Pterins.† Part 9.[1] The Structure of Quinonoid Dihydropterins [2-Amino-7,8-dihydropteridin-4(6*H*)-ones]

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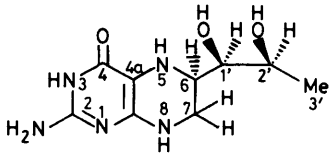
A comparative study of the rates of oxidation of 2-amino-*cis*-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (6), its 2-methylamino (7), 2-dimethylamino (8), 8-methyl (9), and 3-methyl (10) derivatives, and their 6,7-dideuterio-derivatives by molecular oxygen to the corresponding quinonoid 7,8-dihydro-6*H*-pterins, the rates of rearrangement of these to the respective 7,8-dihydro-3*H*-pterins, and the reduction of these quinonoid dihydropterins by NADH in Tris chloride buffer at pH 7.6 and 25° are reported. These data together with the lack of significant u.v. spectral shifts in the bands of the transient spectra of quinonoid 7,8-dihydro-6*H*-pterin, its 6-methyl and 2-dimethylamino-6,7-dimethyl derivatives in buffers between pH 6.8 and 11.6 strongly support the '*ortho*' quinonoid dihydropterin structure (3) over the 'exocyclic *para*' quinonoid (2) and 'endocyclic *ortho*' quinonoid (4) structures. A mechanism whereby hydride from NADH is transferred to N(5) and a proton to N(3) of quinonoid dihydropterin substrates in the enzymic reduction by dihydropteridine reductase is proposed.

QUINONOID dihydropteridine reductase (DHPR) (E.C. 1.6.99.7) catalyses the reduction of quinonoid dihydrobiopterin,‡ formed during enzymic hydroxylation of phenylalanine, tyrosine, and tryptophan by the respective hydroxylases, to tetrahydrobiopterin (1) which is



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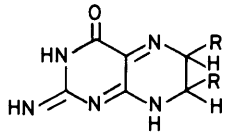

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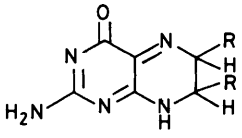

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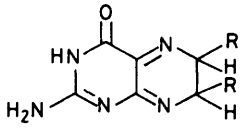

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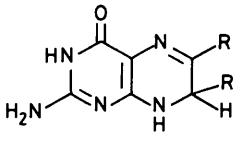
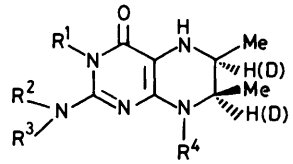


(5)

the essential cofactor for these hydroxylations.[2] The enzyme may also play a part in other biological processes mediated by these reduced pterins.[3] The elegant studies of Kaufman have shown that quinonoid dihydropterins have a double bond between C(4a) and N(5) [for numbering see structure (1)],[4] but the tautomeric form of the rest of the molecule is not known. Based on some spectral evidence [4-6] and molecular orbital calculations [7] the '*para*' tautomer (2) has been favoured, but the '*ortho*' tautomers (3) and (4) cannot be ruled out from these data. Indeed, Pfleiderer,[8] Rhembold,[9] and Scrimgeour [10] draw these pterins in the tautomeric form (3).

Quinonoid dihydropterins are the first identifiable products of oxidation of simple tetrahydropterins. They cannot be characterised in conventional ways because they very rapidly rearrange to the thermodynamically more stable 7,8-dihydro-3*H*-isomers (5) which are not substrates for DHPR. Our attempts to isolate milligram quantities of quinonoid dihydropterins using specific conditions gave only mixtures of the 7,8-dihydro-3*H*-(5) and the fully oxidised pterins. We have prepared a series of four methyl derivatives (7)—(10) of *cis*-6,7-dimethyl-5,6,7,8-tetrahydropterins (and the corresponding 6,7-dideuterio-compounds), and made a comparative study of the oxidation of these compounds and their parent (6) to the quinonoid forms together with the subsequent rearrangement and reduction by NADH in order to distinguish unequivocally between the three likely tautomeric forms (2)—(4). Evidence is presented for the '*ortho*' quinonoid structure (3).



(6) $R^1 = R^2 = R^3 = R^4 = H$
(7) $R^1 = R^3 = R^4 = H$, $R^2 = Me$
(8) $R^1 = R^4 = H$, $R^2 = R^3 = Me$
(9) $R^1 = R^2 = R^3 = H$, $R^4 = Me$
(10) $R^2 = R^3 = R^4 = H$, $R^1 = Me$

RESULTS

The oxidation to the quinonoid form and rearrangement to the 7,8-dihydro-3*H*-isomers of compounds (6)—(10) can be conveniently followed by changes in u.v. spectra (see

† Pterin is 2-aminopteridin-4(3*H*)-one.
‡ The systematic name is 1'*R*,2'*S*,6*R*-2-amino-6-(1,2-dihydroxypropyl)-7,8-dihydropteridin-4(6*H*)-one.[1]

Experimental section). Table 1 contains the data for the oxidation of the tetrahydropterins (6)—(10) to the respective quinonoid 7,8-dihydro-6*H*-pterins * in 0.1M-Tris buffer. In all cases except for compound (10) the 6,7-dideuterio-compounds were used. This eliminated any complications due to subsequent rearrangement since the rearrangement of quinonoid (6) exhibits a large deuterium isotope effect

TABLE 1

Aerial oxidation of 6,7-dimethyltetrahydropterins in oxygenated Tris chloride buffer at pH 7.6 and 25°

Compound	k_{obs}/min^{-1}	$t_{1/2}$/min
(6) *	0.129	5.4
(7) *	0.165	4.2
(8) *	0.431	1.6
(9) *	0.218	3.2
(10)	0.059	11.6

* These are 6,7-dideuterio-derivatives.

(see Table 3).[11] The relative rates of oxidation and rearrangement in these cases are at least 1 : 20 [for (6)] and 1 : 350 [for (8)]. The oxidation and the rearrangement reactions can thus be examined separately. The rearrangement of quinonoid (10) is exceptionally fast (see later) and the rate-limiting step in the formation of the 7,8-dihydro-3*H*-pterin from the tetrahydropterin (10) is the aerobic oxidation to quinonoid (10). This is the value

TABLE 2

Effect of Tris chloride concentration on the aerial oxidation of *cis*-6,7-dimethyl-5,6,7,8-tetrahydropterin at 25° [a]

k_{obs}/min^{-1}	[Tris]/M
1.520	0.01
0.585	0.05
0.329	0.10
0.160	0.20

[a] In Tris chloride buffer saturated with oxygen at pH 7.6 and constant ionic strength of 1M by addition of KCl.

given in Table 1. In all cases the rates of oxidation followed pseudo-first-order kinetics with respect to pterin concentration for at least 70% or more of the reaction. The first-order nature of the oxidation with respect to pterin was confirmed in the case of compound (6) by a plot of ln-[tetrahydropterin] against ln[initial velocity] which gave a typical straight line with a slope of 0.96 for the experimentally determined order. Deviations from first-order rates after 70% of the reaction were always towards larger values of k_{obs} probably reflecting an autocatalytic process (see later).

In the course of the study we found that the rates of oxidation of the parent compound (6) varied inversely as the concentration of the buffer (at constant ionic strength) and the data are in Table 2. Blair and Pearson [12] also noticed a buffer effect in the oxidation of tetrahydrobiopterin and attributed it to the presence of varying concentrations of Cu^{2+} ions which are known to accelerate oxidation. Such an explanation is not consistent with our data. A plot of $1/k_{obs}$ against [Tris] is linear (Figure 1), and reflects the participation of the buffer in the oxidation process, and not the catalytic effect of metal ions because the rate decreases with increasing concentration of Tris in the buffer (see Discussion section). Although we also found

* Throughout this paper quinonoid (6)—(10) will refer to the quinonoid 7,8-dihydro-6*H*-pterins derived from the tetrahudropterins (6)—(10).

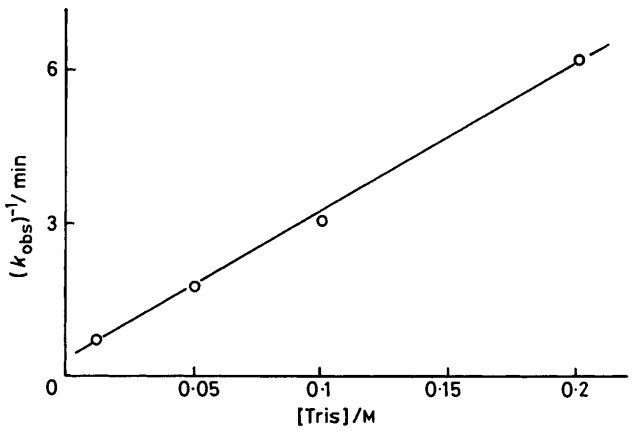


FIGURE 1 Effect of concentration of Tris on the rate of oxidation of *cis*-6,7-dimethyl-5,6,7,8-tetrahydropterin at ionic strength 1M (adjusted by addition of potassium chloride) and 25° (see Table 2)

that Cu^{2+} ions (as $CuSO_4$ at 10^{-4}M final concentration) do greatly accelerate the oxidation (>50 fold), there was no effect of added EDTA (at 10^{-4}M) on the normal aerobic oxidation presumably because dissolved Cu^{2+} ions were almost absent in our buffer. The oxidations were greatly accelerated by peroxidase alone (*ca.* 12 fold at an enzyme concentration of 8.0 μg ml^{-1}), but were unaffected by excess of hydrogen peroxide (compare ref. 13), and by catalase (at 270 units ml^{-1}). On the other hand catalase at concentrations above 0.1 mg ml^{-1} (1 500 unit ml^{-1}) decreased the rate of aerobic oxidation by *ca.* 40%, as did about twice this concentration of bovine serum albumin.[14] It must be pointed out that catalase has a k_{cat} value of 0.4×10^8 s^{-1} for the decomposition of hydrogen peroxide which is close to being diffusion controlled.[15] The rates of oxidation were retarded to the extent of *ca.* 30% by phenol (at 0.5×10^{-4}M) in agreement with the observations of Blair and Pearson.[12]

TABLE 3

Rearrangement of quinonoid dihydropterins at 25° [a]

7,8-dihydro-6*H*-pterins derived from:	[10^2 k_{obs}/min^{-1} ($t_{1/2}$/min)] 6,7-H_2	6,7-D_2	k_H/k_D
(6)	5.87 (11.8)	0.63 (112)	9.5
(7)	2.00 (34.6)	0.23 (300) [b]	9.0
(8)	1.37 (50.5)	0.1 (690) [b]	10
(9)	6.39 (10.8)	1.14 (60.8)	6.0
(10)	>70 (<1) [c]	*d*	

[a] In 0.1M-Tris chloride buffer at pH 7.6. [b] *ca.* 20% Error. [c] k_{obs} was 0.198 min^{-1} ($t_{1/2}$ 3.5 min) at 5°. [d] Deuterio-compound gives 7,8-dihydro-3*H*-pterin and unidentified products.

The rates of rearrangement of quinonoid (6)—(10) for the protio- and 6,7-dideuterio-compounds are in Table 3. The quinonoid compounds were generated using an excess of peroxidase, and the rates were followed spectrophotometrically. Excellent first-order rates were observed for the protio-compounds up to 90% of the rearrangement reactions, and the values are estimated with an accuracy of 6%. The measured rates of rearrangement of 6,7-dideuterioquinonoids (7) and (8) are less accurate (20%) because in these cases the slow rearrangement of the

deuteriated compounds is accompanied by further oxidation of the resulting 7,8-dihydro-3*H*-pterins to the respective pterins. The rates for these compounds were measured only over the first 20% of reaction, and were calculated using the A_∞ values based on the known extinction coefficients of the protio-compounds. The presence of deuterium atoms has an insignificant effect on the values of the extinction coefficients. The quinonoid dihydropterin derived from the 3-methyl compound (10) was undetectable at 25°, and the formation of the 7,8-dihydro-3*H*-isomer was complete within 2 min. However, quinonoid (10) can be generated at 5°; consequently the rate of rearrangement at this temperature can be measured. In this case the deuteriated derivative of the quinonoid (10) was not used because the slow rearrangement allowed side reactions to become significant.

TABLE 4

Initial rates of oxidation of NADH by quinonoid dihydropterins in 0.1M-Tris at pH 7.6 [a]

Quinonoid dihydropterin derived from:	Rate (μmoles NADH/min × 10^3)
(6)	7.6 (25°)
	1.9 (5°)
(7)	5.2 (25°)
(8)	1.3 (25°)
	<0.3 (5°)
(10)	15 (5°)

[a] [Pterin] 1 × 10^{-4}M; [NADH] 1.5 × 10^{-4}M.

Quinonoid dihydropterins are reduced nonenzymically by NADH.[4,16] The 7,8-dihydro-tautomers (5), on the other hand, either resist reduction by NADH or are reduced at extremely slow rates. We have measured the initial rates of oxidation of NADH by the quinonoid dihydropterins (6)—(8) and (10) under identical conditions, and these are listed in Table 4. The rates were measured by following the loss of absorbance of NADH at a predetermined wavelength corresponding with an isosbestic point in the interconversion of the tetrahydro- and quinonoid pterins (see Experimental section). The rates of reduction of quinonoid (10) could not be measured at 25° because it rapidly rearranged (see above). A rate at 5°, however, can be measured and is compared in Table 4 with the values for the parent and the 2-dimethylamino-compound at this temperature.

The u.v. spectra of compounds (6)—(10) and of the corresponding quinonoid derivatives are in Table 5.

TABLE 5

U.v. spectra of quinonoid dihydro- and tetrahydro-pterins in aqueous solution at 25°

Compound [a]	Species [b]	$\lambda_{max.}$/nm	log ε
T-(6)	+	266	4.13
	○	244,	4.19,
		300	3.94
Q-(6)	○	250,	3.75,
		306	3.86
T-(7)	+	270	4.14
	○	244,	3.97,
		308	4.08
Q-(7)	○	244,	3.84,
		324	4.03
T-(8)	+	274	4.04
	○	304	3.87
Q-(8)	○	344 [c]	3.96
T-(9)	+	270	4.06
	○	304	3.95
Q-(9)	○	308	3.84
T-(10)	+	266	4.07
	○	304	3.95
Q-(10)	+ [d]	302 [e]	3.82

[a] T = tetrahydropterin, Q = quinonoid dihydropterin. [b] + is in 0.004M-HCl, pH 2.2; ○ is in 0.1M-Tris chloride at pH 7.6 [quinonoid dihydropterins (pK_a 5) [11] are neutral species]. [c] Reported value [4] of 320 nm is incorrect; see also ref. 5. [d] Cation at pH 7.6. [e] Approximate values measured within 1 min of mixing at 5°.

DISCUSSION

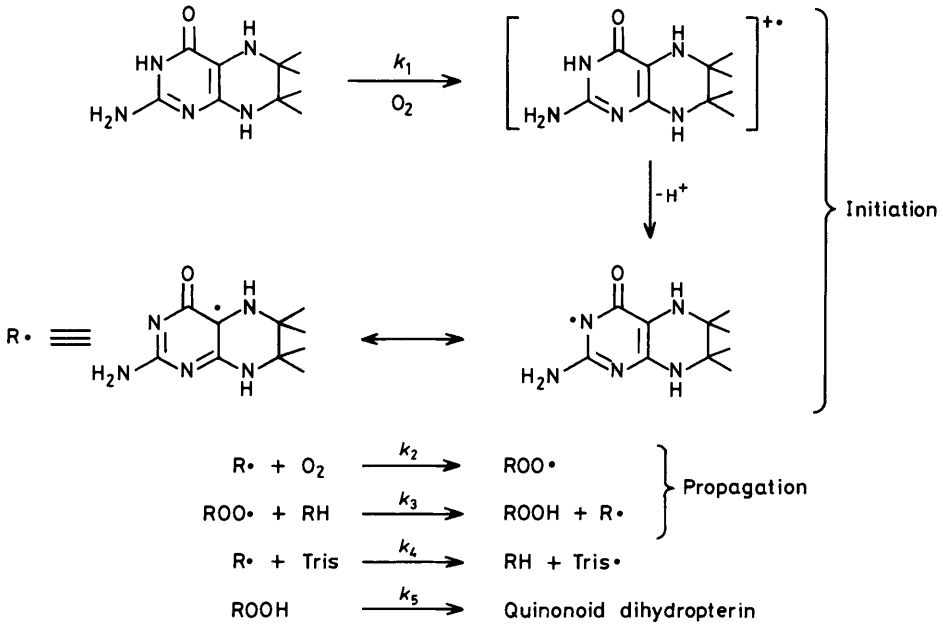
Our first observation which made us favour the quinonoid structure (3) came from measuring the u.v. spectra of the quinonoid dihydropterins derived from 5,6,7,8-tetrahydropterin, and its 6,7-dimethyl and 6,7-dimethyl-2-dimethylamino derivatives in buffers with pH values varying between 6.8 and 11.6. We found that the typical quinonoid dihydropterin spectra with $\lambda_{max.}$ 302, 303, and 344 nm (broad peaks) respectively from these compounds at pH 6.8 were almost identical at all pH values up to 11.6 except for very small solvent shifts (*ca.* 2—6 nm). These spectra altered with time as the rearrangements proceeded. Structures (2) and (4), but not (3), should each have an acidic pK_a in this region of pH (because of the lactam hydrogen atom), and should exhibit substantial spectral differences between neutral species and anions.[17] Further support for structure (3) from comparative rate studies is discussed below.

The autoxidation of tetrahydropterins has been the subject of some interest because of their cofactor activity in enzymic hydroxylations, but it was not our aim to undertake a detailed study of the aerobic oxidation of tetrahydropterins. However, we are reporting some of our findings because they shed some light on the structure of the intermediate quinonoid dihydropterins. Mager and his co-workers have examined the aerial oxidation of tetrahydropterins and related systems, and favour a hydroperoxy-intermediate.[18] Blair and Pearson,[12] on the other hand, proposed a radical mechanism in which peroxy-intermediates do not play kinetically significant parts, and this is proposed by others.[10] In the absence of conclusive evidence to the contrary, we have formulated a mechanism of oxidation which involves radical and peroxy-intermediates as shown in the Scheme. This is similar to the usually formulated scheme for autoxidation of organic compounds.[19] By applying the usual steady-state assumptions with respect to the intermediates, the rate of the reaction is given by equation (1), and is first-order with respect to tetrahydropterin.

$$\text{Rate} = \frac{k_1 k_2 [O_2]^2}{k_4 [\text{Tris}]} . [\text{Tetrahydropterin}] \quad (1)$$

Retardation of the reaction at higher Tris concentrations is explained by abstraction of a hydrogen atom from Tris by the intermediate tetrahydropterin radical (see Table 2). Competitive abstraction from solvent water (O–H bond strength *ca.* 504 kJ mol^{-1}) would be unfavourable given the likely smaller bond energy of a secondary C–H bond (*ca.* 415 kJ mol^{-1}) as in Tris.[20] As

the reaction proceeds this simple scheme breaks down because other reactive intermediates (*e.g.* Tris radicals) accumulate and the process becomes autocatalytic. Other intermediates, *e.g.* a 4a-hydroxy-compound, may be involved but have been omitted for simplicity. Equation (1) accounts for the observed first-order kinetics in tetrahydropterin and the inverse effect of



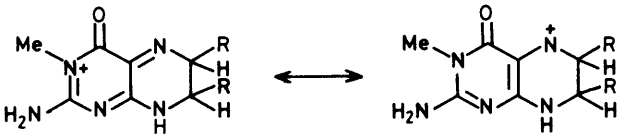
SCHEME

Tris concentration. Additional evidence for the hydroperoxy-intermediate comes from our observation that peroxidase, in the absence of hydrogen peroxide, accelerates the formation of the quinonoid dihydropterin, and the initial rate of this enzymic oxidation is unaffected by catalase (at 216 units ml^{-1}, *cf.* k_{cat}). It suggests that a peroxy-intermediate may be acting as a substrate for the peroxidase, and will have the effect of accelerating cleavage of the O–OH bond.[21] This process is analogous to the oxidation of indol-3-ylacetic acid by peroxidase alone.[22] The oxidation of indol-3-ylacetic acid, like other substituted indoles, almost certainly proceeds through a hydroperoxy-intermediate.[23]

The effect of alkyl groups on the rates of oxidation is consistent with the Scheme, and with the '*ortho*' quinonoid structure (3) for the product of oxidation because the rate of oxidation of compound (10) is significantly slower than those of compounds (6)—(9). The rate of oxidation of the 8-methyl derivative (9) is of the same order as those of compounds (6)—(8), and is further evidence against structure (4). Aerial oxidation of 5,6-dimethyl-5,6,7,8-tetrahydropterin at pH 7.0 in the absence or presence of peroxidase does not give a quinonoid dihydropterin. The u.v. spectrum of the oxidation product has one maximum at 235 nm (compare with Table 5).

The polar effects of alkyl groups in accelerating free radical reactions is well known,[24] and the above can be explained in terms of the more favourable formation of the radical cation intermediate (see Scheme) before proton loss. The decreased rate of oxidation of compound (10) clearly shows that proton loss is normally from N(3) (consistent with refs. 10 and 12) with consequent formation of structure (3). The inhibiting effect of *N*-methyl groups on the rates of oxidation of dihydropyridines[25] and substituted indoles[26] is in agreement with this. The formation of the '*para*' quinonoid structure (2) should not be seriously affected by a 3-methyl group.

In the rearrangement of quinonoid dihydropterins to 7,8-dihydro-3*H*-pterin *N*-methyl groups should not greatly perturb the quinonoid forms unless the methyl groups are on nitrogen atoms which form part of the conjugated system. *N*-Methyl groups do not enhance the basic strength by more than 0.5 pK_a units[27] and cannot be the reason for the greatly enhanced rate of rearrangement of quinonoid (10). In any case, the quinonoid compounds derived from (7) and (8) rearrange slower than the parent (6). The abnormal behaviour of quinonoid (10) is not consistent with the '*para*' structure (2). It can be explained if the initial oxidation product of compound (10) has the '*ortho*' structure (3) with the result that a positive charge builds up on N(3), *i.e.* a pterinium cation (11) is formed. Such a molecule would be subject to very rapid rearrangement since it experiences the equivalence of a full acid catalysis. The rearrangement of quinonoid dihydropterins was shown to be subject to general acid and general base catalysis.[11] If the quinonoid form has the structure (3) it then becomes necessary to explain the slower rates of rearrangement of quinonoids (7) and (8). The fact that the rates of rearrangement have been shown to be acid catalysed and that progressive methylation of the 2-amino-group



(11) (12)

reduces the rates suggested steric hindrance to general acid catalysis. Archer and Scrimgeour [11] measured the relative contributions to acid and base catalysis according to equation (2), where k_{obs} is the pseudo-first-order rate constant of rearrangement, and [B] and [BH] are the concentrations of free base and protonated base

$$k_{obs} = k_B[B] + k_{BH}[BH] \quad (2)$$

$$k_2 = \frac{k_{obs}}{[Tris]_T} = (k_B - k_{BH})\alpha + k_{BH} \quad (3)$$

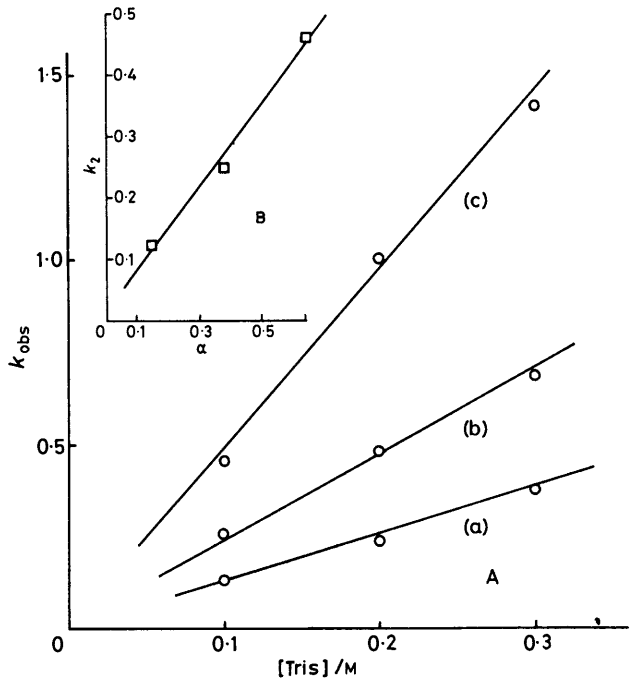


FIGURE 2 A, Plots of the first-order rate constants for the rearrangement of 2-dimethylamino-*cis*-6,7-dimethyl-7,8-dihydropteridin-4(6*H*)-one (8) *versus* the Tris concentration at constant ionic strength of 1M (adjusted by addition of potassium chloride). (a) pH 7.32; (b) pH 7.88; (c) pH 8.29 at 25°. B, Plot of the slopes of the lines in A, *i.e.* second-order rate constants k_2 against α the molar fraction of Tris at 25° [see equation (3)]

respectively in the buffer. On dividing equation (2) by the total buffer concentration and rearranging one obtains equation (3),[28] where α is the proportion of free base and $[Tris]_T$ is the total concentration of Tris buffer. The value of the second-order rate constant for varying pH values (*i.e.* varying α) can be found by plotting k_{obs} *versus* $[Tris]_T$. This is shown for three pH values in Figure 2A for the 2-dimethylamino-compound. A plot of k_2 against α (obtained from pH, the value of the pK_a of 8.4 for Tris at 25°, and the Henderson–Hasselbach equation) [28] shown in Figure 2B gives the values of k_B of 0.69 l mol^{-1} min^{-1} and k_{BH} of 0.01 l mol^{-1} min^{-1} for the rearrangement of quinonoid (8) in Tris buffer. These should be compared with the corresponding values obtained for the parent quinonoid (6) under the same conditions which are 1.5 l mol^{-1} min^{-1} and 0.1 l mol^{-1} min^{-1}.[11] Clearly there is greatly reduced acid catalysis in the rearrangement of quinonoid (8). This is probably due to steric effects to protonation of N(3). Steric effects in such general acid catalysis are known to occur.[29] Interestingly there is also significantly reduced base catalysis perhaps due to the acid weakening effects of a 2-dimethylamino-group compared with a 2-amino-group (σ_{NH_2} −0.66, σ_{NMe_2} −0.83) [30] on 6-H of the quinonoid form transmitted through the conjugated system. These results are inconsistent with a '*para*' quinonoid structure (2) for quinonoid (8) which requires a positive charge on the exocyclic nitrogen atom.[4,5] Such a compound would be expected to rearrange faster than the parent. The fact that the rate of rearrangement of quinonoid (7) is intermediate between that of the parent and the 2-dimethylamino-compound, and that an 8-methyl group has little effect on the rate is consistent with the above interpretation.

The deuterium isotope effects in Table 3 are in general agreement with the value previously obtained for quinonoid (6).[11] The 6,7-dideuterio-analogues of quinonoids (7) and (8) rearrange much too slowly for accurate rate measurement. Although not easily detectable by u.v. spectroscopy, significant amounts of fully oxidised pterin are formed even during the rearrangement of 6,7-dideuterioquinonoid (6). This can be followed by h.p.l.c. The further oxidation of 6,7-dideuterioquinonoid (7) and quinonoid (8) after the rearrangement makes accurate rates difficult to measure. The deuterium isotope effects (k_H/k_D) are however sound. The lower value for k_H/k_D of 6.0 for quinonoid (9) is as yet unexplained (buttressing effect of the 8-Me group?).

The data for the initial rates of oxidation of NADH by quinonoid dihydropterins (6)—(8) and (10) (Table 4) support the '*ortho*'-quinonoid structure (3). The rate of reduction of quinonoid (10) was almost eight times that of the parent quinonoid (6) and 50-fold faster than the dimethylaminoquinonoid (8) at 5°. These ratios can be out by a factor of two or more because the initial concentration of quinonoid (10) alters rapidly during the first minute due to its much faster rate of rearrangement. In all cases except for quinonoid (10), the 6,7-dideuterio-derivatives were used. The faster rate of reduction of quinonoid (10) is consistent with structure (11). Although five bonds away, the accelerating effect of a positive charge on N(3) would be transmitted through the conjugated bonds to N(5) *via* resonance contributions from a nitrenium ion (12). The effect of protonation (and methylation) on the rates of reduction of imines is well known.[31] The difference between the rates of reduction of quinonoids (6) and (8) can be seen more clearly in the data at 25°. Although we have not confirmed it, the difference in rate of reduction upon progressive methylation of the 2-amino-group may be due to differ-

ences in general acid catalysis during hydride transfer. The effect of *N*-methyl groups on the oxidation of NADH parallels the effect on rearrangement.

In the enzymic reduction of quinonoid dihydropterins by DHPR and NADH, a hydride ion is transferred from NADH to the pterin, and for reduction to be complete protonation of the pterin must take place. The exact sequence of these reactions is not known, but the effect of a positive charge on N(3) on the rates of reduction of the quinonoid dihydropterins non-enzymically by NADH raises the possibility that for efficient catalysis N(3) of the pterin must bear a positive charge (*i.e.* be protonated). These results also show the preferred polarity of the substrate and that transfer of hydride from NADH to N(5) of the quinonoid dihydropterin must occur. The measure of the effect of protonation, with respect to enzyme catalysis, should be taken as a comparison between quinonoid dihydropterins (10) and (8), *i.e.* catalysed and only weakly catalysed reduction, since during measurements of enzymic reduction rates [32] the non-enzymic oxidation of NADH is taken into account. Quinonoids (6)—(8) are known substrates for DHPR but the activity decreases sharply in this order. The activity of quinonoid (10) towards DHPR, however, cannot be assessed because it rapidly oxidises NADH during the assay.

The most noteworthy features of the spectral data of tetrahydropterins and quinonoid dihydropterins (Table 5) are the substituent effects of the methylamino- and dimethylamino-groups on the quinonoid forms. The shifts of the spectral maxima of the long wavelength band, relative to the parent, are 18 and 38 nm respectively, *i.e. ca.* 18 nm per methyl group. Because of the large shift in the spectrum of quinonoid (8), Kaufman [4] suggested the '*para*' quinonoid structure (2) with a positive charge on the exocyclic nitrogen atom. Although large, the effects are apparently normal substituent effects reflecting a considerable electronic interaction with the quinonoid conjugated double bond system. Such large effects of dimethylamino-groups on the u.v. spectra are known.[33] A methyl group on a nitrogen atom of the pteridine ring, however, barely alters the position of this band.

EXPERIMENTAL

Compounds (6) [34] and (9) [35] were prepared by literature methods. Compounds (7), (8), and (10) were made by catalytic reduction of the corresponding pterins in 0.5M-HCl using platinum oxide. The 6,7-dideuterio-compounds were similarly prepared using 0.5M-DCl and deuterium gas at atmospheric pressure and 25°. Under these conditions incorporation of deuterium was 100% (by ^1H and ^{13}C n.m.r.). In the course of this work we found that catalytic reduction of 6,7-dimethylpterin in 0.5M-DCl using hydrogen gas gave (6) with 85% incorporation of deuterium at C(6) and C(7). Presumably exchange of deuterium with hydrogen bound to the catalyst is faster than reduction. The converse experiment, *i.e.* reduction in 0.5M-HCl with deuterium gas, gave very little incorporation.

6,7-*Dimethyl*-2-*methylamino*-5,6,7,8-*tetrahydropteridin*-4(3H)-*one* (7) *Hydrochloride*.—The pterin [35] (100 mg) was shaken with hydrogen in 0.5M-hydrochloric acid (10 ml) containing pre-reduced platinum oxide (50 mg). At the end of the reduction (as monitored by the u.v. spectrum of a portion in *N*-hydrochloric acid) the solution was evaporated to dryness at 30° and *ca.* 18 mmHg. The solid was dried overnight at 20° and 0.1 mmHg over phosphorus pentaoxide, dissolved in methanol and precipitated with ether to give the *hydrochloride*, m.p. 235—239° (decomp.) (Found: C, 35.3; H, 6.2; N, 23.3; Cl, 22.0. $C_9H_{15}N_5O.1.9HCl.1.5H_2O$ requires C, 35.3; H, 6.6; N, 22.9; Cl, 22.0%). The 6,7-dideuterio-derivative was prepared in a similar manner and checked by n.m.r. spectroscopy.

2-*Dimethylamino*-6,7-*dimethyl*-5,6,7,8-*tetrahydropteridin*-4(3H)-*one* (8) *hydrochloride* had m.p. 240—245° (decomp.) (Found: C, 40.8; H, 5.8; N, 23.4; Cl, 20.1. $C_{10}H_{17}N_5O.1.7HCl.0.9H_2O$ requires C, 40.0; H, 6.8; N, 23.3; Cl, 19.7%) and 2-*amino*-3,6,7-*trimethyl*-5,6,7,8-*tetrahydropteridine*-4(3H)-*one* (10) *hydrochloride* had m.p. 286—289° (decomp.) (Found: C, 41.2; H, 6.5; N, 26.3; Cl, 16.9. $C_9H_{15}N_5O.1.25HCl.0.5H_2O$ requires C, 41.0; H, 6.6; N, 26.5; Cl, 16.8%), and their 6,7-dideuterio-derivatives were made similarly from the corresponding pterins.[35,36]

All kinetic measurements were carried out on a Unicam SP 1800 recording spectrophotometer, and the cells were thermostatted within *ca.* 0.2° using Coolonics (Komatsu-Yamato) model CTR-1B and CTE-1B thermostat units. Catalase (bovine liver) and peroxidase (horse radish) were purchased from Sigma and Boehringer respectively.

Oxidations.—Stock solutions of 5,6,7,8-tetrahydropterins were made in 0.004M-hydrochloric acid. Oxidation was initiated by pipetting portions (50 μl) of this solution into a thermostatted cuvette containing oxygenated Tris chloride buffer (3.2 ml). This buffer was oxygenated by bubbling oxygen through it for 15 min, and the rates were reproducible to within 8%. The oxygen concentration in the buffer was 0.8mM (Winkler determination). The final concentration of pterin was 0.8×10^{-4}M in all cases, and gave a 10-fold excess of dissolved oxygen. The oxidations were followed by observing the increase in absorbance at 370 nm except for compound (10). In the latter case the decrease in absorbance at 306 nm was monitored. Values of A_∞ were measured after six half-lives. First-order rates were calculated using standard equations, and points were fitted to a straight line by a simple least-squares procedure using a Texas Instrument TI-55 calculator.

Rearrangement Reactions.—The quinonoid dihydropterins were generated as above except that the cuvettes contained 3.1 ml of buffer and 100 μl peroxidase (stock solution had 0.2 mg ml^{-1}). Oxidation was complete within 1 min and the rates were measured from the decrease in absorbance at 370 nm in all cases. The values for A_∞ were calculated as above.

Reduction of NADH.—The isosbestic points for the interconversion of compounds (6)—(8) and (10) to the respective quinonoid dihydropterins were found to be at 316, 314, 314, and 326 nm, respectively. These were determined accurately by measuring the rate of change of optical density (conversion of tetrahydropterin into quinonoid dihydropterin) at various wavelengths near the isosbestic points. At the isosbestic points the optical densities did not alter with time, but at wavelengths on either sides of the isosbestic points increased or decreased with time. The values of ε for NADH at 316, 314, and 326 nm were found as 4 250, 4 550 and 5 750 respectively based on a value of 6 560 at

340 nm.[37] The quinonoid dihydropterins were generated as described above and stock NADH (50 μl) was added rapidly giving a final concentration of 1.5×10^{-4}M. Oxidation of NADH was followed for the first 1—2 min by the decrease in absorbance at the above stated wavelengths. The traces were linear over this period and represented 5—10% reaction. Initial rates were calculated using the above ε values.

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