Tetrahydrobiopterin Analogues: Solution Conformations of 6-Methyltetrahydropterin, 7-Methyltetrahydropterin, and *cis*- and *trans*-6,7-Dimethyltetrahydropterins As Determined by Proton Nuclear Magnetic Resonance†

Thomas C. Williams‡ and Carlyle B. Storm*

Department of Chemistry, Howard University, Washington, DC 20059
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ABSTRACT: The conformation of tetrahydrobiopterin analogues in aqueous solution at 23 °C has been determined by analyzing the 200-MHz ^{1}H NMR spectral parameters of the enzymatically active 6-methyltetrahydropterin, 7-methyltetrahydropterin, and *cis*- and *trans*-6,7-dimethyl-5,6,7,8-tetrahydropterins. Each of these cofactors, with the exception of the *cis*-6,7-dimethyl isomer, exhibited an unusually small trans 6H–7H spin–spin coupling (8.5–9.1 Hz). An empirical equation that accounts for the effects of substituent electronegativity and orientation on vicinal couplings [Haasnoot, C. A. G., deLeeuw, F. A. A. M., & Altona, C. (1980) *Tetrahedron 36*, 2783–2792] predicted this coupling to be 11.3–11.6 Hz. We attribute the discrepancy between the calculated and experimentally observed values of this coupling to hyperconjugation of the axially oriented C_7–H bond with the π orbital of the vinylogous amide protein of the pterin ring (N_8—C_{8a}=C_{4a}—C_4=O) rather than to conformational averaging. The trans 6H–7H interproton distance in the 6-methyl analogue is calculated to be 3.0 Å from the measured decrease in the spin–lattice relaxation rate of the axially oriented C_7 proton after specific deuteration at C_6. This is consistent with the single-conformer interpretation. Chemical shift comparisons of the methyl resonances of these analogues, NOE measurements from selectively deuterated analogues, and the differential sensitivities of axially vs. equatorially disposed ring protons to protonation at N_5 all indicate that (i) the methyl substituents at both the C_6 and C_7 positions markedly prefer equatorial-like orientations and (ii) the tetrahydropterin ring is, with the exception of a pronounced pucker at C_6, nearly planar. In addition, the cofactor characteristics of *trans*-6,7-dimethyltetrahydropterin in the phenylalanine hydroxylase enzyme system are reported. Its relative V_{max} is 3.6 times as great as that of the cis isomer, its apparent K_M is the same, and its enzymatic oxidation is fully coupled to phenylalanine hydroxylation.

Aromatic amino acid hydroxylases (monooxygenases) require the cofactor 5,6,7,8-tetrahydrobiopterin (H_4biopterin)[1] to function normally (Kaufman, 1971; Gunsalus et al., 1975; Benkovic, 1980). This reduced pterin is believed to be intimately involved both in the activation of molecular oxygen prior to the hydroxylation of the amino acid substrate and in the reduction to water of the oxygen atom which remains after hydroxylation is complete (Hamilton, 1974). Although H_4biopterin is the only known cofactor for this family of enzymes that fully preserves their allosteric regulatory properties as well as their catalytic activities (Parniak & Kaufman, 1981), structurally simpler analogues such as 6-methyl-5,6,7,8-tetrahydropterin (6-CH_3-H_4pterin) and *cis*-6,7-dimethyl-5,6,7,8-tetrahydropterin [*cis*-6,7-$(CH_3)_2$-H_4pterin] serve quite well as catalytically functional cofactors (Kaufman, 1958) obeying the normal stoichiometry which yields one molecule of hydroxylated amino acid for each molecule of cofactor oxidized to the dihydro level. However, in the case of phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1; PAH) several cofactor analogues uncouple this enzyme's functions, yielding less than one molecule of tyrosine for each molecule of oxidized cofactor: 7-methyl-5,6,7,8-tetrahydropterin (7-CH_3-H_4pterin), 5,6,7,8-tetrahydropterin (H_4pterin), and several substituted pyrimidines all cause varying degrees of uncoupling in the PAH system (Storm & Kaufman, 1968; Ayling & Helfand, 1975; Bailey & Aylin, 1978; Kaufman, 1979). Because differences in their aqueous solution conformations may contribute to their markedly different cofactor activities, we chose to study 6-CH_3-H_4pterin, 7-CH_3-H_4pterin, and the cis and trans isomers of 6,7-$(CH_3)_2$-H_4pterin by ^{1}H NMR techniques to determine whether or not such conformational differences do indeed exist.

A ^{13}C NMR investigation (Armarego & Waring, 1980) and several ^{1}H NMR studies (Storm & Chung, 1976; Weber & Viscontini, 1977; Armarego & Schou, 1977; Ganguly et al., 1981) of H_4pterins and the closely related tetrahydrofolates (Poe & Benkovic, 1980; Poe et al., 1979a,b; Poe & Hoogsteen, 1978; Furrer et al., 1978) have concluded that the tetrahydropyrazine ring assumes one of two essentially equivalent cyclohexene-like half-chair conformations which differ only in the preferred orientations of their C_6 and C_7 substituents.

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‡ Present address: Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

[1] Abbreviations: NMR, nuclear magnetic resonance; H_4biopterin, 5,6,7,8-tetrahydrobiopterin; 6-CH_3-H_4pterin, 6-methyltetrahydropterin; 7-CH_3-H_4pterin, 7-methyltetrahydropterin; *trans*-6,7-$(CH_3)_2$-H_4pterin, *trans*-6,7-dimethyltetrahydropterin; *cis*-6,7-$(CH_3)_2$-H_4pterin, *cis*-6,7-dimethyltetrahydropterin; PAH, phenylalanine hydroxylase; TSP, 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate (sodium salt); D_2O, deuterium oxide; NADH, nicotinamide adenine dinucleotide (reduced form); *d*, deuterium (as used in naming the specifically deuterated analogues); DDQ, dichlorodicyanobenzoquinone; pH*, pH uncorrected for the deuterium isotope effect; FID, free induction decay; TCA, trichloroacetic acid; Hz, hertz; NOE, nuclear Overhauser effect.

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However, recent X-ray diffraction studies of the crystal structures of several tetrahydropterin salts (Bieri & Viscontini, 1977; Bieri, 1979) and a tetrahydrofolate (Fontecilla-Camps et al., 1979) show notable differences from the previously proposed solution conformation. Unlike the half-chair conformation which forces both the C_6 and C_7 ring atoms to opposite faces of the pyrimidine ring plane, these crystal studies indicate that C_7 is nearly coplanar with the pyrimidine ring and that the N_8 atom is essentially sp^2 hybridized due to a vinylogous amide resonance interaction.

From the ^1H NMR studies of the methyl-substituted tetrahydropterins examined herein, we propose that the flattened conformation of the tetrahydropyrazine ring which is observed in the crystal state is preserved in aqueous solution.

Experimental Procedures

Analogue Syntheses

The tetrahydrobiopterin analogues used in this study were prepared from the appropriate pterins: 6-CH_3-pterin, 7-CH_3-pterin, and 6,7-$(CH_3)_2$-pterin were synthesized from the Isay condensation of 2,4,5-triamino-6-hydroxypyrimidine sulfate with pyruvic aldehyde (or 2,3-butanedione, in the case of the 6,7-dimethyl analogue) as described by Storm et al. (1971); 6-CH_3-7,8-dihydropterin and 6,7-$(CH_3)_2$-7,8-dihydropterin were prepared by partial reduction of the indicated pterins with Zn dust in 1.0 M NaOH as described by Whitely & Huennekens (1967).

6-CH_3-H_4pterin, 7-CH_3-H_4pterin, and cis-6,7-$(CH_3)_2$-H_4-pterin were isolated as acetone-precipitated sulfate salts after catalytic reduction (PtO_2/H_2 gas, 2 atm) of the appropriate pterin or 7,8-dihydropterin in 250 mM H_2SO_4. This method is a variation of published procedures that yield these tetrahydropterins as their hydrochloride salts (Armarego & Schou, 1977; Gangulay et al., 1980).

trans-6,7-$(CH_3)_2$-H_4pterin was prepared as its chloride salt by reduction of an aqueous suspension of 6,7-$(CH_3)_2$-pterin or 6,7-$(CH_3)_2$-7,8-dihydropterin over 3% sodium amalgam. Complete reduction of 250 mg of the oxidized pterin required 4–5 days at 23 °C. The reduced pterin was isolated as a 3:1 trans–cis isomeric mixture by extracting the precipitated salt [formed by H_2SO_4 acidification of the crude reaction mixture) with hot concentrated HCl, evaporating the extract to dryness, redissolving the residue in a minimum volume of distilled water, and then collecting the acetone-precipitated chloride salt.

Most deuterated tetrahydropterins were prepared by substituting the appropriate deuterated reagents, i.e., D_2O, D_2 gas, D_2SO_4, or NaOD, for their protio counterparts in the methods referenced above or described below.

6-Methyl-d_3-H_4pterin-7,7-d_2 and 6-methyl-d_3-H_4pterin-7-(50%R,50%S)-d were prepared as follows: the 6-CH_3 and 7-CH_2 protons of 6-CH_3-7,8-dihydropterin were exchanged for deuterium by pretreatment in 1.0 M NaOD at 95 °C; the dihydropterin, isolated by precipitation at neutral pH, was either (i) catalytically reduced (PtO_2/H_2 gas, 2 atm) in 250 mM H_2SO_4 to yield 6-methyl-d_3-H_4pterin-$_m$,7-d_2 or (ii) oxidized to 6-methyl-d_3-pterin-7-d with DDQ and then reduced catalytically in H_2SO_4 to give 6-methyl-d_3-H_4pterin-7(50%-R,50%S)-d.

NMR Methods

^1H NMR samples were prepared by dissolving 10 mg of the D_2O-lyophilized tetrahydropterin salts in 1.0 mL of D_2O to which 50 μL of 1% TSP in D_2O was added. These solutions were adjusted to the desired pH* with microliter additions of 40% NaOD or concentrated D_2SO_4 (or DCl). ^1H NMR spectra were acquired at 23 °C by using a Nicolet NTC-200 MHz spectrometer operating in the conventional pulsed Fourier-transform mode with quadrature detection. One-pulse experiments (90°; 6.1 μs) were used to collect the FIDs (16K data points; ±700 Hz sweep width; 40–100 transients) which were then exponenitally multiplied by a line-broadening constant of 0.25 Hz. All chemical shifts were referenced to the principal resonance of TSP.

pH Titrations.* A series of spectra was recorded for each sample after first bringing it to pH* 0.00 and then progressively adjusting its pH* upward in increments of 0.25–0.50 pH unit using microliter additions of 40% NaOD. Half of the sample was reserved for use at the highest pH* values since the acid-stable H_4pterins were quickly oxidized to their 7,8-dihydro forms in base.

^1H T_1 and NOE Determinations. Samples were thoroughly vacuum degassed in septum-sealed 5-nm NMR tubes. The phase-shifted modified version of the inversion–recovery pulse sequence (180°–τ–90°; Cutnell et al., 1976) was used to collect a set of 10–15 spectra half of which showed inverted resonances (Akasaka et al., 1975a). For any given sample, the longest τ delay in each T_1 experiment was set to 10× the length of the longest approximated T_1 value. T_1 values were subsequently determined from the slopes ($1/T_1$) of log $[(I_0 - I)/I_0]$ vs. τ plots.

For each homonulear Overhauser experiment, two separate spectra were acquired. In the first, the methyl singlet resonance was irradiated; in the second, the decoupling frequency was shifted to a position as far upfield from the TSP resonance as was the methyl signal downfield from it. Integrations of the 6H and 7H resonances were normalized to the internal TSP resonance, NOEs being expressed as percent enhancements of these integrated areas.

^{13}C T_1 Determinations. 6-CH_3-H_4pterin (100 mg) was dissolved in 6.0 mL of 2:1 H_2O–D_2O. The solution's pH was adjusted to 0.00 by using concentrated HCl, and 25 μL of *p*-dioxane was added to serve as an internal secondary reference (67.4 ppm referenced to external tetramethylsilane). In order to eliminate dissolved oxygen, N_2 gas was bubbled through the solution. Broad-band (noise-modulated) heteronuclear ^1H decoupling was used in conjunction with the progressive saturation method (90°–τ; Freeman & Hill, 1971) to determine the ^{13}C T_1 values of this analogue. For each τ value in a series of seven (from 0.5 to 40 s), 5000 FIDs of a 16K data-point spectrum (±5000 Hz sweepwidth) were collected. The T_1 values were then determined from semilog plots of difference magnetization (i.e., $I_\infty - I_\tau$) vs. τ delay.

$^3J_{HH}$ Calculations. Proton vicinal spin–spin coupling constants were calculated for the methyl-substituted H_4pterins by the following equation (Haasnoot et al., 1980):

$$^3J_{HH} = P_1 \cos^2 \phi + P_2 \cos \phi + P_3 + \sum \Delta\chi_i [P_4 + P_5 \cos^2 (\xi_i \phi + P_6 |\Delta\chi_i|)]$$

where ϕ is the dihedral angle formed by the vicinal C–H bond axes, $\Delta\chi_i$ is the electronegativity difference between substituent i and hydrogen, ξ_i is the orientation factor (either +1 or −1, depending on the substituent position relative to the geminal hydrogen involved in the coupling), and P_1 through P_6 are constants taken from empirically determined parameter sets. For the methyl-substituted H_4pterins examined in this study, the following parameter set was used (Haasnoot et al., 1980): $P_1 = 13.22°$, $P_2 = -0.99°$, $P_3 = 0°$, $P_4 = 0.87°$, $P_5 = -2.46°$, and $P_6 = 19.9°$.

Because a nitrogen group's effective electronegativity strongly depends on its charge, orbital hybridization, and the nature of its other substituents, the precise electronegativity

Table I: ^{1}H NMR Chemical Shifts of Selected Methyl-Substituted Tetrahydropterins[a]

H_4pterin	$6H_e$	$6H_a$	$7H_e$	$7H_a$	$6CH_3$	$7CH_3$
6-methyl[b]		3.663	3.638 (*S*)	3.331 (*R*)	1.427	
7-methyl[b]	3.603 (*R*)	3.096 (*S*)		3.817		1.315
trans-6,7-dimethyl		3.300		3.522	1.431	1.312
cis-6,7-dimethyl[c]	*3.781*		*3.810*		*1.301*	*1.241*

[a] In ppm; pH* 3.50 in D_2O at 23 °C; referenced to TSP. [b] Italicized letters indicate the proton's prochiral designation as discussed in the text. [c] Italicized values indicate that these chemical shifts represent conformationally averaged environments.

values (on the Pauling scale) for the N_5 and N_8 groups of H_4pterins are unknown. Therefore, an array of $^3J_{HH}$ values was calculated by varying the effective electronegativities of both the N_5 and N_8 groups between 2.5 (an estimated lower limit for an uncharged, sp^3 hybridized secondary amine; Huheey, 1965) and 3.5 (an estimated upper limit for a charged, sp^2 hybridized imine; Huheey, 1965, 1983). From this array, the couplings which were calculated by using an $\chi_{N5} = 3.05$ (the estimated electronegativity of N_5) and an $\chi_{N8} = 3.05$ (the estimated electronegativity of N_8) were used for comparison with the experimentally observed H_4pterin couplings.

Cofactor Assay Methods

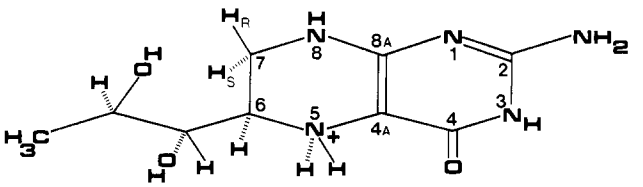
The K_m's, relative V_{max}'s, and coupling characteristics of the cis and trans isomers of 6,7-$(CH_3)_2$-H_4pterin were determined for the phenylalanine hydroxylase system by standard methods (Parniak & Kaufman, 1981).

K_m and V_{max} Determinations. The *cis*- and *trans*-6,7-$(CH_3)_2$-H_4pterins were assayed in a concentration range of 20–300 μM by using a mixture of NADH (0.4 mM), dihydropteridine reductase (0.4 mg/mL), catalase (0.2 mg/mL), phenylalanine (20 mM), and potassium phosphate buffer, pH 6.8 (100 mM) at 26 °C. After a 5-min thermal equilibration period, 10 μL of a phenylalanine hydroxylase solution (4 μg/mL) was added to the assay mixture; enzymatic oxidation of NADH was monitored by following the time-dependent decrease in absorbance at 340 nm. Double-reciprocal (Lineweaver–Burk) plots of $1/v_0$ vs. 1/[cofactor] were used to determine the K_m and relative V_{max} values for these two analogues.

Coupling Characteristics. To determine the coupling characteristic of *trans*-6,7-$(CH_3)_2$-H_4pterin in the PAH enzyme system, an assay mixture identical with the one used for the K_m and V_{max} determinations was employed. The cofactor concentration was chosen such that, after a 30-min incubation at 26 °C, the absorbance at 340 nm had decreased by approximately 0.5 *A* unit. A measured aliquot was then withdrawn from the assay mixture and emptied directly into 0.5 mL of 12% TCA. After this suspension was centrifuged, the supernatant was assayed for tyrosine by using the nitrosonaphthol method (Waalkes & Udenfriend, 1957). The amount of NADH consumed during the assay was calculated from the decrease in A_{340} and a molar absorbtivity (ϵ_{340}) of 6220 cm^{-1} M^{-1}. The molar ratio of NADH consumed to tyrosine formed provided a measure of the coupling characteristic of the cofactor. The coupling characteristics of 6-CH_3-H_4pterin, 7-CH_3-H_4pterin, and *cis*-6,7-$(CH_3)_2$-H_4pterin were determined concurrently to provide comparison values.

Results

Figure 1 illustrates the structure of tetrahydrobiopterin, the appropriate substitutions required to generate those methyl-substituted analogues used in this study, and the ring-atom numbering system adopted by the Commission on Biochemical Nomenclature (1966). Wherever appropriate, the methylene ring protons of 6-CH_3-H_4pterin and 7-CH_3-H_4pterin are differentiated following Hanson's prochiral designation



	R_6	6H	$7H_R$	$7H_S$
I	CH_3	H	H	H
II	H	H	CH_3	H
III	CH_3	H	H	CH_3
IV	CH_3	H	CH_3	H

FIGURE 1: Structure of (6*R*)-5,6,7,8-tetrahydropterin; the numbering system is the one recommended by the IUPAC. The table beneath the structure indicates the substitutions required to form the following analogues: I, 6-methyltetrahydropterin; II, 7-methyltetrahydropterin; III, *trans*-6,7-dimethyltetrahydropterin; IV, *cis*-6,7-dimethyltetrahydropterin. R_6 represents the 1,2-dihydroxypropyl side chain of tetrahydrobiopterin.

(Bentley, 1969); 7*HR* (or 6H*R*) designates that proton which, if replaced by deuterium, converts C_7 (or C_6) to an absolute *R* configuration, whereas 7H*S* (or 6H*S*) refers to that proton which, if replaced by deuterium, converts C_7 (or C_6) to an absolute *S* configuration. Although all of the cofactors studied herein were racemic mixtures, only the 6*S* stereoisomer is depicted in Figure 1 since (a) the 6*S* and 6*R* forms of these analogues are not resolvable by NMR spectroscopy in an achiral solvent such as D_2O and (b) the 6(*S*)-methyl analogues correspond to the absolute 6*R* configuration of naturally occurring tetrahydrobiopterin (Matsuura et al., 1980). In Figure 1 and the assignments of resonance positions and coupling constants which follow, 7-CH_3-H_4pterin was arbitrarily taken as its 7*R* enantiomer.

Resonance Assignments. The isolated upfield doublet—at 1.427 ppm in the ^{1}H NMR spectrum of 6-CH_3-H_4pterin and at 1.315 ppm in the spectrum of 7-CH_3-H_4pterin (not shown)—was, in each case, readily assigned to the methyl group protons: the chemical shift and doublet apperance of this resonance are characteristic of a methyl group adjacent to a –CH–function. Similarly, the two closely spaced upfield doublets found in the spectra of the *cis*- and *trans*-6,7-$(CH_3)_2$-H_4pterins were assigned to the protons of the two methyl groups in each of these analogues (see Table I); however, specific assignment of these resonances to the 6-CH_3 and 7-CH_3 substituents required comparison with specifically deuterated forms. In the spectra of both *cis* and *trans* isomers of 6,7-$(CH_3)_2$-H_4pterin-*7-d*, the upfield methyl resonance (at 1.241 and 1.312 ppm, respectively) appeared as a singlet, an indication that it arose from the 7-CH_3 protons. Since, in each case, the downfield methyl resonance (at 1.301 and 1.431 ppm, respectively) was still present as a doublet, it was assigned to the 6-CH_3 protons.

The tightly coupled spins of the ring-bound protons generated a somewhat more complex series of multiplets in the

3–4 ppm region of the spectrum. However, several guidelines proved useful in the assignment of these resonances: (I) due primarily to anisotropic shielding from adjacent sigma bonds, axially oriented protons tend to resonate upfield of equatorial ones (Lemieux et al., 1958; Jackman et al., 1962); (2) trans-configured vicinal ring-bound protons usually show larger spin–spin couplings than do analogous pairs of cis-configured protons, a combined result of the dependence of this parameter on the H–C–C–H dihedral angle (Karplus, 1959; Haasnoot et al., 1980) and the probability that the ring will assume a suitable staggered conformation; (3) due to additional vicinal spin–spin interactions, a methine proton adjacent to both methylene and methyl groups gives rise to a resonance significantly more complex than that of a proton in the neighboring methylene group.

Thus, of the three well-resolved multiplets found between 3 and 4 ppm in the spectrum of 7-CH_3-H_4pterin (not shown), the most upfield resonance, a doublet of doublets centered at 3.096 ppm, was readily assigned to the C_6 proton which was, on the average, axially disposed and trans to the C_7 methine proton (6H*S* for the 7*R* enantiomer; 6H*R* for the 7*S* enantiomer). The doublet of doublets centered at 3.603 ppm and exhibiting the smaller vicinal coupling was assigned to the C_6 proton oriented cis to 7H and equatorially disposed (6H*R* for the 7*R* enantiomer; 6H*S* for the 7*S* enantiomer). The complex multiplet centered at 3.817 ppm was assigned to the C_7 methine proton (Table I).

By similar reasoning, the doublet of doublets centered at 3.407 ppm in the spectrum of 6-CH_3-H_4pterin (Figure 2A) was assigned to the axially disposed C_7 proton (7H*R* for the 6*S* enantiomer; 7H*S* for the 6*R* enantiomer). However, the multiplet resonances of the equatorially oriented C_7 methylene proton and the C_6 methine proton so severely overlapped that their precise chemical shifts were determined by other means. Decoupling the C_6 methine proton by time-shared irradiation of the 6-CH_3 doublet resonance at 1.427 ppm greatly reduced the complexity of these overlapping multiplets (Figure 2B). The resultant decoupled spectrum, treated as an ABC spin system, yielded the three-component resonance positions and three spin–spin couplings when simulated by ITRCAL [Nicolet's iterative spectrum calculation routine based on the LAOCOON III program of Castellano & Bothner-By (1964)]. This method indicated that 6H, although upfield of 7H*S* in the pH* ranges 0–1.5 and 5–14, was slightly downfield of 7H*S* in the pH* range 1.5–5.0 (see Table I). To support these resonance asssignments, the relatively slow spin–lattice relaxation rate of 6H was used to produce a spectrum by the inversion–recovery pulse sequence in which the resonance of this particular proton was effectively nulled (Figure 2C). In addition, the difference in sensitivity of 6H and 7H*S* to N_5 deprotonation was used to resolve these resonances: at pH* values >6, the 6H and 7H*S* resonances are completely free of overlap (Figure 3A).

Final confirmation of the 6H, 7H*R*, and 7H*S* resonance positions for 6-CH_3-H_4pterin came from comparisons with the spectra of two specifically deuterated forms: the two singlets of 6-CH_3*d*-H_4pterin-*m*(50%*R*,50%*S*)-*d* at 3.436 and 3.020 ppm (pH* 8.58) confirmed the 7H*S* and 7H*R* resonance positions, respectively (Figure 3B), and the singlet resonance of 6-methyl-d_3-H_4pterin-7,7-d_2 at 3.140 ppm (pH* 8.50) confirmed the 6H resonance position (Figure 3C).

Similarly, comparisons of the spectra of the deuterated analogues *cis*-6,7-$(CH_3)_2$-H_4ptern-7-*d* and *trans*-6,7-$(CH_3)_2$-H_4pterin-7-*d* (not shown) with the spectra of the all-protio *cis*- and *trans*-6,7-$(CH_3)_2$-H_4pterins (Figure 4) indicated that, in each case, 6H resonated upfield of 7H.

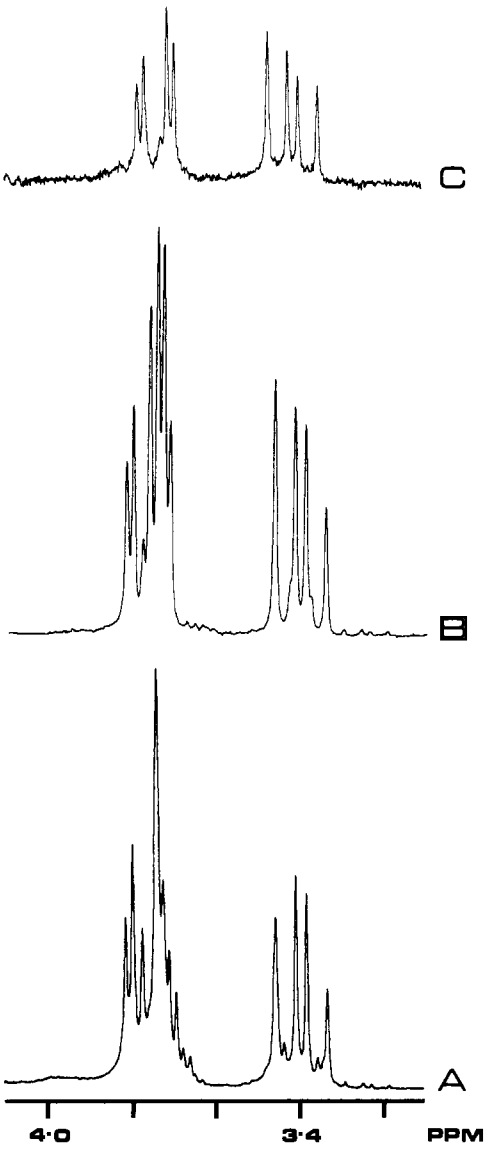


FIGURE 2: Partial 200-MHz ^{1}H NMR spectra of 6-methyltetrahydropterin (pH* 0.0, 23 °C, 25 mM in D_2O) showing the 6H (3.73 ppm), 7H*R* (3.40 ppm), and 7H*S* (3.75 ppm) resonances. A, fully coupled; B, 6-CH_3 decoupled by irradiation of the upfield doublet at ~1.4 ppm (not shown); C, partially relaxed (6H resonance nulled) by allowing τ = 600 ms in 180°–τ–90° pulse sequence.

Coupling Constant Determinations. Measured directly from the doublet splitting of the methyl resonance, the spin–spin interaction between the methyl group's protons and its adjacent ring-bound methine proton was virtually identical in all of the analogues: 6.4–6.6 Hz. Although characteristic of an interaction in which the rotation of the methyl group is free and unhindred, this coupling is independent of the endocyclic torsion angles and was, therefore, not sensitive to conformational variations in the tetrahydropyrazine ring.

As indicated in the previous section, the coupling constants which arose from the various pairwise spin–spin interactions of the ring-bound protons were determined by a combination of decoupling, spectral simulation, and specific deuteration techniques. Irradiation of either methyl doublet resonance in the spectrum of *cis*- or *trans*-6,7-$(CH_3)_2$-H_4pterin caused the downfield resonance multiplet of one or the other ring-bound protons to collapse to a doublet; direct measurement of these doublet splittings yielded a 6H–7H coupling constant of 3.1 Hz for the cis isomer and 8.3 Hz for the trans isomer (Table III). Spectral simulation was used to extract from the 7-

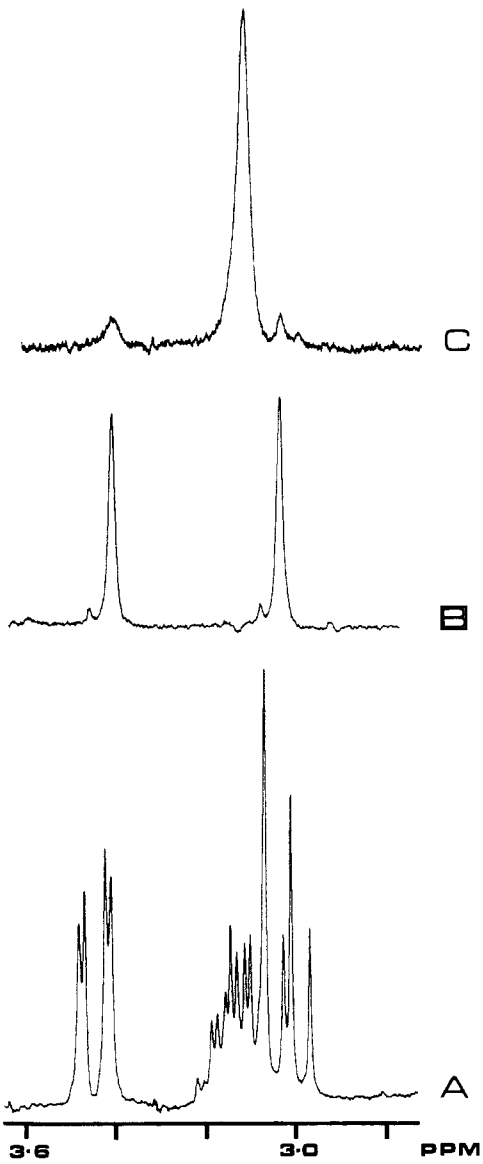


FIGURE 3: Partial 200-MHz ^{1}H NMR spectra of 6-methyltetrahydropterin (23 °C) showing comparisons with three specifically deuterated forms. A, 6-CH_3-H_4pterin at pH* 8.46 (6H, 3.14 ppm; 7H*R*, 3.02 ppm; 7H*S*, 3.44 ppm); B, 6-CH_3-*d*-H_4pterin-7(*R*)-*d* (7H*S*, 3.44 ppm) and 6-CH_3-*d*-H_4pterin-7(*S*)-*d* (7H*R*, 3.02 ppm) at pH* 8.58; C, 6-methyl-d_3-H_4pterin-7,7-d_2 (6 H, 3.14 ppm) at pH* 8.50. The unresolved doublet resonance at 3.44 ppm and the resolved doublet resonance at 3.02 ppm in C indicate the presence of small amounts (~5%) of the 6-methyl-d_3-7(*R*)-*d* and 6-methyl-d_3-7(*S*)-*d* analogues, respectively.

CH_3-decoupled spectrum of 7-CH_3-H_4pterin (which consisted of three sets of nonoverlapping doublet of doublets) one geminal and two vicinal spin–spin couplings; these values are listed in Table II.

For 6-CH_3-H_4pterin, in addition to spectral simulation techniques, direct measurements of the doublet splittings from several specifically deuterated forms yielded the pertinent spin–spin couplings. The spectrum of 6-CH_3-*d*-H_4pterin (not shown) consisted of an upfield singlet (due to 6-CH_3) and two downfield doublets (due to 7H*S* and 7H*R*) from which the geminal 7H*S*–7H*R* coupling constant was measured directly. The spectrum of an equimolar mixture of 6-methyl-d_3-H_4-pterin-7(*R*)-*d* and 6-methyl-d_3-H_4pterin-7(*S*)-*d* (the uppermost trace of Figure 6) consisted only of two pairs of doublets: the 6H–7H*S* coupling (3.3 Hz) was measured from the splitting of the most downfield doublet [i.e., the 7H*S* resonance of the 7(*R*)-*d* analogue; satisfactorily resolved at pH* >7]; the 6H–7H*R* coupling (8.8 Hz) was measured from the splitting of the most upfield doublet [the 7H*R* resonance of the 7(*S*)-*d* analogue]; the resonance positioned between these two doublets came from the superposition of the 6H doublets of the two constituent deuterated forms.

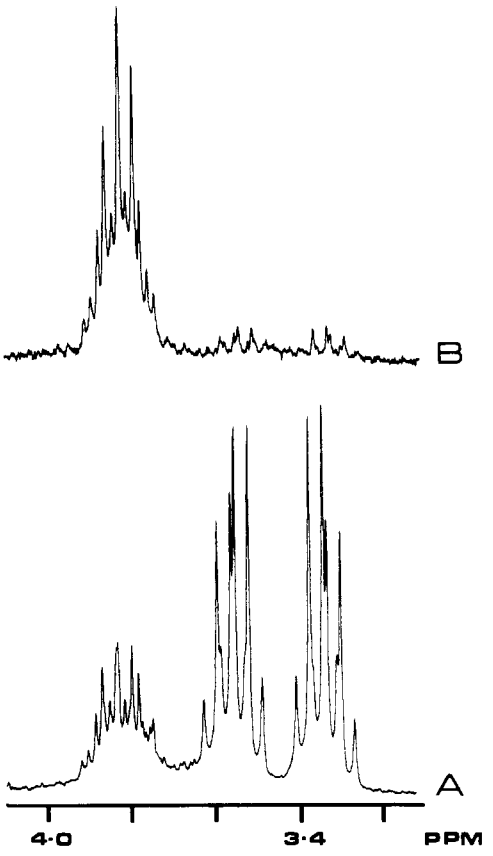


FIGURE 4: Partial 200-MHz ^{1}H NMR spectra of the 6,7-$(CH_3)_2$-H_4pterins (pH* 1.6, 23 °C, 25 mM) showing the 6H and 7H resonances of the trans isomer (3.36 and 3.54 ppm, respectively) and the cis isomer (3.79 and 3.84 ppm, respectively). A, 76:24 molar ratio of trans:cis isomers; B, 5:95 molar ratio of trans:cis isomers.

Table II: ^{1}H NMR Spin–Spin Couplings of Selected Methyl-Substituted Tetrahydropterins[a]

H_4pterin	cis $^3J_{67}$	trans $^3J_{67}$	^2J
6-methyl	(+0.2) 3.3 (−0.4) *3.6*	(+0.3) 8.8 (−0.3) *11.6*	(−0.3) −14.0 (+1.6)
7-methyl	(+0.1) 3.3 (+0.0) *3.6*	(−0.6) 9.1 (−1.5) *11.6*	(−0.3) −12.3 (+0.4)
trans-6,7-dimethyl		(+0.1) 8.3 (−0.8) *11.3*	
cis-6,7-dimethyl	(+0.1) 3.1 (+0.0) *3.6*		

[a] In hertz; pH* 3.50 in D_2O at 23 °C. Values in italics indicate the spin–spin couplings as calculated from the equation of Haasnoot et al. (1980). See text for details. Values in left-hand parentheses indicate changes (in Hz) of the observed couplings associated with protonation of the N_5-protonated form; values in right-hand parentheses indicate changes associated with deprotonation of the N_5-protonated form.

The coupling constants calculated for these H_4pterins using the equation of Haasnoot et al. (1980) are also listed in Table II. Figure 5 illustrates the dependence of these calculated values on the electronegativities chosen for the N_5 and N_8 substituents of the C_6–C_7 ring fragment. The calculated values appearing in Table II were determined for $\chi_{N5} = \chi_{N8} = 3.05$ because (i) N_5 was assumed to be charged and sp^3 hybridized and (ii) N_8 was assumed to be uncharged and sp^2 hybridized.

Spin–Lattice Relaxation (T_1) and Nuclear Overhauser Enhancement (NOE) Studies. The proton spin–lattice re-

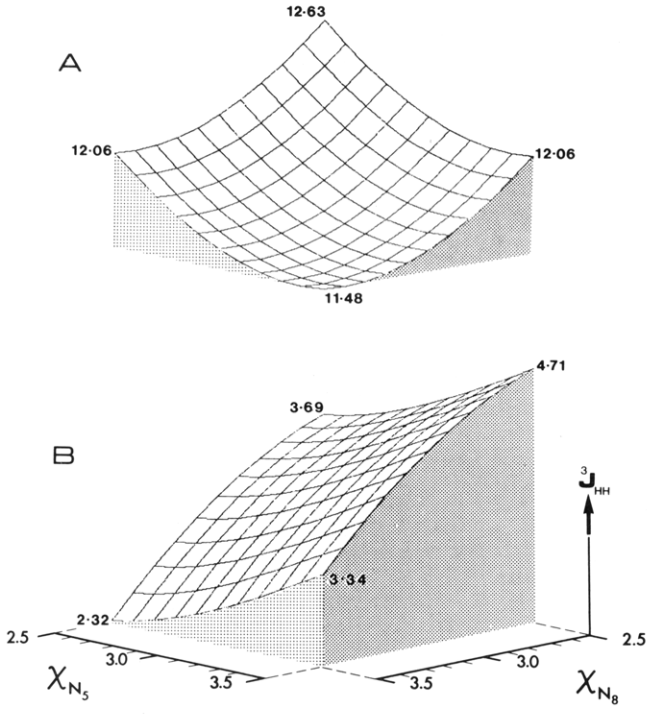


FIGURE 5: Surface representations of the calculated vicinal couplings of 6-CH_3-H_4pterin (equatorial CH_3 conformer) as a function of the electronegativities of N_5 and N_8. The numbers at the vertexes of each surface are the calculated spin–spin couplings (in Hz) for the indicated electronegativity limits. A, 6H–7H*R* (trans; $\phi = 174°$) coupling; B, 6H–7H*S* (cis; $\phi = 62°$) coupling.

Table III: ^{1}H Spin–Lattice Relaxation Times of Selected Methyl-Substituted Tetrahydropterins[a]

H_4pterin	6H	7H*R*	7H*S*	$6CH_3$
6-methyl	*0.9*	0.592	*0.6*	0.741
6-methyl-*d*		0.606	0.621	0.833
6-methyl-d_3-7(*S*)-*d*[b]	(*2.6*)	4.525		
6-methyl-d_3-7(*R*)-*d*[b]	(*2.0*)		2.890	
6-methyl-d_3-7,7-d_2	7.463			
	7.407[c]			
	1.775[d]			

[a] In seconds; at pH* 0.0 and 23 °C unless otherwise indicated. Italicized values are best approximations. [b] Determined at pH* 6.1 to avoid overlap of the 6H and 7H*S* resonances. [c] Determined at pH* 3.7. [d] Determined at pH* 8.6.

laxation times for 6-CH_3-H_4pterin and several of its selectively deuterated analogues are listed in Table III. As determined from the singlet resonance of 6-methyl-d_3-H_4pterin-7,7-d_2, the T_1 for 6H, though essentially constant below the pK_a of N_5, was remarkably sensitive to N_5 deprotonation; in addition, the proton homonuclear Overhauser enhancements measured for the 6H and 7H spins were also sensitive to N_5 deprotonation (see Table V), being reduced to little more than half their values by this pH change. For these reasons, the proton T_1 values of the *all-protio* form and the *6-d* analogue were determined at pH* 0.00.

The T_1 values of the *6-d* analogue were particularly informative since the 6H-specific dipolar contributions to the relaxation of 7H*R* and 7H*S* could be used to calculate the 6H–7H*R* and 6H–7H*S* interproton distances. Deuterium substitution effects on relaxation times (DESERT; Akasaka et al., 1975a,b) can be formalized as follows:

$$R = [(1/T_1)_{\text{normal}} - (1/T_1)_{\text{deuterio}}]/0.937$$

where R is the calculated fractional reduction in the relaxation rate, $(1/T_1)_{\text{normal}}$ is the relaxation rate of the all-protio compound, $(1/T_1)_{\text{deuterio}}$ is the relaxation rate of the specifically deuterated compound, and the factor 0.937 is derived from the relative ability of ^{2}H to contribute to dipole–dipole relaxation. If the correlation time for overall molecular tumbling, τ_c, is known, R can then be used to calculate an interproton distance via the following equation:

$$r_{HH} = [1.5(\gamma_H{}^2\hbar^2\tau_c)/R]^{1/6}$$

where r_{HH} is the calculated interproton distance. γ_H is the proton magnetogyric ratio, and $\hbar$ is Planck's constant divided by 2π. Because tetrahydropterins are small (M_r <500), we assumed that the rate of their overall tumbling was fast and fell within the extreme narrowing limit; further, we assumed that the spin–lattice relaxation of C_6 was dominated by the dipolar interaction with its directly bonded proton and that the dominant correlation time for the reorientation of the C_6–H bond stemmed from this molecular tumbling. In such cases, τ_c can be calculated from the following equation:

$$\tau_c = (1/T_1)_{^{13}C}[r^6/(\gamma_H{}^2\gamma_C{}^2\hbar^2)]$$

where r is the C–H bond length (which, for the C_6–H of these tetrahydropterins, was taken as 1.020 Å; Bieri & Viscontini, 1977) and γ_H, γ_C, and $\hbar$ have their usual meanings. When entered into the equation above, the spin–lattice relaxation time of C_6 (0.85 s; see Table IV) yielded the value of 3.7×10^{-11} s for τ_2. In turn, R [calculated to be 0.042 s^{-1} from T_1 (normal) = 592 ms and T_1(deuterio) = 606 ms] and τ_c were entered into the equation above to yield 3.0 ± 0.2 Å for the 6H–7H*R* interproton distance.

Table IV: ^{13}C Spin–Lattice Relaxation Times for 6-Methyltetrahydropterin[a]

C_2	C_4	C_{4a}	C_6	C_7	C_{8a}	$6CH_3$
3.4	8.9	6.1	0.85	0.53	5.6	0.89

[a] In seconds as measured in an N_2-purged aqueous solution (67% H_2O–33% D_2O) at pH 0.0, ambient temperature.

Table V: ^{1}H Homonuclear Overhauser Enhancements[a]

H_4pterin	pH*	7H*S*	7H*R*	6H*S*
6-methyl-*d*-7-*d*	0.00	12.0	11.2	
	7.09	7.3	6.3	
7-methyl-*d*-6-*d*	0.00			11.6
	7.47			6.5

[a] Percent enhancements following irradiation of the methyl group resonance (see text).

Near-complete overlap of the 6H and 7H*S* resonances of the all–protio form rendered precise values for the T_1 values of these spins indeterminable; consequently, the measured increase in the T_1 of 7H*S* following deuterium substitution at 6H was, at best, a poor approximation. Because the DESERT technique required that R be determined precisely, no value for the 6H–7H*S* interproton distance was calculated. However, a lower limit of relative (6H–7H*R*):(6H–7H*S*) interproton distances was estimated from the T_1 values of 7H*R* and 7H*S* in the 6-methyl-d_3-7(50%*R*,50%*S*)-*d* analogue mixture. From the inversion–recovery spectra of this sample (Figure 6), the slower relaxation of the 7H*R* spin (evidenced by the delayed τ_{null} > 3 s of the most upfield doublet resonance) compared to the faster relaxtion of the 7H*S* spin (whose downfield doublet resonance showed a τ_{null} < 2 s) indicated clearly that $r_{6-7R} > r_{6-7S}$. On the basis of the r^6 dependence of this parameter, these T_1 values (4.525 s for 7H*R* and 2.890 s for 7H*S*; see Table III) yielded a minimum (6–7*R*):(6–7*S*) interproton distance *ratio* of 1.6.

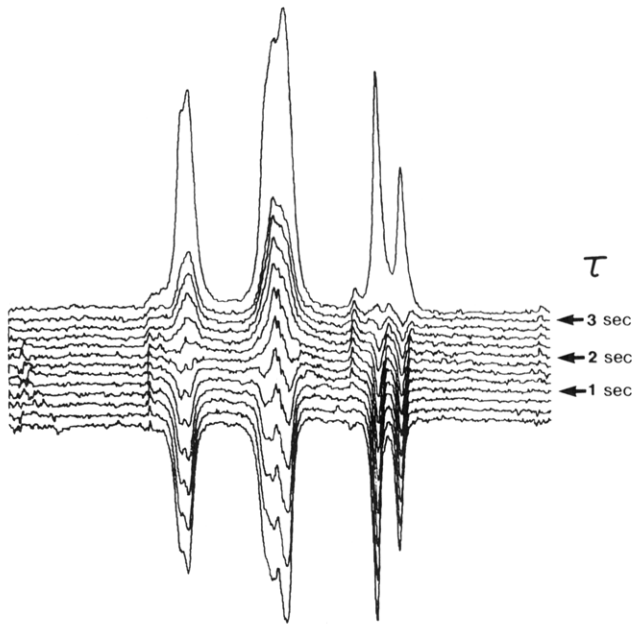


FIGURE 6: Inversion–recovery spectra of 6-methyl-d_3-H_4pterin-7-(50%*R*,50%*S*)-*d*. Resonance assignments: 7H*R*, right-most doublet; 7H*S*, left-most doublet; 6H, broad center resonance (superposition of the two different *J*-coupled doublet resonances of the indicated deuterated forms). τ was incremented in 250-ms steps from 250 ms to 3.0 s; the uppermost trace was acquired with τ = 30 s.

Additional distance constraints were also gleaned from several homonuclear proton NOEs (see Table V). By use of the following equation derived by Bell & Saunders (1976)

$$1/\mathrm{NOE} = Ar_{\mathrm{IS}}^{6}$$

[where the constant A was given as 0.98×10^{-2} for a CH_3–H interaction and r_{IS} is the through-space distance between the irradiated methyl group (centroid model) and the proton of interest], the 12.0% NOE experienced by 7H*S* in the 6,7(*R*)-d_2 analogue and the 11.2% NOE experienced by 7H*R* in the 6,7(*S*)-d_2 analogue yielded $6CH_3$–7H*S* and $6CH_3$–7H*R* interproton distances of 3.1 Å each. As also indicated in Table V, the axially oriented proton of the 7-CH_3-6(*R*),7-d_2 analogue (i.e., 6H*S*) experienced an 11.6% NOE. The $7CH_3$–6H*S* interproton distance calculated from this enhancement was also 3.1 Å.

Protonation Effects on Chemical Shifts. Without exception, the resonances of the C_6, C_7, and methyl group protons on all of the analogues shifted upfield in response to N_5 deprotonation. Although the C_6 protons were most sensitive to this protonation as measured by these upfield shifts, the relative 7H*S*–7H*R* sensitivity to N_5 deprotonation was conformationally more descriptive (Figure 7). From the upfield shift of 0.20 ppm for 7H*S* and 0.29 ppm for 7H*R* in 6-CH_3-H_4-pterin, the relative 7H*S*–7H*R* sensitivity was determined to be 0.69. If one assumes that these N_5 deprotonation induced changes in chemical shift arise predominantly from the electric field effects of a point charge (Buckingham, 1960; Batchelor, 1975), then the deprotonation sensitivities reflect the relative distances of these protons from N_5 according to an r^{-2} dependence of the field. On this basis, the ratio of (7H*R*–N_5):(7H*S*–N_5) distances is predicted to be 0.83. Calculated from the relevant bond lengths and bond angles determined by the X-ray analysis of several tetrahydropterins (Bieri & Viscontini, 1977; Bieri, 1979), the ratio of (7H*R*–N_5):(7H*S*–N_5) distances in the methyl-equatorial conformer of 6-CH_3-H_4pterin is 0.80.

Cofactor Studies. Table VI lists the kinetic parameters

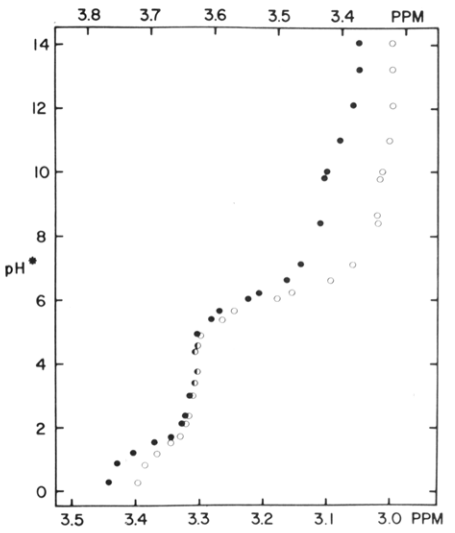


FIGURE 7: Titration data of the 7H*R* (○) (3.0–3.5 ppm) and 7H*S* (●) (3.3–3.8 ppm) resonances of 6-CH_3-H_4pterin. For comparison of relative deprotonation-induced chemical shift excursions, the upper and lower axes were shifted to align the chemical shifts of these resonances at pH* 3.5.

Table VI: Kinetic Data from PAH Assays of H_4pterin Cofactors[a]

H_4pterin	app K_M (μM)	rel V_{max}	coupling
6-methyl	*61*	*3.0*	1.02
7-methyl	*60*	*0.8*	2.72
trans-6,7-dimethyl	70	3.6	1.04
cis-6,7-dimethyl	70	1.0	1.07
biopterin[b]	4.5	0.3	0.97

[a] At 26 °C. Italicized values are from Storm & Kaufman (1968). [b] From Kaufman (1971).

determined for the cis and trans isomers of the 6,7-$(CH_3)_2$-H_4pterin cofactor when assayed in the phenylalanine hydroxylase enzyme system. Also included in this table are the coupling characteristics of the 6-CH_3-H_4pterin and 7-CH_3-H_4pterin cofactors as determined from this study. A double-reciprocal Lineweaver–Burke plot was used to graphically determine the apparent K_m's and relative V_{max}'s for the 6,7-$(CH_3)_2$-H_4pterin cofactors, taking into account the 5% trans impurity in the cis sample and the 24% cis impurity in the trans sample.

Discussion

The pyrimidine porition of the tetrahydropterin ring is planar due to its conjugated system of π bonds. The sp^2 hybridization of C_{4a} annd C_{8a} effectively transmits the planarity of the pyrimidine ring to N_5 and N_8 of the tetrahydropyrazine portion of the tetrahydropterin. However, several tetrahedrally hybridized atoms in the N_5–C_6–C_7–N_8 half-ring allow the structure a limited degree of conformational freedom. Because each ring geometry is defined by a unique set of torsional angles and interproton distances, the predominant solution conformation of the pterin ring may be described completely by the torsional angles of the N_5–C_6, C_6–C_7, and C_7–N_8 bonds. Although the facile ^1H–^2H exchange at N_5 and N_8 virtually eliminated proton spin–spin couplings as a probe of the N_5–C_6 and C_7–N_8 torsional angles, the spin–spin couplings between the carbon-bound protons at C_6 and C_7 were extremely informative. These couplings revealed not only the dihedral angles of the H–C_6–C_7–H ring segment but also, because of their dependence on substituent effects, the relative orientation of the adjacent ring segments, particulary C_7–N_8.

For 6-CH_3-H_4pterin, 7-CH_3-H_4pterin, and *trans*-6,7-$(CH_3)_2$-H_4pterin the $^3J_{HH}$ between protons with trans orien-

tation across the C_6–C_7 ring bond was remarkably similar, varying only from 8.3 Hz for the *trans*-6,7-$(CH_3)_2$ analogue to 9.1 Hz for the 7-CH_3 analogue (Table II). However, in no case was the comparison between observed and calculated $^3J_{HH}$ close (Table II; Figure 5). Though the observed difference of this coupling between the monomethyl- and dimethyl-substituted forms (~0.5Hz) agreed well with the calculated difference, a discrepancy of 2.8–3.0 Hz between the observed and calculated results persisted even after substituent electronegativities and orientations were taken into account.

For several reasons, conformational averaging was not considered the likely cause of this discrepancy. By comparison with *trans*-2,3-dimethyltetrahydroquinoxaline, a tetrahydropterin model compound with equatorially oriented methyl groups (Archer & Mosher, 1967), we tentatively assumed that both methyl groups of *trans*-6,7-$(CH_3)_2$-H_4pterin occupied equatorial positions. Comparison of the chemical shifts of the methyl protons of 6-CH_3-H_4pterin and 7-CH_3-H_4pterin to those of the *trans*-6,7-$(CH_3)_2$ analogue (Table I) indicated that the monomethyl analogues also existed in predominantly equatorial conformations. By contrast, the cis isomer cannot simultaneously position its methyl groups in equatorial environments; the predicted exchange between axial and equatorial orientations of both methine and methyl group protons was verified by the relative upfield shifts of the 6-CH_3 and 7-CH_3 resonances and the downfield shifts of the 6H and 7H resonances. Furthermore, the CH_3–7H*R* and CH_3–7H*S* interproton distances calcualted from the NOEs for the 6-CH_3 analogue also support the conclusion that the C_6 methyl group is equatorially oriented: these distances are not only virtually identical with each other (3.1 ± 0.2 Å) but are also equivalent (within experimental error) to the calculated interproton distances (2.9 Å) for the CH_3-equatorial conformation (given the bond lengths and bond angles from the crystal structures of similar tetrahydropterins; Bieri & Viscontini, 1977; Bieri, 1979). In addition, the 6H–7H*R* interproton distance calculated from the DESERT results of the 6-CH_3-*d* analogue (3.0 Å) is equivalent to the 6H–7H*R* distance (calculated from the X-ray data) for the diaxial orientation of these protons. Lastly, the ratio of (N_5–7H*R*):(N_5–7H*S*) inernuclear distances (0.80) calculated from the N_5-deprotonation induced chemical shift excursions agrees quite well with the ratio fo these distances calculated for the CH_3-equatorial conformer (0.83).

With regard to the trans $^3J_{HH}$ values of the monomethyl analogues, rapid interconversion between CH_3(equatorial) and CH_3(axial) conformations would reduce this vicinal coupling to 6.8 Hz (Table II), significantly less than what was observed. Therefore, consideration of the inductive effects of electronegative substituents neither alone nor in combination with conformational averaging could account for the abnormally low axial–axial interaction observed for these tetrahydropterins. Crist et al. (1980), in an attempt to separate the lone pair effects from the inductive effects of heteroatoms in three-membered heterocycles, having shown that a lone pair which eclipses one of the C–H bonds involved in a vicinal H–H interaction decreases the observed coupling by 2.5–2.7 Hz. The −2.8 to −3.0 Hz discrepancy between observed and calculated vicinal couplings in reduced pterins may indicate that the C_7–H*R* bond is involved in a hyperconjugative interaction with an electron pair on N_8. A comparison of the geminal couplings of the 7-CH_2 group in 6-CH_3-H_4pterin (−14.0 Hz) with the 6-CH_2 group in 7-CH_3-H_4pterin (−12.3 Hz; Table II) further supports the idea that the C_7–H*R* bond is aligned with the electron pair on the sp^2 hybridized N_8. Barfield & Grant (1963) have studied the effect of hyperconjugation on geminal spin–spin coupling constants. They find that alignment of one C–H bond of a CH_2 group with an adjacent π orbital contributes about −1.7 to −2.0 Hz to a geminal coupling. In a twist–chair conformation the CH_2 groups in 6-CH_3-H_4pterin and 7-CH_3-H_4pterin would be in very similar environments and predict nearly identical geminal coupling constants. The −1.7-Hz difference observed here (Table II) is consistent with the "puckered" conformation observed in the solid state. In Figure 8, the solution conformation of the tetrahydropterin ring is illustrated for the 6-CH_3 analogue.

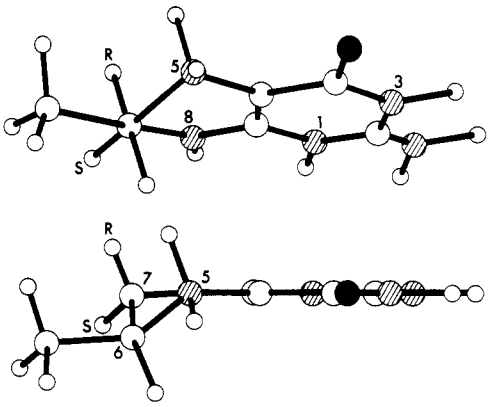


FIGURE 8: Schematic drawings of 6-CH_3-H_4pterin in the equatorial CH_3 conformation showing the staggered orientations of the C_6–C_7 substituents (upper) and the "pucker" of the otherwise flat tetrahydropterin ring (lower). Atom designations: H, small unshaded; C, large unshaded; N, shaded; O, black.

CONCLUSION

The trans and cis isomers of 6,7-$(CH_3)_2$-H_4pterin are both excellent catalytically active cofactors for PAH, the oxidation of each being fully coupled to tyrosine formation; this suggests that the uncoupling quality of the H_4pterin and 7-CH_3-H_4-pterin cofactors stems from their lack of a C_6 substituent rather than from the presence of a C_7 substituent. The role of the C_6 substituent woould, therefore, appear to be basically a steric one, stabilizing the ternary *pteridine–phenylalanine–enzyme* complex during the reaction with molecular oxygen and the subsequent hydroxylation of the amino acid substrate.

The importance of the resonance delocalization of the C_{8a}=C_{4a}—C_4=O_π system is supported by the fact that 6-CH_3-8-deazaH_4pterin serves neither as a cofactor nor as an inhibitor for PAH whereas the 5-deaza analogue is a good inhibitor (Lazarus et al., 1981). This suggests that the sp^2 hybridized N_8 is important in binding the cofactor to the enzyme.

Recently ^{1}H NMR has been used to determine the structure of the unstable quinonoid dihydropterin intermediate generated by the enzymatic or chemical oxidation of tetrahydropterins (Lazarus et al., 1982b). In the quinonoid pterin 6H is allylic to C_{4a}=N_5 and moves downfield by 1.08 ppm from its position in the tetrahydropterin. The C_7 protons are much less affected by conversion to the quinonoid form, the axial 7H moving 0.27 ppm downfield and the equatorial 7H moving 0.18 ppm downfield (Lazarus et al., 1982b). It was argued from this shift differential that the ortho quinonoid isomer (Lund, 1975), having an N_8=C_{8a} double bond, could be excluded from consideration and that only para quinonoid forms need be considered (Kaufman, 1971; Lazarus et al., 1982b). Consideration of the N_8-to-C_4 vinylogous amide delocalization, supported by the X-ray structure and our results reported here, shows that there is already substantial double bond character

at the $N_8{=}C_{8a}$ position in the tetrahydropterin form. Thus, the C_7 protons are already pseudo allylic, and a much smaller downfield shift than for 6H would be expected. We think it premature to dismiss the ortho quinonoid form based on current ^1H NMR results.

ACKNOWLEDGMENTS

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Registry No. 6-CH_3-H_4pterin, 942-41-6; 7-CH_3-H_4pterin, 3116-66-3; *cis*-6,7-$(CH_3)_2$-H_4pterin, 60378-42-9; *trans*-6,7-$(CH_3)_2$-H_4pterin, 91379-84-9; 6-methyl-*d*-H_4pterin, 94202-47-8; *trans*-6-methyl-d_3-H_4pterin-7-*d*, 94202-48-9; *cis*-6-methyl-d_3-H_4pterin-7-*d*, 94202-49-0; 6-methyl-d_3-H_4pterin-7,7-d_2, 94202-50-3; 6-methyl-*d*-H_4pterin-7-*d*, 94202-51-4; 7-methyl-*d*-H_4pterin-6-*d*, 94202-52-5; phenylalanine hydroxylase, 9029-73-6.

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