Autoxidation of 5-Methyl-5,6,7,8-tetrahydrofolic Acid

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The autoxidation of 5-methyl-5,6,7,8-tetrahydrofolic acid has been studied by kinetic methods and product analysis. The yields of the two major products, 5-methyl-5,6-dihydrofolic acid and 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydrofolic acid, were determined under a variety of conditions. Attempts to observe or isolate possible intermediate organic hydroperoxides failed. A mechanistic interpretation is considered.

We have recently reported [1] a kinetic study of the autoxidation of tetrahydrobiopterin and tetrahydrofolic acid, and have proposed a mechanism involving oxygen attack at C(4a) and implicating the hydroperoxyl radical ($HO_2\cdot$) as chain carrier. This paper reports a similar study on the related compound 5-methyl-5,6,7,8-tetrahydrofolic acid (1) (the major mammalian folate monoglutamate), which carries an N(5) substituent and might therefore be expected to reflect this in its autoxidation. Gapski *et al.*[2] have shown that (1) may be oxidised by hydrogen peroxide to yield 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydrofolic acid (2) and 5-methyl-5,6-dihydrofolic acid (3). We now report the characterisation of these compounds during the autoxidation reaction, together with their yields under various conditions.

RESULTS AND DISCUSSION

Products.—5-Methyl-5,6,7,8-tetrahydrofolic acid (barium salt) was autoxidised as described in the Experimental section. After complete oxidation, (2) and (3) were found to account for 99% of the starting material, estimated from areas under chromatography elution profiles using molar extinction coefficients of $17{\cdot}8 \times 10^3$ for the 4a-alcohol (2) (at 281 nm) and $31{\cdot}2 \times 10^3$ for 5-methyl-5,6-dihydrofolic acid (3) (at 292 nm). Yields of these compounds are given in Table 1. The ratio of (2) : (3) is seen to increase when autoxidation is continued for longer periods (*cf.* reactions A and C in Table 1). Also, deoxygenation of an oxidised solution, containing mainly (3), and standing leads to an increase in the alcohol content of the mixture, so it appears that this compound may be formed in part by addition of water to 5-methyl-5,6-dihydrofolic acid. The slight reduction in alcohol content of the products when catalase is incorporated indicates that it is also formed by reaction of (1) with hydrogen peroxide (see later), in agreement with the findings of Gapski *et al.*[2] A third route for the formation of the 4a-hydroxy-compound is the aquation of a quinonoid dihydrofolate intermediate [1,3] which in the case of 5-methyl derivatives would be a quaternary nitrogen compound (4) and would readily form the corresponding pseudo-base. The possible reaction path-

TABLE 1

Product analysis

Method of oxidation *	4a-Hydroxy-5-methyl-4a,5,6,7-tetrahydrofolic acid (%)	5-Methyl-5,6-dihydrofolic acid (%)
A	59·9	40·1
B	54·0	46·0
C	13·8	86·2
D	47·3	52·7

* As in Experimental section.

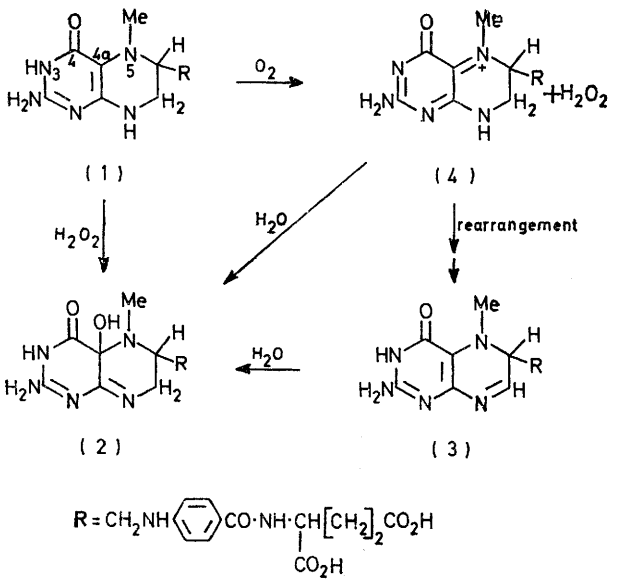
[1] J. A. Blair and A. J. Pearson, *J.C.S. Perkin II*, 1974, 80; A. J. Pearson, *Chem. and Ind.*, 1974, 233.

[2] G. R. Gapski, J. M. Whitely, and F. M. Huennekens, *Biochemistry*, 1971, **10**, 2930.

[3] M. C. Archer and K. G. Scrimgeour, *Canad. J. Biochem.*, 1970, **48**, 278; S. Kaufman, *J. Biol. Chem.*, 1961, **236**, 804; 1964, **239**, 332.

ways are summarised in Scheme 1, and these are essentially in agreement with our findings [1,4] on the autoxidative introduction of oxygen functions in other methylated reduced pteridines.

Attempts to isolate organic hydroperoxides on t.l.c. were unsuccessful, though hydrogen peroxide could be



SCHEME 1 Reaction pathways during autoxidation of 5-methyl-5,6,7,8-tetrahydrofolic acid

detected in distillates (Experimental section), in agreement with earlier observations. Consequently, it would

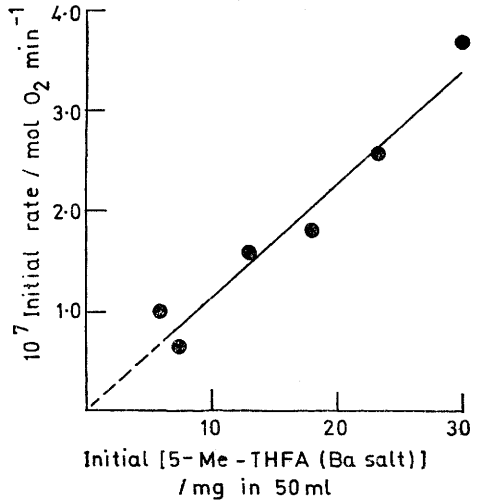


FIGURE 1 Dependence of autoxidation rates on concentration of 5-methyltetrahydrofolic acid (5-Me-THFA) (in 0·1M-phosphate buffer, pH 7, oxygen gas phase, 25°)

appear unlikely that (2) arises by reduction or decomposition of a 4a-hydroperoxide. Oxygen-18 labelling experiments were not undertaken owing to the low volatility and poor mass spectra of folate derivatives.

Kinetics.—The kinetics of the reaction are essentially parallel to those of the non-methylated compounds reported earlier [1] (though the data are rather less precise

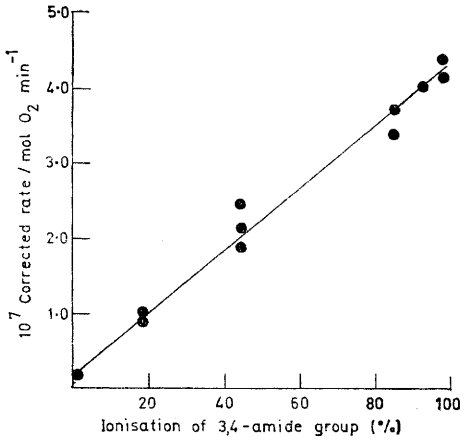


FIGURE 2 Plot of corrected rate (text) against % ionisation of 3,4-amide group (0·93mM-5-methyltetrahydrofolic acid, 25°, oxygen gas phase)

owing to the slowness of reaction leading to larger corrections due to atmospheric pressure fluctuations during the time of an experiment). Thus, the reaction is first order in (1) (Figure 1) and the rate increases in the region of pH corresponding to deprotonation of the 3,4-amide group (Experimental section). Assuming the rate equation (1),

$$\text{Rate} = k_a\,[\text{non-ionised 5-methyl tetrahydrofolate}] + k_b\,[\text{derived imino-enolate anion}] \quad (1)$$

estimating the first term on the right hand side at each pH and correcting the overall rate by subtraction of this, a linear dependence of corrected rate on per cent ionisation of the amide group (and therefore imino-enolate anion concentration) was found (Figure 2).

The reaction rate was also found to decrease markedly from pH 5·6 to 4, the region in which N(5) becomes protonated [5] (pK_a 5·2); below pH 4 it was too slow to be determined manometrically with any precision.

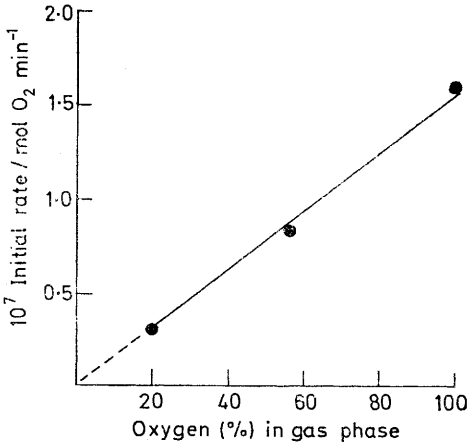


FIGURE 3 Dependence of autoxidation rates on % oxygen in gas phase (14 mg 5-methyltetrahydrofolic acid, barium salt, in 50 cm³ 0·1M-phosphate buffer, pH 7, 25°)

A first-order dependence on oxygen gas-phase concentration was found (Figure 3), a behaviour slightly dissimilar to the non-methylated compounds, which show a

[4] J. A. Blair and A. J. Pearson, *Tetrahedron Letters*, 1973, 1681.
[5] J. M. Whitely, J. H. Drais, and F. M. Huennekens, *Arch. Biochem. Biophys.*, 1969, **133**, 436.

distinct combination of first- and zero-order effects, the relative contributions of which are affected by visible light.[6] These phenomena are not completely understood at present.

Table 2 shows the effects of catalysts and inhibitors.

TABLE 2

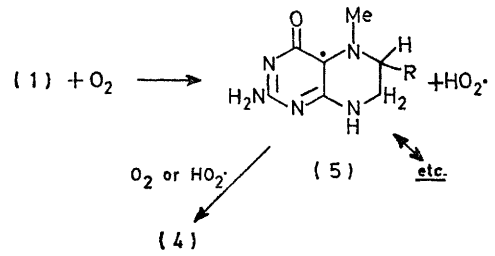
Effect of additives on autoxidation rates under oxygen gas phase (using 0·93 mM-5-methyl-5,6,7,8-tetrahydrofolic acid, unless otherwise stated)

Buffer solution (temperature 25° unless otherwise stated)	Additives or other modifications	10^7 Initial rate/ mol $O_2 min^{-1}$ (gas uptake)
0·1M-Sodium phosphate, pH 7	None	2·1
0·1M-Sodium phosphate, pH 7, 34°	None	3·7
0·1M-Sodium phosphate, pH 7	2mM-Phenol	1·4
0·1M-Sodium phosphate, pH 7	2mM-EDTA	2·0
0·1M-Sodium phosphate, pH 7	Light excluded	1·8
1·0M-Ammonium acetate, pH 7	None	4·4
1·0M-Ammonium acetate, pH 7	0·1mM-$FeCl_3$	4·1
1·0M-Ammonium acetate, pH 7	2mM-EDTA	2·7
Water	None	1·3
Water	0·1mM-$CrCl_3$	1·5
Water	0·1mM-$CuSO_4$	20·0
Water	0·1mM-$CuSO_4$, light excluded	19·0
0·1M-Sodium hydroxide, pH 13	None	4·4
0·8mM-Tetrahydrofolic acid in 0·1M-sodium phosphate, pH 7	None	62·8 *
0·8mM-Tetrahydrofolic acid in 0·1M-sodium hydroxide, pH 13	None	122·8*

* These determinations for solution volume of 100 cm^3, all others using 50 cm^3.

Copper at low concentrations accelerates the reaction, but the apparent specificity for copper catalysis indicates that this is not due to chain initiation but rather a reduction to copper(I) by (1) *and* by the free radical (5), followed by its rapid reoxidation to copper(II). The apparent inhibition by EDTA in some buffers is due to removal of copper impurities by complex formation, whilst inhibition by phenol and the involvement of ground state oxygen indicate a free radical chain process.

The overall activation energy is estimated to be 55—58 kJ mol^{-1} (temperature data in Table 2). An interesting observation is that the 5-methyl compound is autoxidised approximately ten times more slowly than is tetrahydrofolic acid itself (data in Table 2; note that solution volumes are different). This is consistent with our proposals that oxygen attack occurs at C(4a), when the presence



SCHEME 2 Proposed mechanism of autoxidation of 5-methyl-5,6,7,8-tetrahydrofolic acid

of an N(5) substituent would lead to either steric hindrance to oxygen approach or steric compression in any transition state for electron transfer from the reduced compound or radical (5) to oxygen or chain carrier. It is possible that the presence of the methyl group might lead to slight changes in mechanism, favouring addition of oxygen at C(4a) in (5) rather than electron abstraction, but in the absence of any evidence for the formation of hydroperoxides, we propose that the mechanism is analogous to that for the non-methylated compounds, as in Scheme 2. This is supported by the parallel overall kinetics, though we have been unable to determine the kinetic behaviour of the free radical, owing to interference by visible absorption from products (see ref. 1).

EXPERIMENTAL

U.v. spectra were recorded on Perkin-Elmer 137UV or Unicam SP 700 spectrophotometers and n.m.r. spectra for solutions in trifluoroacetic acid with Perkin-Elmer R14 or Varian HA 100D spectrometers. Folic acid was purchased from Koch–Light.

5-Methyl-5,6,7,8-tetrahydrofolic Acid.—This was prepared as the barium salt by the method of Blair and Saunders,[7] except that antioxidants were omitted from the final stages of isolation to prevent any interference in the kinetic studies. The product gave $\lambda_{max.}$ (log ε) 270 and 293 nm at pH 1, 292 (4·48) nm at pH 7, and 290 nm at pH 13, τ 2·0 and 2·5 (each 2H, d, *J* 8 Hz, benzene ring), 4·9br (1H, α-CH of glutamate), 5·7 (1H, m, 6-H), 5·9br and 6·1br (each 2H, 7- and 9-H_2), 6·7 (3H, s, 5-NMe), and 7·2 and 7·6 (2H each, CH_2 groups of glutamate) (Found: C, 35·65; H, 4·7; N, 14·7. Calc. for $C_{20}H_{23}BaN_7O_6 4H_2O$: C, 36·0; H, 4·3; N, 13·6%).

4a-Hydroxy-5-methyl-4a,5,6,7-tetrahydrofolic Acid.—The barium salt was obtained for characterisation of reaction products, using the method of Gapski *et al.*,[2] and gave $\lambda_{max.}$ (log ε) 279 and 395 nm at pH 1, 281 (4·24) nm at pH 7, and 281 nm at pH 13, τ 2·1 and 3·0 (each 2H, d, *J* 8 Hz, benzene ring), 5·0br (1H, α-CH of glutamate), 5·2 (1H, d, J_{gem} 14 Hz, 7-H), 5·6 (1H, d, J_{gem} 14 Hz, 7-H), 5·8br (1H, 6-H), 6·0 (2H, 9-H_2), 6·6 (3H, s, 5-NMe), and 7·26 and 7·5 (2H each, CH_2 groups of glutamate) (Found: C, 35·45; H, 4·25; N, 13·7. Calc. for $C_{20}H_{23}BaN_7O_7,4H_2O$: C, 35·15; H, 4·55; N, 14·4%). (N.m.r. assignments based on Gapski *et al.*[2])

5-Methyl-5,6-dihydrofolic Acid, Barium Salt.—This compound was isolated from reaction C (see product analysis section) by column chromatography and gave $\lambda_{max.}$ (log ε) 267 and 290sh nm at pH 1, 251 (4·34) and 292 (4·48) nm at pH 7, and 282 nm at pH 13 (Found: C, 35·3; H, 4·25; N, 13·95. Calc. for $C_{20}H_{21}BaN_7O_6,4H_2O$: C, 35·2; H, 4·5; N, 14·3%), n.m.r. not obtainable owing to the rearrangement of this compound to the 5,8-dihydro-isomer in trifluoroacetic acid; in D_2O there is considerable overlap from a HDO peak.[8]

Product Analysis.—Oxidations and yield determinations were as follows. (A) 5-Methyl-5,6,7,8-tetrahydrofolic acid, barium salt (40 mg) was stirred in distilled water (50 ml) under oxygen for 15 h at 25°. (B) As (A) with catalase 1,200 Keil units g^{-1}; 25 mg) present in the oxidation medium. (C) The tetrahydrofolate (40 mg) was oxidised in 0·1mM-copper sulphate solution (50 ml) at 25° for 1 h. (D) As (C) except that, subsequent to the 1 h oxidation, the solution was deoxygenated with a stream of nitrogen and left under nitrogen for 14 h.

Each reaction solution was then lyophilised, the residue

[6] J. A. Blair and A. J. Pearson, *J.C.S. Perkin II*, 1974, 1786.
[7] J. A. Blair and K. J. Saunders, *Analyt. Biochem.*, 1970, **34**, 376.
[8] A. J. Robb, unpublished data.

dissolved in 0·1M-ammonium acetate (1·5 ml), and placed on a DEAE Sephadex (A25) column [10 × 8 mm; previously swollen, equilibrated, and washed with 0·1M ammonium acetate (500 ml)]. Elution was carried out with 0·13M-ammonium acetate, the first 120 ml discarded, and subsequent 15 ml fractions collected automatically. The u.v. spectrum of each fraction was recorded after accurate dilution with 0·1M-ammonium acetate, and the yields of both products, (2) and (3), estimated from the elution profile peak areas, using the appropriate extinction coefficients (Results section).

Search for Peroxides.—Solutions of oxidised material were subjected to t.l.c. (cellulose MN 300 layer) in three solvents (0·1M-sodium phosphate buffer, pH 7; n-propanol–water–ammonia (*d* 0·88) (200 : 100 : 1 v/v); organic phase of n-butanol–acetic acid–water (4 : 1 : 5 v/v)], and the developed chromatograms sprayed with 1% aqueous *NN*-dimethyl-*p*-phenylenediamine hydrochloride. In no case were positive results given. Hydrogen peroxide was detected as follows. Oxidation solutions were chilled and submitted to distillation under high vacuum, the distillate being collected in a dry ice–acetone trap. The resulting distillate reacted with ferrous ammonium thiocyanate solution giving a red colour, thus indicating the presence of hydrogen peroxide.

Kinetics.—Initial rate data were obtained using the manometric technique previously described.[1]

pK_a *of 3,4-Amide Group.*—This was determined as described previously [1] giving a value of 10·9.

Characterisation of Products.—Products were identified by comparison with authentic materials (t.l.c. in three solvents as above, and u.v. spectra at pH 1, 7, and 13).

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