Tautomeric Nature of Quinonoid 6,7-Dimethyl-7,8-dihydro-6*H*-pterin in Aqueous Solution: A ^{15}N NMR Study

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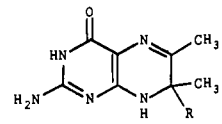
Abstract: The ^{15}N chemical shifts of N-1, N-3, N-5, and the NH_2 in the parent 6,7-dideuterio-5,8-dihydro-6,7-dimethylpterin, its cation, the two-electron oxidation product quinonoid 6,7-dideuterio-6,7-dimethylpterin, its cation, the rearrangement product 7-deuterio-8-hydro-6,7-dimethylpterin, and fully oxidized 6,7-dimethylpterin were assigned from the ^{15}N NMR spectra of the pterins with the ^{15}N isotope in N-5 (95%), N-1 (33%), N-3 (33%), and NH_2 (33%) and the pterins labeled singly in the NH_2 (95%). The ^{15}N spectrum of the unstable quinonoid dihydropterin was obtained by using dilute Bicine buffer at low temperature combined with deuterium substitution at carbons 6 and 7 to slow rearrangement to the 7,8-dihydro derivative. The spectral shifts of the ^{15}N resonances observed upon oxidation of the parent compound to the two-electron oxidation product established that the endocyclic quinonoid 6,7-dimethyl-7,8-dihydro-6*H*-pterin tautomer **5** is the predominant form in aqueous solution at near neutral pH. We propose that in future the structure of the quinonoid species of 7,8-dihydro-6*H*-pterins which are not further substituted in the pyrimidine ring should be drawn with the 2-NH_2 group and an endocyclic double bond as in **5**.

Tetrahydrobiopterin is the naturally occurring coenzyme utilized by enzymes that hydroxylate aromatic amino acids (phenylalanine, tyrosine, and tryptophan). These hydroxylases have strict requirement for oxygen and 5,6,7,8-tetrahydropterin,[1] but the natural cofactor is 5,6,7,8-tetrahydrobiopterin.[2] Direct evidence has been obtained for the formation (from using the reduced 6-methyl-5,6,7,8-tetrahydropterin as substrate) of a 4a-hydroxy adduct such as **2**,[3] concomitant with tyrosine production for the reaction catalyzed by phenylalanine hydroxylase (PAH, EC 1.14.16.1) (Scheme I). This product can undergo spontaneous dehydration or catalyzed dehydration by 4a-hydroxycarbinolaminetetrahydropterin dehydratase to form a quinonoid 7,8-dihydropterin (q-$DMPH_2$) such as **3**.[4] The latter rearranges non-enzymically to the thermodynamically more stable 7,8,-dihydro derivative **4**.[5] Q-$DMPH_2$ in turn is a substrate for dihydropteridine reductase (DHPR, EC 1.6.99.7), an enzyme that utilizes NADH to re-form $DMPH_4$ **1**.[2]

The hydroxylase oxidation product **3** was originally recognized 20 years ago,[6] but its structure has only been partially elucidated. The presence of a 4a-5 double bond in the structure has been fully established by the preservation of a tritium label at C-7 in the cycle **1** → **2** → **3** → **1**,[7] by the retention of the chiral center at C-6 in the same cycle,[8] by the DHPR activity of quinonoid 6,6-dimethyl-7,8-dihydro-6*H*-pterin,[9] and by the large downfield shift (1.08 ppm) of H-6 but not H-7 (0.26 ppm) in the ^1H NMR spectrum of **1** when it is oxidized to **3**.[10] The tautomeric structure of the pyrimidine ring, however, has not been settled. There are minimally three possible tautomeric structures for Q-$DMPH_2$ (**3**, **5**, and **6**). Each of these structures has been supported, in some measure, by experimental work. The exocyclic tautomer **3** was favored on the basis of UV spectral data of **3** (R = H) and its 2-methylamino and 2-dimethylamino derivatives,[7,11,12] on the basis of differences in the rates of oxidation of various N-methylated tetrahydropterins,[13] and has been inferred from molecular orbital calculations.[14] Arguments for the endocyclic tautomer **5** were derived from the Q-$DMPH_2$ acid-base behavior[15] and more recently from the rates of aerobic oxidation of several N-methylated tetrahydropterins, rearrangements of their quinonoid oxidation products to the respective 7,8-dihydro derivatives, and the reduction of these quinonoid species by NADH.[16] Support for the endocyclic ortho tautomer **6** was based on electrochemical data.



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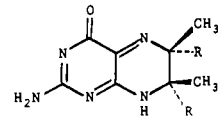

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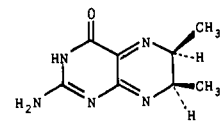

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(1) Abbreviations: Pterin is the generic name for 2-aminopteridin-4-one; biopterin, 6-(L-*erythro*-1′,2′-dihydroxypropyl)pterin; $DMPH_4$, 6,7-dideuterio-5,8-dihydro-6,7-*cis*-dimethylpterin; Q-$DMPH_2$, quinonoid 6,7-dideuterio-6,7-*cis*-dimethyl-8*H*-pterin; 7,8-$DMPH_2$, 7-deuterio-8-hydro-6,7-dimethylpterin; DMP, 6,7-dimethypterin.

(2) Kaufman, S.; Fisher, D. B. In "Molecular Mechanisms of Oxygen Activation"; Hayaishi, O., Ed.; Academic Press: New York, 1974; pp 285-369.

(3) Proposed originally by: Kaufman, S. In "Chemistry and Biology of Pteridines"; Pfleiderer, W., Ed.; W. de Gruyter: Berlin, 1973; pp 291-304; and recently demonstrated by: Lazarus, R. A.; DeBrosse, C. W.; Benkovic, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 6869-6871.

(4) This protein originally characterized by Kaufman (Kaufman, S. *J. Biol. Chem.* **1970**, *245*, 4751-4759) and called phenylalanine hydroxylase stimulator protein has recently been revealed as a dehydratase. See: Lazarus, R. A.; Benkovic, S. J.; Kaufman, S. *J. Biol. Chem.* **1983**, *258*, 10960-10962.

(5) Archer, M. C.; Scrimgeour, K. G. *Can. J. Biochem.*, **1970**, *48*, 278-287.

(6) The first suggestion of a *p*-quinonoid tautomer with an exocyclic imino structure **3** was presented by Hemmerich, P. In "Pteridine Chemistry (Proceedings of the Third International Symposium, 1962 Stuttgart)"; Pfleiderer, W., Taylor, E. C., Eds.; Pergamon Press: Oxford, 1964, 323 and the first enzymatic production by phenylalanine hydroxylase was by Kaufman (see ref 7).

(7) Kaufman, S. *J. Biol. Chem.* **1964**, *239*, 332-338.

(8) Hasegawa, H.; Imaizumi, A.; Ichiyama, A.; Sugimoto, T.; Matsuura, S.; Oka, K.; Kato, T.; Nagatsu, T.; Akino, M. In "Chemistry and Biology of Pteridines"; Kisliuk, R. L., Brown G. M., Eds.; Elsevier: New York, 1979; pp 183-188.

(9) Armarego, W. L. F.; Waring, P. In "Chemistry and Biology of Pteridines"; Blair, J. A., Ed.; W. de Gruyter: Berlin, 1983; pp 429-433.

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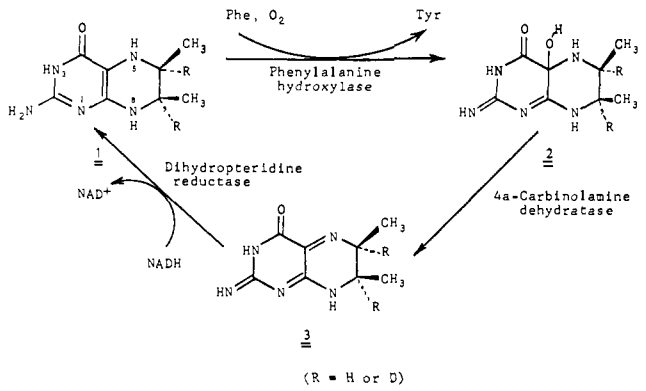
Scheme I



However, recent ^1H NMR analysis of the quinonoid 6-methyl-7,8-dihydro-6*H*-pterin,[10] although not distinguishing tautomer **3** from **5**, has ruled out the ortho isomer **6**. Attempts to stabilize the quinonoid form have involved the synthesis of 6,6-dimethyl derivatives that are unable to rearrange to the 7,8-dihydro form, but information about the tautomeric structure of the pyrimidine ring in solution is still lacking.[18] An alternative possibility for the structure of Q-$DMPH_2$ is that tautomers **3** and **5** both contribute, to some degree, to an equilibrium mixture.

In order to determine unequivocally whether one particular tautomer predominated as the quinonoid tetrahydropterin, we have undertaken an analysis of the structure by ^{15}N NMR. The cofactor analogue 6,7-dimethylpterin was synthesized for this purpose with ^{15}N isotope specifically incorporated in N-5 (95%), N-1 (33%), N-3 (33%), and the 2-NH_2 (33%). This material was then catalytically reduced with $^2H_2$ to form 6,7-dideuterio-5,8-dihydro-6,7-dimethylpterin ($DMPH_4$). The incorporation of deuterium at the C6 and C7 positions takes advantage of the large isotope effect ($k_H/k_D \sim 10$) for the rearrangement of the quinonoid dihydropterin (**3**) to 7,8-dihydropterin (**4**)[5,19] to increase the stability of the quinonoid species. The reduced pterin with a ^{15}N label only on the exocyclic NH_2 was also prepared for the purpose of assigning the signal and because the shifts of the resonance of the nitrogen atom of this group will provide important information regarding the preferred tautomer.

^{15}N labeling permits the use of low reagent concentrations to slow intermolecular catalyzed rearrangement of the quinonoid to 7,8-dihydropterin as well as providing the sensitivity required to obtain in a shorter time the spectrum of the quinonoid species before substantial rearrangement could occur. Additional spectral acquisition time was obtained by working at lower temperatures in dilute buffered solution in the more stable neutral pH range.[5] Another benefit of using low concentrations of pterin is that intermolecular effects, i.e., stacking, dimers, etc., become very weak.[20]

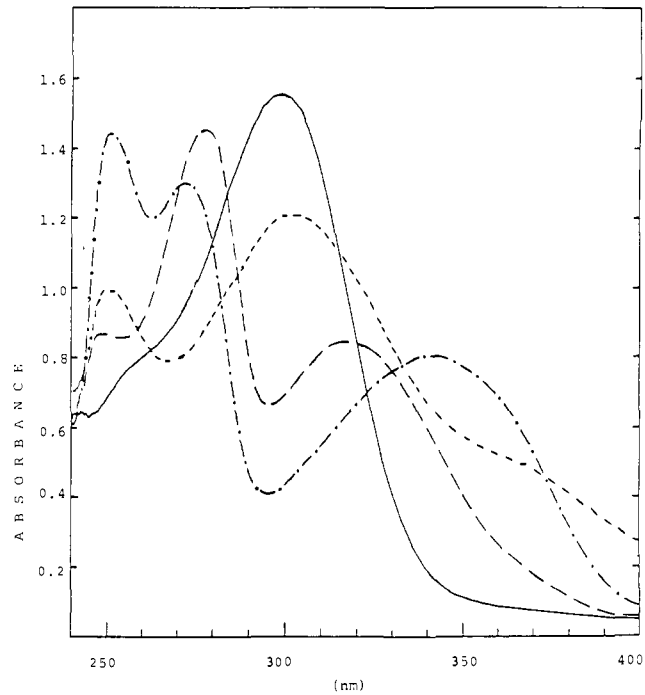


Figure 1. UV spectra taken on small aliquots of samples used for ^{15}N NMR spectra: (a) (—) $DMPH_4$ spectrum obtained from samples used for Figure 2; (b) (- - -) Q-$DMPH_2$ spectrum obtained from samples used for Figures 4 and 5; (c) (– – –) 7,8-$DMPH_2$ spectrum obtained from samples used for Figure 9; (d) (- · - ·) DMP spectrum obtained from samples used for Figure 10.

Experimental Section

Materials and Methods. ^{15}N NMR spectra were collected on a WM 360-MHz Bruker spectrometer at 36.49 (20-mm probe), on a WX 200-MHz Bruker spectrometer at 20.28 MHz (10-mm probe), and on an FX 90Q Jeol spectrometer at 9.11 MHz (10-mm probe). Decoupled spectra at 36.49 MHz were obtained by using an inverse gated decoupling sequence to remove any possible ^{15}N–^1H nuclear Overhauser effects.[21,22] A 40° pulse flip angle was used with a 68-ms acquisition time followed by a 2-s relaxation delay so the ratio of decoupler off to decoupler on time was 29.3.[23] The collected FID's were left shifted four data points to prevent base line rolling in the Fourier-transformed spectra; a line broadening of 5 Hz per data point was applied before zero filling from 2 to 8 K (see Table I). Samples were prepared without deuterium-labeled solvent to prevent quadrupolar dipole–dipole interactions that would decrease the ^{15}N sensitivity; consequently, spectra were collected unlocked. Spectral reference was done by first obtaining a natural abundance ^{15}N spectrum of 6,7-dimethylpterin in trifluoroacetic acid. The NH_2 resonance was assigned a value of 60.8 ppm downfield from $^{15}NH_4NO_3$ in 2 M HCl. The value was obtained by correcting the assignment of Schwotzer et al.[24] by adding 357.1 ppm.[25] Hence in the spectra presented, NH_4^+ is assigned a value of 0 ppm, and downfield is the positive direction. The ^{15}N chemical shifts given here for compounds **11–14** are values obtained by subtracting Stefaniak's values[26] from 357 ppm. For further details see Table I.

Samples of the pterin hydrochloride for the 36.49-MHz spectra were weighed and then flushed extensively with argon in a serum-stoppered 20-mm NMR tube. Solutions of 50 mM Bicine (pH 8.62, Sigma, mp 196 °C, $\Delta pK_a/\Delta T = 0.018/°C^{25}$), methanol (HPLC grade Baker), 1.0 M NaOH, and 1.0 M HCl were all extensively alternatively degassed by exposing to vacuum and then repressurized with argon (in doubly ser-

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(11) Armarego, W. L. F.; Waring, P. *J. Chem. Soc., Perkin Trans. 2* **1982**, 1227–1233.
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Table I. ^{15}N Chemical Shifts[a] of Pterins

pterin[b]	delay[c]	resolution, Hz/data pt	temp, °C	N-1	N-3	N-5	2-NH_2	pH	species[d] and solvent
			36.49 MHz (8.45 T)[e]						
$DMPH_4$ (Figure 2)	2 s	7.4[f]	4	g	114.8	21.4	45.2	8.6	N[h]
$DMPH_4$ (Figure 3)	2 s	7.4[f]	−30	147.6	113.7[i]	20.7	45.9[j]	8.6	N[k]
Q-$DMPH_2$ (Figure 4)	2 s	14.7[l]	4	157.9	173.0	337.4	f	8.6	N[h]
Q-$DMPH_2$ (Figure 6)	2 s	7.4[f]	−30 and 0	153.2	118.9	342.0	82.4[m]	2.5–4.5	C[h]
7,8-$DMPH_2$ (Figure 9)	2 s	7.4[l]	4	150.7	171.0	252.7	48.9	13	A[m]
DMP (Figure 10)	2 s	7.4[f]	4	157.6	187.6	292.2	54.4	12.8	A[n]
			20.28 MHz (4.7 T)[e]						
$DMPH_4$	20 ms	2.5[l]	5	151.3	117.3	23.8	g	8.1	N[o,p]
$DMPH_4$	3 and 10 s	2.5[f]	5	g	117.5	23.9	47.7	7.4	N[h,p]
$DMPH_4$	3 s	2.5[l]	<4[q]	150.85	117.0	23.4	g	8.4	N[h,p]
Q-$DMPH_2$	4 s	1.9[f]	4	g	g	341.8	86.7	7.5	N[r,p]
			9.11 MHz (2.11 T)[s]						
$DMPH_4$ (Figure 7b)	100 ms	0.6[l]	30	118.5	115.9	22.9[t]	57.8[u]	~1	C[w]
$DMPH_4$	100 ms	2.5[v]	20	150.8	117.5	~24[x]	47.3	7.1	N[y]
$DMPH_4$	100 ms	2.5[l]	4	g	117.0	g	47.6	8.0	N[h]
$DMPH_4$[m]	6.5 s	0.6[l]	30	g	obs[z]	obs[z]	obs[z]	8.4	N[y]
$DMPH_4$	3.6 s	0.6[l]	30	obs[p]	obs[z]	g	obs[z]	8.3	N[y]
$DMPH_4$	100 ms	2.5[v]	4	g	116.9	g	47.3	8.5	N[h]
Q-$DMPH_2$[aa] (Figure 5)	200 ms	2.5[l]	4	aa	aa	aa	86.4	7.5	N[h,bb]
Q-$DMPH_2$	200 ms	2.5[v]	4	g	g	341.7	87.1	7.5	N[h,bb]

[a] Downfield shifts in ppm from ^{15}N standard in internal capillary. [b] 6,7-Dimethyl-6,7-dideuterio-5,8-dihydropterin ($^{15}N_4$) and its oxidation products (see abbreviations). These pterins have ^{15}N labels on N-1 (0.33), N-3 (0.33), N-5 (0.95), and 2-NH_2 (0.33). [c] Delay between last acquisition of data and next pulse. [d] N = neutral species, C = cation, A = anion. [e] 2 M $^{15}NH_4{}^{15}NO_3$ in D_2O standard in capillary with internal lock; $^{15}NH_4{}^+$ and $^{15}NO_3{}^-$ are at δ 0 and 353.2 ppm; ~40° pulse flip angle. [f] 400–1000 acquisitions. [g] Signal not observed. [h] 50 mM Bicine–HCl in H_2O. [i] Doublet $J_{^{15}N\text{-}^1H}$ 85.4 Hz. [j] Triplet $J_{^{15}N\text{-}^1H}$ 88.2 Hz. [k] 30 mM Bicine–HCl in 40% MeOH–H_2O. [l] 2000–6000 acquisitions. [m] Triplet $J_{^{15}N\text{-}^1H}$ 92.4 Hz. [n] 24 mM Bicine–HCl in 32% MeOH–H_2O. [o] 50 mM Bicine–HCl in 20% D_2O–H_2O. [p] Contains 0.2 mg of $Cr(acac)_3$. [q] Sample froze during run. [r] 30 mM Bicine–HCl in H_2O. [s] 0.5 M $^{15}NH_4Cl$ in H_2O in capillary with external lock; ~20° pulse flip angle. [t] Doublet $J_{^{15}N(5)\text{-}^1H(6)}$ = 3.1 Hz in the coupled spectrum. [u] Triplet (ill-defined) in coupled spectrum. [v] 20 000–96 000 acquisitions. [w] 1 M HCl–H_2O. [x] Very broad signal. [y] 0.1 M Tris-HCl–H_2O. [z] Signal observed, no ^{15}N standard added. [aa] Only the 2-NH_2 nitrogen is labeled with ^{15}N (95%). [bb] Buffer contains 8–10% MeOH.

um-stoppered flasks) to remove the last traces of oxygen. All solution transfers were done in gas-tight syringes through serum stoppers under positive argon pressure. For the spectra at 9.11 MHz solutions were made up as above but were degassed in 10-mm NMR tubes and repressurized with nitrogen. The UV spectra were measured before and after the NMR experiments to confirm that the species had not altered. UV spectra were measured on a Perkin-Elmer scanning spectrophotometer. Small aliquots (10–20 μL) were removed and diluted into 1.0-mL stoppered cuvettes containing argon- or nitrogen-saturated 50 mM Bicine (pH 8.62) and recorded within 5 min. Typical spectra are shown in Figure 1. For the preparation of the quinonoid species bromine was dissolved fresh in methanol for each oxidation. The concentration was determined by making serial dilutions into H_2O and observing the absorption at 393 nm, ϵ = 198 M^{-1} cm^{-1}.[27]

Syntheses. 6,7-Dideuterio-5,8-dihydro-6,7-dimethylpterin Hydrochloride (1-, 3-, 5-, and 2′-$^{15}N_4$). Ammonium chloride (250 mg, 4.6 mmol, 99.2% ^{15}N labeled, Merck and Co.) was ground with cyanoguanidine (160 mg, 1.9 mmol), fused at 220 °C for 1–2 min and then kept at 190 °C for 2.5 h. Ammonium chloride that sublimed onto the colder surface of the flask was periodically scraped into the melt. The melt was cooled, dissolved in water (5 mL), and filtered. The filtrate was evaporated in vacuo and the residue was dried at 100 °C to give guanidine hydrochloride (335 mg, 81%, ca. 33% labeled on each nitrogen atom; literature[28] yield was 85–92% using $^{14}NH_4NO_3$ which is dangerous to use in this preparation). The quality of the hydrochloride was tested by condensation with diethyl malonate to give 2-aminopyrimidine-4,6-dione (70% yield) similar to authentic unlabeled material (literature[29] yield 81%). Sodium ethoxide (from 180 mg, 7.9 mmol, of sodium in 8 mL of ethanol) was added to ethyl cyanoacetate (890 mg, 7.9 mmol). A second quantity of sodium ethoxide was added to guanidine hydrochloride (760 mg, 7.9 mmol, above ^{15}N labeled), sodium chloride was filtered off, and the filtrate was run into the sodium ethyl cyanoacetate solution. The mixture was boiled for 2 h and evaporated, and the residue was dissolved in hot water (5 mL). Acidification with acetic acid gave a solid which was collected and washed with ice-cold water by centrifugation and dried to give 2,4-diaminopyrimidin-6(1*H*)-one (690 mg, 69%, with ca. 33% ^{15}N on each nitrogen atom) which was spectroscopically similar to an authentic unlabeled sample.[30]

Sodium nitrite (390 mg, 6 mmol, 95% ^{15}N labeled) in water (2 mL) was added (30 min) to a vigorously stirred suspension of the labeled diaminopyrimidinone (690 mg, 6 mmol) in acetic acid (10 mL) and water (10 mL) overnight. The pink solid was collected and washed with water (2 × 20 mL) and ethanol (30 mL) by centrifugation and dried (20 °C in vacuo, then at 100 °C) to give the labeled 5-nitroso derivative (720 mg, 83%).

The nitroso compound (530 mg) suspended in 0.5 M HCl (20 mL) containing platinum oxide (100 mg, prereduced) was shaken under hydrogen for 16 h (720 mmHg) during which time the theoretical volume of hydrogen was absorbed. The solution was filtered and gave, after evaporation to dryness in vacuo and drying at 100 °C, 2,4,5-triaminopyrimidin-6(1*H*)-one hydrochloride (595 mg, 96%, ca. 33% labeled with ^{15}N on N-1, N-3, and 2-NH_2, and 95% on 5-NH_2) as a pale yellow solid with UV spectra identical with those of the unlabeled authentic sample.

The labeled pyrimidinone hydrochloride (580 mg) was converted into labeled 6,7-dimethylpterin (450 mg, 78%) by reaction with butane-2,3-dione using a previous method.[31] The labeled pterin (100 mg) in 1 M DCl (20 mL) was shaken with deuterium in the presence of prereduced platinum oxide (50 mg). The theoretical volume was absorbed in 1 h (at 720 mmHg), but shaking with D_2 was continued for a further 1 h. The solution was filtered and the filtrate evaporated in vacuo to give 6,7-dideuterio-5,8-dihydro-6,7-dimethylpterin hydrochloride (140 mg, 33% ^{15}N labeled on N-1, N-3, and 2-NH_2, 95% labeled on N-5, and 100% $^2H_2$ labeled on C_6 and C_7) which had UV absorption identical with that of an authentic sample. In a separate similar reduction with unlabeled pterin, deuterium incorporation at C6 and C7 was shown by ^1H NMR to be almost quantitative. To avoid NMR signal broadening due to traces of platinum, the pterin hydrochloride was dissolved in a small volume of water, passed through acid-washed Chelex-100 (2 mL, 100–200 mesh, Bio-Rad Laboratories), eluted with water, and evaporated to dryness in vacuo before storage. A molecular weight of 350 (consistent with ca. 3HCl,2.5H_2O) was obtained by measuring the UV absorption in 0.1 M HCl at 268 nm (ϵ_{268} = 16 000 M^{-1} cm^{-1}).

6,7-Dideuterio-5,8-dihydro-6,7-dimethylpterin Hydrochloride (2′-^{15}N). 6,7-Dimethyl-2-methylthiopteridin-4(3*H*)-one[32] (100 mg, 0.48 mmol)

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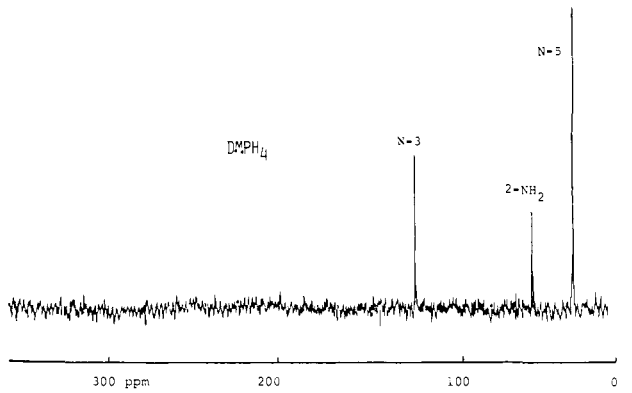


Figure 2. ^{15}N NMR spectrum (36.49 MHz, NOE suppressed, decoupled) of $^{15}N_4$ $DMPH_4$·HCl (56 mg, 8 mM) in 50 mM Bicine buffer pH 8.6 (see Table I).

and ammonium acetate (500 mg, 95% ^{15}N, CEA, Gif-sur-Yvette, France) were thoroughly mixed and heated with stirring in a wax bath at 160–165 °C. After 5 min the mixture fused to a pale yellow liquid with liberation of methanethiol (odor). It began to set to a yellow solid after 15 min, but heating was continued for 2.5 h. The solid was washed with cold water (3 × 7 mL), ethanol (3 × 4 mL), and ether (2 × 4 mL) by centrifugation and dried in vacuo to give pure 6,7-dimethylpterin (2′-^{15}N) as a pale yellow solid (86 mg, 90%) with UV, TLC, and ^1H NMR properties identical with those of an authentic unlabeled sample (the yield was 70% after heating for 1.5 h; literature[33] yield of unlabeled pterin using 1 g of ammonium acetate and heating for 1 h was 93%).

The labeled pterin (85 mg) in 3 M DCl (8 mL) was added to prereduced platinum oxide (150 mg) in the same solvent (5 mL) and shaken with $^2H_2$ as above. The filtrate gave sharp ^1H NMR signals for the methyl groups (indicating the absence of Pt contamination and >97% deuterium at C6 and C7) and was lyophilized to give the title compound (130 mg) which had identical physical properties with those of authentic unlabeled compound. A molecular weight of 344 (consistent with ca. $3HCl,3H_2O$) was obtained by measuring the UV absorption at 268 nm and pH 2.5 (ϵ_{268} at pH 2.5 is 13 600 M^{-1} cm^{-1}).

Results and Discussion

Assignments of ^{15}N Signals. The ^{15}N resonances of derivatives of 6,7-dimethylpterin at various field strengths are in Table I together with some of the spectrometer settings. The spectrum of the neutral species of the parent compound $DMPH_4$ (**1**) at 36.49 MHz is shown in Figure 2. The pyrazine 5-nitrogen (95% ^{15}N) is easily assigned to the most intense resonance at 21.4 ppm and correlates well with reported data.[21,24,34] The NH_2 (33% ^{15}N) on the pyrimidine ring is assigned to the 45.2 ppm signal.[24,35] The signal at 114.8 ppm is from N-3 (33% ^{15}N), but the signal from N-1 (also 33% ^{15}N) is clearly missing unless two signals are superimposed. It is not unusual in ^{15}N NMR spectroscopy for some signals to disappear under certain spectrometer settings and reappear under others because several factors affect the amplitude and phase of the signals, e.g., nuclear Overhauser effect (NOE) and chemical shift anisotropy (CSA).[36,37] Prior to oxidation to Q-$DMPH_2$, the pH was raised to 9.1, but the only difference in the spectrum was signal broadening. The fully coupled spectrum was also measured, but no clear coupling could be observed from the slightly broadened signals. The spectrum of the neutral species was re-run on a solution of $DMPH_4$ in 30 mM Bicine buffer and 40% (v/v) methanol at −30 °C or in 50 mM Bicine buffer and 40% (v/v) methanol at 4 °C (Figure 3), and this showed all the signals in Figure 2 with approximately the same chemical shifts together with the missing signal for N-1 which now appears at

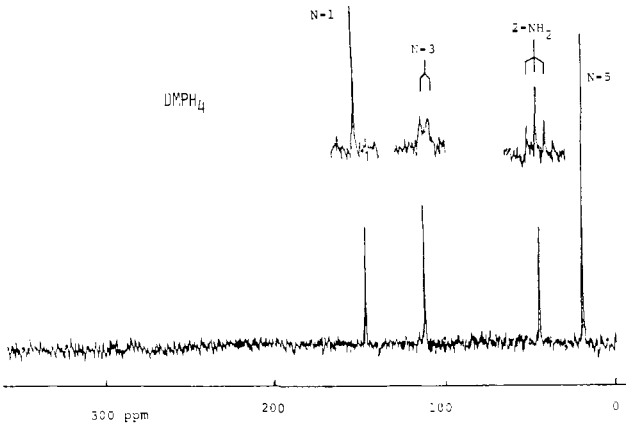


Figure 3. Inverse-gated decoupled ^{15}N NMR spectrum (36.49 MHz) of $DMPH_4$·HCl (70 mg, 10 mM) in 30 mM Bicine and 40% MeOH (v/v) at pH 8.6. Inserts are from the coupled spectrum (see Table I).

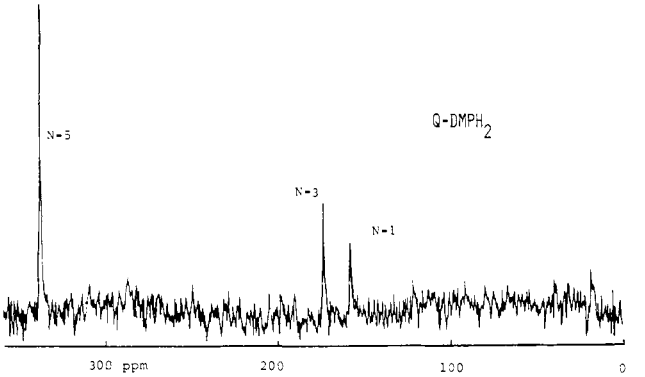


Figure 4. ^{15}N NMR spectrum (36.49 MHz, NOE suppressed, decoupled) of $^{15}N_4$ Q-$DMPH_2$ generated from the sample used in Figure 2 by addition of Br_2–MeOH (190 μL, 1.1 M). The final pH was 8.6. To ensure solubility of the sample at 4 °C more 50 mM Bicine (5 mL, pH 8.6) was added (pterin, 6.3 mM). The ^{15}N–^1H coupled spectrum was unresolved.

147.6 ppm. Also in the solution at −30 °C the ^{15}N–^1H single bond coupling constants were clearly observed (but not at 4 °C) in the proton coupled runs and confirm the assignments in Figures 2 and 3. The resonances of all four labeled nitrogen atoms were, on the other hand, observed in the neutral species in aqueous Tris buffer at 9.11 MHz (fast pulsing) except that the signal from N-5 was rather broad (ca. 24 ppm). The other signals had chemical shifts similar to the above. The broadening of the N-5 signal may be due to NOE and/or the exchange rate of the proton on N-5. At the pH of this solution (7.1) about 3% of $DMPH_4$ (pK_a = 5.6)[38] is in the species protonated on N-5. At 20.28 MHz all the resonances were observed at one spectrometer setting or another (see Table I).

The signals from the cation of $DMPH_4$ containing more than 50% of dication can be assigned to N-5, 2-NH_2, N-3, and N-1 in increasing downfield shift from the standard ($^{15}NH_4Cl$ at 9.11 MHz). The most intense signal is that of N-5 (95% ^{15}N; 22.9 ppm), which is a doublet in the proton-coupled spectrum due to coupling with H6 (J = 3.1 Hz). In the coupled spectrum the NH_2 signal at 57.8 ppm is an ill-defined triplet, and N-1 and N-3 are assigned by comparison with the neutral species. However, because these two signals are very close together (Δ 2.6 ppm) it is conceivable that the assignments may be reversed, i.e., N-1 at 115.9 and N-3 at 118.5 ppm.

Preliminary experiments showed that in 50 mM Bicine buffered at pH 7.6 and 10 mM Q-$DMPH_2$, about equal amounts of Q-$DMPH_2$ and the rearranged product 7,8-$DMPH_2$ (**4**), as judged by the presence of signals from both species, are obtained after 35 min at 37 °C and 220 min at 15 °C, but the rearrangement

(33) Curran, W. V.; Angier, R. B. *J. Org. Chem.* **1963**, *28*, 2672–2677.
(34) Kawano, K.; Ohishi, N.; Suzuki, A. T.; Kyogoku, Y.; Yagi, K. *Biochemistry* **1978**, *17*, 3854–3860. Franken, H.-D.; Rüterjans, H.; Müller, F. *Eur. J. Biochem.* **1984**, *138*, 481–489.
(35) Stadeli, W.; Philipsborn, W. v. *Org. Magn. Reson.* **1981**, *15*, 106–109.
(36) Levy, G. C.; Lichter, R. L. "Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy"; Wiley: New York, 1979.
(37) Martin, G. J.; Martin, M. L.; Gouesnard, J. P. "^{15}N-NMR Spectroscopy"; Springer-Verlag: Berlin, 1981.
(38) Kallen, R. G.; Jencks, W. P. *J. Biol. Chem.* **1968**, *241*, 5845–5851.

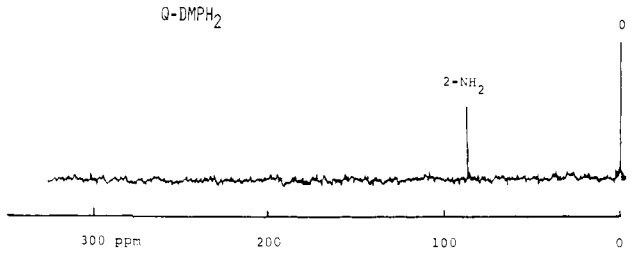


Figure 5. ^{15}N NMR spectrum (9.11 MHz, decoupler on and fast pulsing) of $^{15}N_1$ Q-DMPH$_2$ prepared from $^{15}N_1$ DMPH$_4$·HCl (15.8 mg) in 50 mM Bicine (1.4 mL, pH 7.5) at 4 °C and Br$_2$–MeOH (118 μL, 384 mM). The pH decreased to ~1 but was immediately adjusted to 7.5 (glass electrode) by addition of 1 M NaOH.

is a little faster in Tris-HCl buffer. Q-DMPH$_2$ is stable for long periods (>6 h) at temperatures of 4 °C and below. The NMR sample for Figure 2 was checked for contamination with other speices by a UV scan (Figure 1a) of a small sample and then oxidized to Q-DMPH$_2$ by 1.3 equiv of bromine.[39] The conversion to Q-DMPH$_2$ was immediate as shown by UV monitoring (Figure 1b). The resulting ^{15}N spectrum is shown in Figure 4. Signals from only three of the four ^{15}N labeled atoms are observed. The most obvious change is that the N-5 resonance has shifted downfield by 316 ppm to appear at 337.4 ppm. This is predicted from published data.[24,34] Efforts were made at 36.49 MHz to see all the signals in one spectrum but without success. If either of the signals at 157.9 or 173.0 ppm is from the exocyclic NH$_2$ group, then the signal of this nitrogen atom will have experienced a downfield shift of at least 112.7 ppm upon oxidizing DMPH$_4$ to Q-DMPH$_2$. This would be consistent with the formation of an exocyclic imino structure such as **3**. However, this is not the case (see below).

The absence of a fourth signal in the spectrum of Q-DMPH$_2$ (Figure 4) was disconcerting. In order to make sure of the assignment of the NH$_2$ resonance, the spectra of DMPH$_4$ and Q-DMPH$_2$ in which only the exocyclic amino nitrogen atom is labeled with ^{15}N (99%) were examined. In this case we used a low field (9.11 MHz) and short pulse delays. The spectrum of Q-DMPH$_2$ ($^{15}N_1$) is reproduced in Figure 5. The signal at 86.4 ppm for the NH$_2$ shows that this resonance is missing from the spectrum at high field (Figure 4). This was confirmed when the spectrum of Q-DMPH$_2$ ($^{15}N_4$) was run at 20.28 MHz. The signals from N-5 (at 341.8 ppm) and NH$_2$ (at 86.7 ppm) were observed, although the signals for N-1 and N-3 did not appear. The reason for not observing the NH$_2$ signal at 36.49 MHz is most probably because of chemical shift anisotropy. In one experiment Q-DMPH$_2$ was generated but too much acid was formed. The spectrum obtained (Figure 6) was due mainly to the cation of Q-DMPH$_2$ because the pH of the solution was ~3.5, and the pK_a of Q-DMPH$_2$ is 5.4 ± 0.1.[40] The same NOE-suppressed decoupled spectrum was obtained after the sample was warmed to 0 °C. Here all four resonances were observed and were assigned (see inserts in Figure 6) to NH$_2$ (triplet), N-3 (broad singlet, ca. 90 Hz, from ^{15}N–^1H coupling), N-1 (very sharp singlet), and N-5 on the basis of signal multiplicity, intensity, and/or chemical shift from the coupled spectrum.

Predominant Tautomeric Form. The question of whether the spectra for Q-DMPH$_2$ in Figures 4 and 5 are for only one tautomer or for the average of two rapidly equilibrating tautomers (namely **3** and **5**) is a classical problem amenable to ^{15}N NMR studies.[21,23,26] If we consider the large downfield change in chemical shift of N-5 (316 ppm) when DMPH$_4$ (**1**) is oxidized to Q-DMPH$_2$ (Figures 2 and 4), we should also expect a large downfield shift of the 2-NH$_2$ resonance if the tautomeric structure of Q-DMPH$_2$ had a 2-imino group as in **3**. The experiment revealed a downfield shift of only 39 ppm (see Table I), suggesting a large contribution from the tautomer with the endocyclic double bond

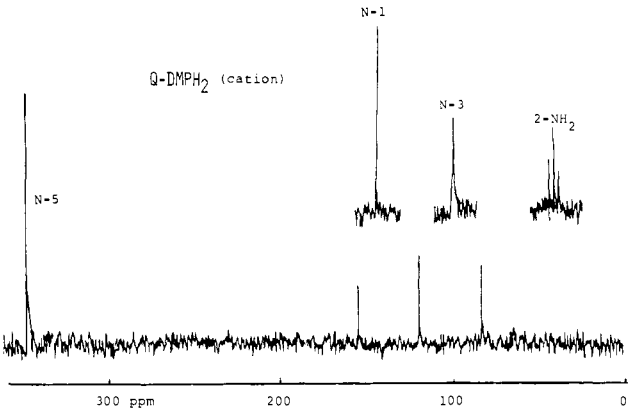
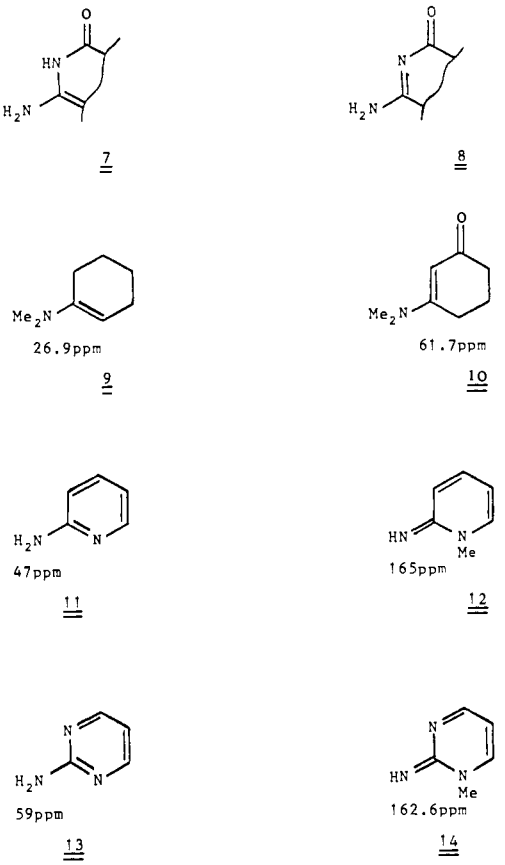


Figure 6. ^{15}N NMR spectrum (36.49 MHz, NOE suppressed, decoupled) of $^{15}N_4$ Q-DMPH$_2$ generated from the sample in Figure 3 by addition of Br$_2$–MeOH (260 μL, 1.05 M) resulting in pH 3.5. Inserts are for the coupled spectrum.

5. For a quantitative estimate of the composition of the tautomeric equilibrium, approximations of the maximum chemical shift changes for rehybridization could be made by using ^{15}N resonances of model compounds. The accuracy of the estimates is only as good as the similarity of the models used to the compounds in question.

If we consider the oxidation of DMPH$_4$ to Q-DMPH$_2$ as the conversion of an amine **7** to the vinylogous amide **8**, insofar as the NH$_2$ is concerned, then we could use the ^{15}N chemical shifts of enamines and enamino ketones as models.[41] The chemical shifts of the nitrogen atoms of the models **9** and **10** are 26.9 and 61.7 ppm, respectively, i.e., a shift of 34.8 ppm. This downfield shift



is not very different from that observed for the 2-NH$_2$ resonances (38.8–39.8 ppm) between DMPH$_4$ and Q-DMPH$_2$ and supports

(39) Preliminary small-scale model work revealed that DMPH$_4$, with Br$_2$–H$_2$O, required 1.3 equivalents for immediate conversion to Q-DMPH$_2$.
(40) Armarego, W. L. F.; Waring, P., unpublished results, 1984.

(41) Schwotzer, W.; Philipsborn, W. v. *Helv. Chim. Acta* **1977**, *60*, 1501–1509; δ values downfield from NH$_4^+$ (δ = 0), i.e., corrected by adding 357 ppm.

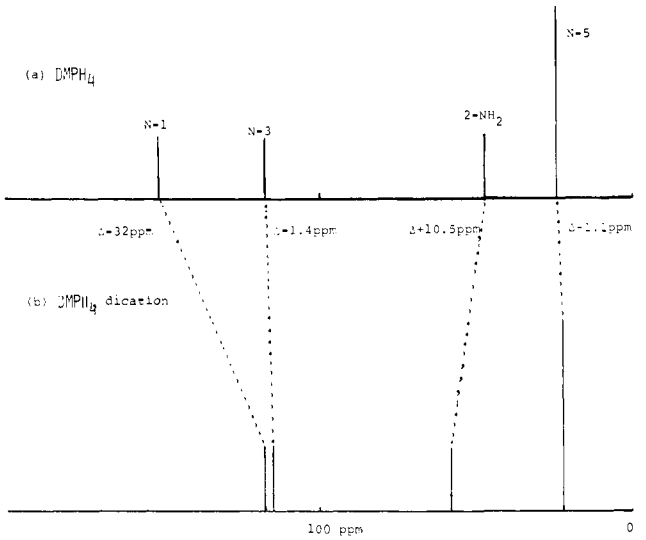


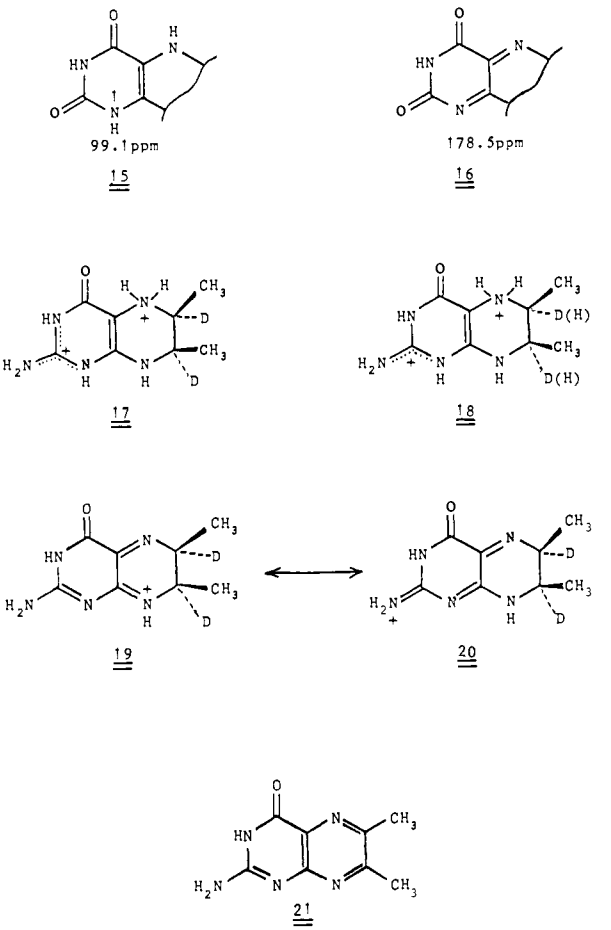
Figure 7. Shifts of ^{15}N resonances when $DMPH_4$ (a) is converted to its dication (b).

the endocyclic tautomer **5** almost completely. In a second estimate we consider the maximum deshielding expected for the exocyclic nitrogen atoms of the models **11** and **12**.[26] For this example methylation of the pyridine ring nitrogen atom has very little effect on the overall deshielding of the exocyclic nitrogen atom, the major contribution being sp^2 hybridization causing a 118 ppm downfield shift. The observed $\Delta\delta$ of 39 ppm for the pterins represents ca. 67% of the endocyclic tautomer **5** and 33% of the exocyclic tautomer **3**. On the other hand, if we use the maximum deshielding from the models **13** and **14** we obtain a value of $\Delta\delta$ 103.5 ppm,[42] which indicates 62.3% of **5** and 37.7% of **3**. In yet another estimate the overall magnitude for shifting the signal N-3 downfield could be obtained from data on riboflavin-2′,3′,4′,5′-tetrabutyrate (in Me_2SO-d_6).[34] The signal from N-1 in the reduced flavin **15**, taken to be analogous to N-3 in $DMPH_4$ (**1**), is deshielded by 79.4 ppm upon oxidation to **16**. Hence the resonance from N-3 in the pterin **1** would be expected to shift from ca. 115 ppm to ca. 194 ppm. The observed value for N-3 obtained on oxidation is 173 ppm (see below for signal assignment), which represents 73% of the tautomer with the exocyclic double bond **5**.

All the models used have some weakness, but they all indicate that the predominant tautomer, and thus the thermodynamically more stable neutral species in solution, of Q-$DMPH_2$ is the one with the endocyclic double bond **5**. This tautomer was previously shown to be the most reactive toward rearrangement to 7,8-$DMPH_2$ and toward oxidation of NADH.[11]

The crossing over, and the large downfield shift, of the resonance from N-5 compared with that from the 2-NH_2 (see Figures 2–6) observed upon oxidation of $DMPH_4$ to Q-$DMPH_2$ is evidence that the relative electronegativities of these two nitrogen atoms have been reversed. Thus, in Q-$DMPH_2$ N-5 has become much less electronegative than the nitrogen atom of the 2-NH_2 group. This is consistent with the known protonation of N-5 in $DMPH_4$ and of the 2-amino group in Q-$DMPH_2$ (see below) and also with the transfer of hydride from NADH to N-5 in the nonenzymic,[7,11,43] and the dihydropteridine reductase catalyzed,[43,44,45] oxidation of NADH.

Spectral Changes on Protonation. ^{15}N NMR resonances are known to exhibit small deshielding (alkylamines), small shielding (arylamines), and large shielding (π-deficient N-heterocycles) upon protonation of the nitrogen atoms.[36,37] The chemical shift changes



on protonation of $DMPH_4$ in aqueous solution (Figure 7) revealed some interesting features. In acidic solution (pH ~ >1) much of the dication is formed and N-5, which is protonated, shifts upfield by a small amount (ca. 2 ppm); the resonance from the NH_2 group moves downfield by 10.5 ppm, N-3 barely moves, and N-1 shifts upfield by 32 ppm. The second proton goes to N-1, but stabilization of the cation by a guanidinium-type resonance, i.e., **17** does not explain the very small shift of the signal from N-3. Resonance stabilization by amidinium-type resonance, i.e., **18**, without involving N-3 (which is involved in amide resonance), is probably operating (cf. ref 24).

The spectrum of Q-$DMPH_2$ (Figure 6) at low pH contrasts with the spectrum of the neutral species (Figure 4) in several ways (Figure 8). The N-5 signal of 6,7-$DMPH_2$ is at 252.5 ppm (see below), and N-5 of 6-methyl-7,8-dihydropterin at pH 10.3 lies at 256 ppm. The N-5 resonance of the latter compound moves upfield by 84 ppm upon protonation.[40] This is also evidence that N-5 of the Q-$DMPH_2$ is not protonated under the conditions in Figure 6. The rather high pK_a (5.4) of Q-$DMPH_2$ implies that there is considerable stabilization of the cation with respect to the neutral species and is consistent with the structures **19** ↔ **20**. The 2-NH_2 resonance is shielded by only 4 ppm on protonation, which is the sum total of a deshielding effect of the partial 2-imino structure **20** and a shielding effect of the partial positive charge. The signal from N-1 is not expected to experience a large shift on protonation of the pterin, and an upfield shift of 4.7 ppm (157.9–153.2 ppm, Figure 4) is observed. The chemical shift of N-3 in the Q-$DMPH_2$ cation at 118.9 ppm is consistent with the structures **19** ↔ **20**, i.e., unprotonated amide nitrogen (see dication of $DMPH_4$ above). The signal at 173 ppm from N-3 of the neutral species then experiences an upfield shift of 54.1 ppm consistent with a predominant tautomeric form with an exocyclic double bond (**5**) for the neutral species.

Further Oxidation of Q-$DMPH_2$. Under the conditions used to generate Q-$DMPH_2$ no 7,8-$DMPH_2$ was observed in the solution that gave the spectrum in Figure 4 after 4 h followed by warming to 0 °C (UV data not shown). Warming the solution

(42) Städeli, W.; Philipsborn, W. v.; Wick, A.; Kampis, I. *Helv. Chim. Acta* **1980**, *63*, 504–520.
(43) Nielsen, K. H.; Simonsen, V.; Lind, K. E. *Eur. J. Biochem.* **1969**, *9*, 497–502.
(44) Craine, J. E.; Hall, E. S.; Kaufman, S. *J. Biol. Chem.* **1972**, *247*, 6082–6091.
(45) Armarego, W. L. F. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 246–249.

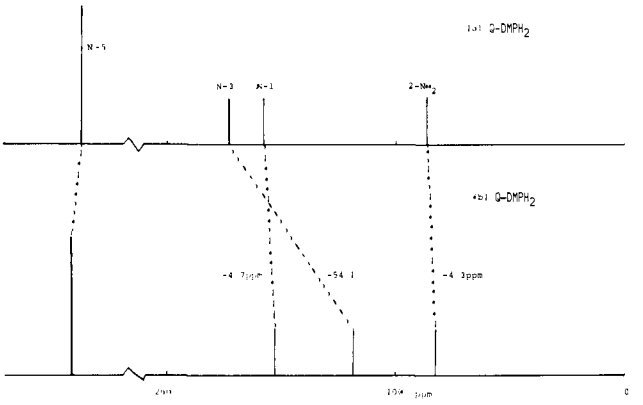


Figure 8. Shifts in ^{15}N resonances when Q-DMPH$_2$ (a) is converted to its cation (b).

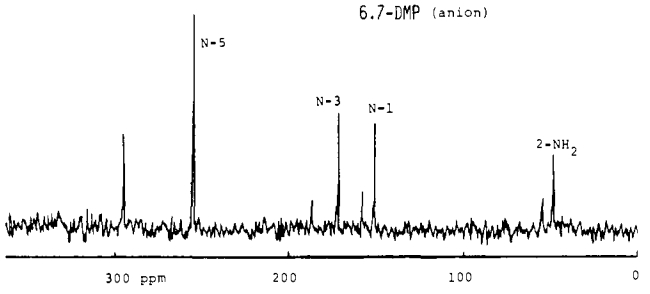


Figure 9. ^{15}N NMR spectrum (36.49 MHz, NOE suppressed, decoupled) of $^{15}N_4$ 7,8-DMPH$_2$ derived from sample in Figure 3 after incubation at 4 °C and pH 13 for 16 h. This solution is now 7.6 mM in pterin and 32% in MeOH (v/v).

in a 50 °C bath did convert the Q-DMPH$_2$ to 7,8-DMPH$_2$ (**4**, Figure 1c); however, the sample precipitated. Dissolution was achieved by adding enough 1.0 M NaOH to raise the pH to 13 and by incubation at 4 °C for 16 h.[46] The NMR spectrum of 7,8-DMPH$_2$ (Figure 9) was taken at 4 °C rather than at −30 °C since no temperature effect was observed for either the Q-DMPH$_2$ or 7,8-DMPH$_2$. This spectrum (Figure 9) does contain signals from the fully oxidized DMP (**21**). The chemical shift assignments for the 7,8-DMPH$_2$ (**4**) are in Table I. The NH$_2$ resonance in the ^{15}N–^1H coupled spectrum of **4** was so broad that it had virtually disappeared into the noise.

The above sample was partially converted to the fully oxidized DMP **21** by a second addition of bromine in methanol and was checked by UV (Figure 1d). The NMR spectrum at pH 12.8 (Figure 10) contains signals from some unreacted 7,8-DMPH$_2$. The only difference in the chemical shifts of the minor peaks in Figure 10 and the major peaks in Figure 9 is a 4 ppm upfield shift of the N-3 signal of the remaining 7,8-DMPH$_2$ anion, most probably signifying a small amount of the neutral species (pK_a of 7,8-DMPH$_2$ is 11.09).[47] The chemical shift assignments for

(46) We were unable to maintain even a 2 mM solution of 7,8-DMPH$_2$ **4** in 50 mM Bicine at pH 8.6.

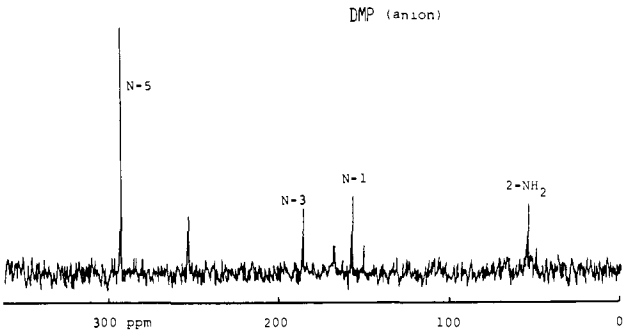


Figure 10. ^{15}N NMR spectrum (36.49 MHz, NOE suppressed, decoupled) of $^{15}N_4$ DMP after the addition of Br_2–MeOH (92 μL, 2.19 M) to the sample in Figure 9; final pH is 12.8.

DMP (**21**) are in Table I. The spectra in Figures 9 and 10 are consistent with an overall deshielding of the resonances as the pyrazine ring takes an aromatic character. The assignment of resonances for N-1 and N-3 is based on the fact that these spectra were measured at high pH, which should have little effect on N-1 but should ionize N-3 to the lactim form.[48]

Conclusion

The spectra collectively provide strong evidence for the formation of tautomer **5**, the endocyclic Q-DMPH$_2$, upon oxidation of DMPH$_4$ (**1**). They do not, however, exclude smaller amounts of the exocyclic tautomer **3** in equilibrium with it in aqueous solution at neutral pH. We propose that the structure of quinonoid 7,8-dihydro-6*H*-pterins unsubstituted in the pyrimidine ring be drawn in the tautomeric form **5** in the future because as far as we know this is the thermodynamically more stable tautomer.

Acknowledgment. We thank Professor L. M. Jackman and Dr. M. D. Fenn for stimulating discussions and constructive criticism.

Registry No. 1, 60378-42-9; **4**, 5977-33-3; **5**, 83650-50-4; **9**, 13815-46-8; **10**, 6135-22-4; **11**, 504-29-0; **12**, 4088-63-5; **13**, 109-12-6; **14**, 1003-58-3; **15**, 67972-77-4; **16**, 752-56-7; [$^{15}N_4$]-DMPH$_4$, 96227-64-4; DMPH$_4$ dication, 68300-02-7; [^{15}N]-6,7-dideuterio-5,8-dihydro-6,7-dimethylpterin cation, 96245-26-0; [^{15}N]-6,7-dideuterio-6,7-dimethylpterin, 96227-65-5; [^{15}N]-7-deuterio-8-hydro-6,7-dimethylpterin, 96227-66-6; [^{15}N]-ammonium chloride, 39466-62-1; cyanoguanidine, 461-58-5; guanidine hydrochloride, 96227-55-3; diethyl malonate, 105-53-3; 2-amino-4,6-pyrimidinedione, 56-09-7; ethyl cyanoacetate, 105-56-6; [^{15}N]-2,4-diaminopyrimidin-6(1*H*)-one, 96227-56-4; [^{15}N]-sodium nitrite, 68378-96-1; [^{15}N]-5-nitrosopyrimidin-6(1*H*)-one, 96227-57-5; [^{15}N]-2,4,5-triaminopyrimidin-6(1*H*)-one hydrochloride, 96227-58-6; [^{15}N]-6,7-dimethylpterin, 96227-59-7; butane-2,3-dione, 431-03-8; [^{15}N]-6,7-dideuterio-5,8-dihydro-6,7-dimethylpterin hydrochloride, 96227-60-0; 6,7-dideuterio-5,8-dihydro-6,7-dimethylpterin hydrochloride-^{15}N, 96227-63-3; 6,7-dimethyl-2-methylthiopterin-4(3*H*)-one, 54030-43-2; [^{15}N]-ammonium acetate, 96227-61-1; [^{15}N]-6,7-dimethylpterin, 96227-62-2; 6,7-dimethylpterin, 611-55-2.

(47) Pfleiderer, W.; Zondler, H. *Chem. Ber.* **1966**, *99*, 3008–3021.
(48) The effect of base also moves the amide nitrogen signal (184 ppm) further downfield than the pyridine-like nitrogen signal (143 ppm) in guanosine-3′-phosphate (Büchner, P.; Maurer, W.; Rüterjans, H. *J. Magn. Reson.* **1978**, *29*, 45–63.