain carrier, and there are three possible ways this 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 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

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)	9·50	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

$$\text{Rate} = (k_2k_3/k_4)^{\frac{1}{2}}[AH_2][O_2] \quad (3)$$

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.

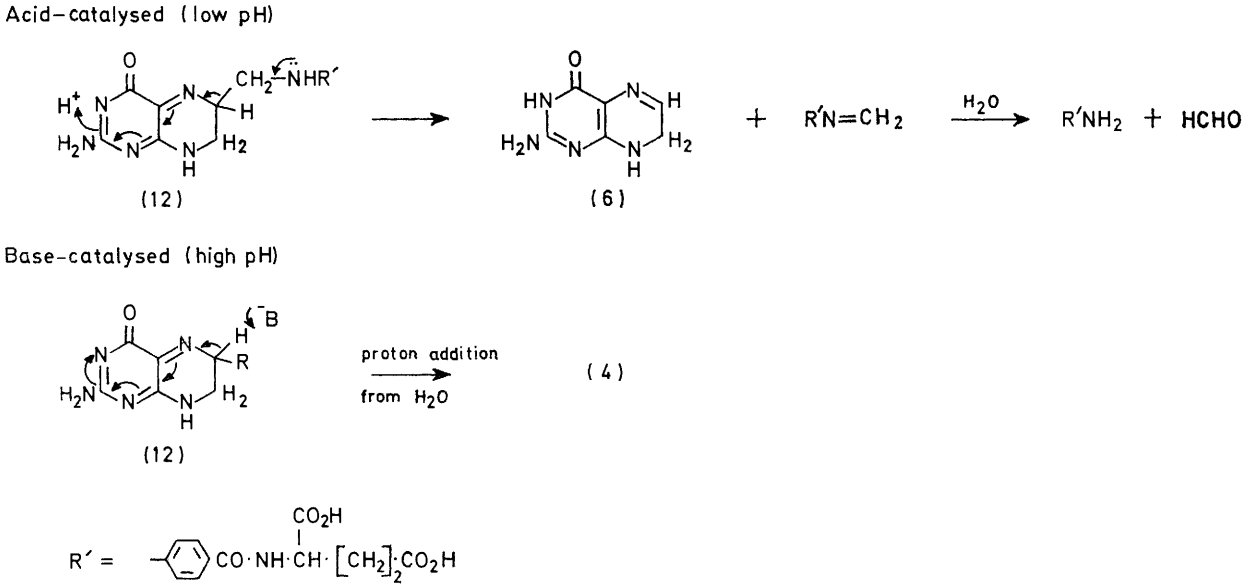
Spectrophotometric observation of the steady-state concentration of free radicals formed during tetrahydrobiopterin autoxidation ($\lambda_{max.}$ 550 nm *), and the corresponding overall rate, over a range of temperatures, shows that the product $k_2[O_2]$ increases considerably as temperature is raised (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.

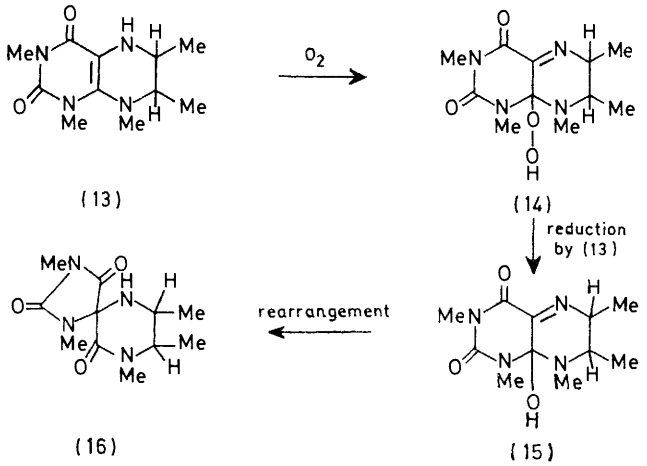
Thus, autoxidation must be as shown in Scheme 3, a possible mechanism recognised [14,15] for other compounds but for which no definite evidence has been described.

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. 5d)

* 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

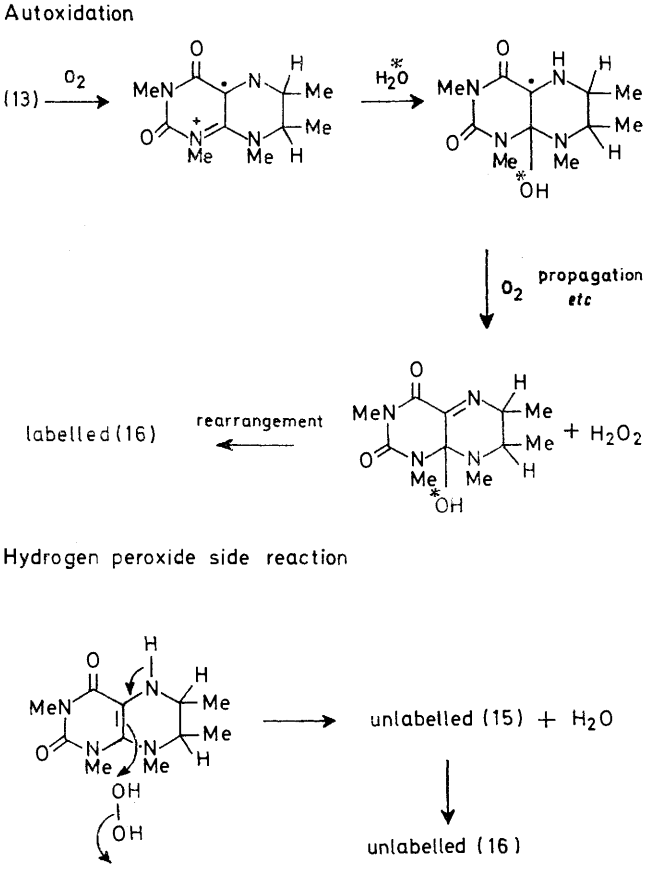
TABLE 6

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 cm^3) 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 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° 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

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[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.

of tetrahydropterin containing 0·1% 2-mercaptoethanol, using appropriate blanks containing 2-mercaptoethanol as reference. Sigmoid curves were obtained between pH 9 and 13 and the pH corresponding to maximum slope was taken as the pK_a of the 3,4-amide group (*cf.* ref. 11).

Kinetic Characteristics of Free Radical.—Tetrahydrobiopterin (25 mg) was stirred rapidly in 0·1M-ammonium acetate buffer solution (25 cm^3), pH 6·8, containing 10^{-4}M-EDTA (to remove effects of copper impurities present in ammonium acetate), and using a continuous stream of air above the solution. The optical density at 550 nm was determined at 1 min intervals, and the steady-state value noted, together with the time for attainment of steady-state conditions. The overall rate at this time was also determined for solutions of 20 mg tetrahydrobiopterin in 100 ml buffer (since absolute values of k_2 were not required this was found convenient), and the data shown in Table 5 for different temperatures were computed.

Autoxidative Ring Contraction of 1,3,6,7,8-*Pentamethyl*-5,6,7,8-*tetrahydropteri*-2,4-*dione in* ^{18}O-*Labelled Water.*—In these experiments the blocked pteridine (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; 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).

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