5,10-Methylene-5,6,7,8-tetrahydrofolate. Conformation of the Tetrahydropyrazine and Imidazolidine Rings†

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ABSTRACT: It is suggested from analysis of its ^{1}H NMR spectrum and from model building that methylenetetrahydrofolate in solution at neutral and alkaline pH has a rigid conformation for its tetrahydropyrazine and imidazolidine rings. The proposed conformation in solution has three noteworthy features: (1) the tetrahydropyrazine ring is in a half-chair conformation with C_6-H axial; (2) the imidazolidine ring is on the same side of the pyrimidine ring as C_7 and is nearly planar, and the plane is at an angle of 120° to the pyrimidine ring plane; (3) the aromatic ring of the *p*-aminobenzoyl-L-glutamate moiety extends away from the pteridine rings, but this possibly may not be true for methylenetetrahydrofolate at neutral pH. The chemical shifts and spin–spin coupling constants of the 15 carbon-bound hydrogens of a diastereomeric mixture of the two C_6 stereoisomers and of the natural C_6 stereoisomer of methylenetetrahydrofolate were measured at 25 °C and 300 MHz in 0.1 M NaOD and in 0.1 M sodium phosphate, pH* 6.79. In 0.1 M NaOD and at pH* 6.79, these compounds had the same coupling constants between their C_6, C_7, C_9, and C_{11} hydrogens. Also examined was a methylenetetrahydrofolate specifically deuterated at C_7. The resonances corresponding to the two hydrogens on C_7 had a geminal coupling to one another of 12 Hz and spin–spin couplings to C_6–H of 10.0 and 3.0 Hz for the hydrogens trans and cis to C_6–H, respectively. The two C_9 resonances had geminal coupling of 9.5 Hz and spin–spin couplings to C_6–H of 6.5 and 1.5 Hz for the hydrogens cis and trans to C_6–H, respectively. The two resonances for the hydrogens on the carbon between N_5 and N_{10}, C_{11}, had a chemical shift difference greater than 1 ppm and a geminal coupling of 5.0 Hz.

The one-carbon adducts of 5,6,7,8-tetrahydrofolate are important in the metabolism of single carbon units at three levels of oxidation (Blakley, 1969). 5,10-Methylene-5,6,7,8-tetrahydrofolate (CH_2-H_4folate)[1] is the carrier of carbon at the formaldehyde level of oxidation. One important enzyme which uses this cofactor is thymidylate synthetase (methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45). Thymidylate synthetase is a useful target in cancer chemotherapy (Heidelberger, 1975); the metabolic product 5-fluoro-2′-deoxyuridylate of the anticancer drug fluorouracil is a potent inhibitor of thymidylate synthetase (Lockshin et al., 1979; James et al., 1976; Leary et al., 1975). Thus, analogues of CH_2-H_4folate could have anticancer activity. Thymidylate synthetase also has an interesting reaction mechanism; the enzyme catalyzes both a redox reaction and a carbon-transfer reaction (Blakley et al., 1963). At the present time, there are sufficient data from enzymatic and appropriate nonenzymatic studies as reviewed by Benkovic (1978) to warrant discussion of some of the more probable of the catalytic mechanisms of enzymes that use CH_2-H_4folate. The present study of the solution conformation of CH_2-H_4folate was undertaken to provide structural insight into these mechanisms and to provide insight into possible conformations of CH_2-H_4folate on enzymes such as thymidylate synthetase.

The absolute configuration of natural 5,10-methenyl-5,6,7,8-tetrahydrofolate has recently been solved; the absolute configuration is equivalent to *S* for H_4folate at both C_6 and the α-carbon of the glutamate carbon (Fontecilla-Camps et al., 1979). Presumably all the natural tetrahydrofolates, including CH_2-H_4folate, have the same absolute configuration at C_6. The 300-MHz ^{1}H NMR studies reported here show resolved resonances for the seven protons on C_6, C_7, C_9, and C_{11} (the carbon between N_5 and N_{10}). It was possible to measure their spin–spin coupling constants and thereby to infer the conformation of the tetrahydropyrazine and imidazolidine rings of CH_2-H_4folate by comparison to model compounds and by building molecular models. The conformation we propose is identical with that proposed earlier by Benkovic (1978) and Benkovic & Tatum (1977) from model systems and enzymatic studies.

Experimental Procedures

(±)-L-5,6,7,8-Tetrahydrofolate was purchased from Sigma Chemical Co. and used without further purification. The natural isomer of tetrahydrofolate, (−)-L-5,6,7,8-tetrahydrofolate, was prepared enzymatically by reduction of 7,8-dihydrofolate with NADPH by dihydrofolate reductase as described by Poe et al. (1979).

(6*R*,*S*)-5,10-Methylene-5,6,7,8-tetrahydro-L-folate and (6*R*)-5,10-methylene-5,6,7,8-tetrahydro-L-folate were prepared nonenzymatically in 20-mg batches from the diastereomeric mixture and natural isomer of tetrahydrofolate, respectively. They were prepared in 1 mL of D_2O near pH* 7 by addition of 2–3 molar equiv of fresh formaldehyde at 25 °C with only 2–3 molar equiv of dithiothreitol present. These conditions are derived from the procedures of Tatum et al. (1977). The time course of CH_2-H_4folate formation was monitored at 294 nm. It was important for the attainment of clean NMR spectra that large excesses of formaldehyde and dithiothreitol not be used. The CH_2-H_4folate was purified by precipitation by addition of 1 mL of 0.1 M DCl, followed by centrifugation at 1000*g* for 1 min at 0 °C. The supernatant fluid was discarded and the precipitate was dissolved in 1 mL of 0.1 M NaOD. Each solution was lyophilized. When possible, handling operations were carried out in the dark and in aluminum foil wrapped vessels. The concentration of a CH_2-H_4folate

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[1] Abbreviations used: CH_2-H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; (6*R*,*S*)-CH_2-H_4-L-folate, diastereomeric mixture of two C-6 stereoisomers of CH_2-H_4folate, both *S* at C-α; (6*R*)-CH_2-H_4-L-folate, natural stereoisomer of CH_2-H_4folate; pH*, direct reading of pH electrode in D_2O solution.

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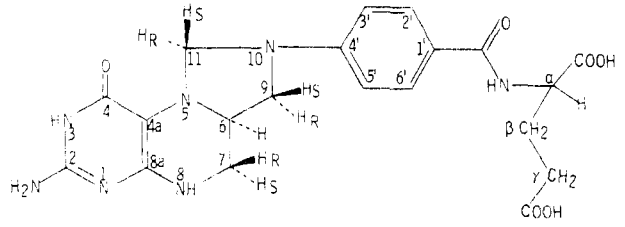

FIGURE 1: Numbering system of CH_2-H_4folate.

solution was standardized by UV absorbance at pH 7.2 by using $\epsilon_{294nm} = 32.0\ cm^{-1}\ mM^{-1}$ (Blakley, 1960).

^1H nuclear magnetic resonance spectra were obtained in D_2O with a Varian SC-300 NMR spectrometer at 25 °C and were internally referenced to the methyl resonance of sodium 3-trimethylsilyl(2,2,3,3-$^2H_4$)propionate with downfield shifts positive. Spectra were accumulated as described by Poe et al. (1979).

The structural formula and numbering system for CH_2-H_4-folate that is used herein is shown in Figure 1. This numbering system is based on that proposed by the Subcommittee on Folates of the Commission on Biochemical Nomenclature (1966). CH_2-H_4folate has two chiral centers, one at C_6 and the other at C_α; the stereoisomers at C_6 are referred to as 6*R* or 6*S* with 6*R* being the natural stereoisomer produced from the natural isomer, (6*S*)-tetrahydro-L-folate. The isomers at C_α are D and L; L is the natural isomer and has the absolute configuration *S*. (6*R*,*S*)-CH_2-H_4-L-folate is a diastereomeric mixture of the two stereoisomers, both having the *S* configuration at the α-carbon, and (6*R*)-CH_2-H_4-L-folate is the natural stereoisomer used by thymidylate synthetase. The stereoisomer that is *R* for C_6 in CH_2-H_4folate has C_6 in the same configuration as the natural stereoisomer of 5,10-methylene-5,6,7,8-tetrahydrofolate (Fontecilla-Camps et al., 1979); this configuration of C_6 is *S* in 5,6,7,8-tetrahydrofolate. The hydrogens in Figure 1 on C_7, C_9, and C_{11} labeled H_R and H_S are the hydrogens that would make the carbon to which they are attached have the absolute configuration *R* and *S*, respectively, if that hydrogen were replaced by deuterium or tritium; i.e., H_R and H_S are the prochiral *R* and *S* hydrogens, respectively, according to the Hanson system as described by Bentley (1969). The designation 7-D (50% D) is herein used to designate a reduced folate which has one hydrogen and one deuterium at C_7.

Molecular models of CH_2-H_4folate were built with Kendrew Skeletal Molecular Models and space-filling Corey–Pauling–Koltun models, both purchased from Ealing. For the ORTEP plot in Figure 3 of the proposed conformation for natural CH_2-H_4folate in solution, the bond lengths and bond angles of the tetrahydropyrazine ring were as measured by Bieri & Viscontinini (1977) for 5-formyl-6,7-dimethyl-5,6,7,8-tetrahydropterin, and the bond lengths and bond angles for the *p*-aminobenzoyl-L-glutamate moiety were as measured by Matthews et al. (1977) for the corresponding moiety in methotrexate. For the imidazolidine ring atoms, the following bond lengths and bond angles were used: bond lengths, C_6 to C_9, 1.52 Å; C_9 to N_{10}, 1.47 Å; N_{10} to C_{11}, 1.47 Å; N_5 to C_{11}, 1.47 Å; bond angles, N_5–C_6–C_9, 111.0°; C_6–C_9–N_{10}, 108.0°; C_9–N_{10}–C_{11}, 108°; N_{10}–C_{11}–N_5, 108°. These bond lengths are standard values for the respective bond types (*Handbook of Chemistry and Physics*, 1971) and the bond angles were chosen to be equal except for the N_5–C_6–C_9 bond which was chosen to be 111.0° to complete the five-membered ring. The nitrogen atoms N_5 and N_{10} were assumed to be tetrahedrally bonded. Dihedral or torsional angles were as measured from the Kendrew skeletal molecular model, with the glutamate moiety in the configuration described by Matthews et al. (1977) for the glutamate moiety of methotrexate in the binary methotrexate complex of *Escherichia coli* MB 1428 dihydrofolate reductase.

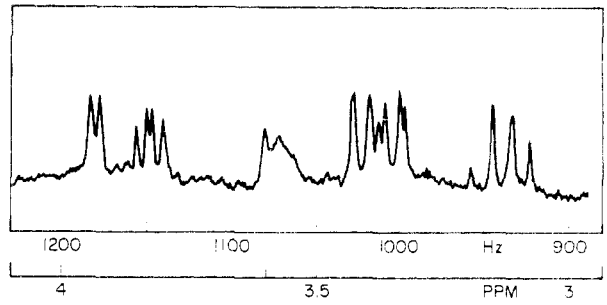

FIGURE 2: Methylene region of 300-MHz ^1H NMR spectrum of (6*R*,*S*)-5,10-CH_2-H_4-L-folate. The spectrum was accumulated at 25 °C in 0.1 M NaOD with 17 mM CH_2-H_4folate.

Table I: Chemical Shifts and Coupling Constants for (6*R*,*S*)-Methylenetetrahydro-L-folate at 25 °C and 17 mM in 0.1 M NaOD

resonance(s) of	chemical shift (ppm)	multiplicity	*J* (Hz)
$C_{2',6'}$-2H	7.800 7.770	d	8.4
$C_{3',5'}$-2H	6.720 6.690	d	8.4
C_{11}	5.050	d	5.0
α-CH	4.317		4.0 and 9.0
C_{11}	3.930	d	5.0
C_9-H cis[a]	3.833	dd	6.5 and 9.5
C_6-H	3.573	m	multiplet
C_6-H trans[a]	3.407	dd	1.5 and 9.5
C_7-H cis[a]	3.347	dd	3.0 and 12.0
C_7-H trans[a]	3.133	dd	10.0 and 12.0
γ-CH_2	2.317	t	7.3
β-CH	2.153	m	multiplet
β-CH	2.033	m	multiplet

[a] Cis and trans refer to hydrogens on the same or opposite face as C_6-H, respectively, of either the tetrahydropyrazine ring for C_7-H or the imidazolidine ring for C_9-H. [b] Center of multiplet.

Results and Discussion

Previous ^1H NMR studies of CH_2-H_4folate by Tatum et al. (1977) have shown that the resonances of the *p*-aminobenzoyl-L-glutamate moiety of CH_2-H_4folate exhibit about the same chemical shifts as corresponding protons of tetrahydrofolate (Poe & Hoogsteen, 1978; Furrer et al., 1978). Importantly, Tatum et al. (1977) demonstrated that the protons of the methylene bridge C_{11} were nonequivalent with a chemical shift difference of 1.21 ppm and geminal spin–spin coupling of 4 Hz. Due to resonance overlap, these constants were not measured for the five protons on C_6, C_7, and C_9. When the ^1H NMR spectrum of CH_2-H_4folate was examined at 300 MHz, the resonances between 3 and 4 ppm exhibited an almost first-order spin–spin coupling pattern, as is shown in the spectrum replotted for Figure 2. Only the C_6 resonance at 3.57 ppm was an unresolved multiplet. This spectrum was of (6*R*,*S*)-CH_2-H_4-L-folate at 17 mM in 0.1 M NaOD. The natural stereoisomer (6*R*)-L-CH_2-H_4folate had the same spin–spin coupling constants in 0.1 M NaOD as shown in Figure 2 and given in Table I for the diastereomeric mixture but the chemical shifts equal to those of the diastereomeric mixture only at infinite dilution. The natural stereoisomer had the same spin–spin coupling constants at pH* 6.79 as in 0.1 M NaOD.

It is readily apparent from the spin–spin splittings in the resonances shown in Figure 2 that the doublet of doublet

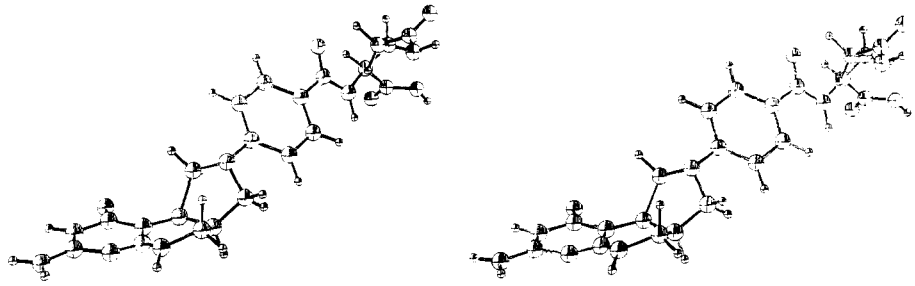

FIGURE 3: ORTEP stereo plot of proposed conformation for CH_2-H_4folate in solution. The absolute configurations at C_6 and C_α are *R* and *S*, respectively; the *R* configuration at C_6 here is equivalent to *S* at C_6 for tetrahydrofolate.

resonance centered at 3.13 ppm is coupled through a $|J| = 12.0$ Hz coupling to the doublet of doublet resonance centered at 3.35 ppm, while the doublet of doublet centered at 3.83 ppm is coupled through a $|J| = 9.5$ Hz coupling to the doublet of doublet resonance centered at 3.41 ppm. The area under each of these resonances is equal and corresponds to one proton, when standardized against the area of the resonances of the aromatic protons of the *p*-aminobenzoyl-L-glutamate moiety of CH_2-H_4folate. The second spin–spin coupling constants for the resonances centered at 3.13, 3.35, 3.41, and 3.83 ppm are 10.0, 3.0, 1.5, and 6.5 Hz, respectively. These second constants presumably reflect coupling to the complex multiplet at 3.57 ppm whose area also corresponds to a single proton. This complex multiplet doubtless corresponds to the C_6–H. The resonances at 3.13 and 3.35 ppm correspond to the C_7 hydrogens. These resonances are doublets with areas corresponding to one-half proton each in 7-D (50% D)-(6*R*)-CH_2-H_4-L-folate. The remaining pair of coupled resonances at 3.41 and 3.83 ppm therefore corresponds to the two C_9 hydrogens. The resonance at 3.13 ppm has a large spin–spin coupling constant to C_6–H of 10 Hz, which is typical for trans hydrogens at C_6 and C_7 in model tetrahydropteridines (Weber & Viscontini, 1975; Storm & Chung, 1976; Bieri & Viscontini, 1974). The small spin–spin coupling of 3 Hz between the other C_7–H and C_6–H is normal for cis hydrogens at C_6 and C_7 in tetrahydropteridines. The C_9 hydrogen that has the spin–spin coupling of 6.5 Hz probably corresponds to the hydrogen cis to C_6–H, the one which has a dihedral angle C_9–H to C_9 to C_6 to C_6–H near 0°. The C_9 hydrogen that has a smaller spin–spin coupling constant of 1.5 Hz to C_6–H thus is the trans hydrogen. The remaining one-proton resonance in Figure 2 centered at 3.93 ppm corresponds to one of the two hydrogens on C_{11}. The other C_{11}–H has its resonance centered at 5.05 ppm; the geminal spin–spin coupling between the two hydrogens is 5.0 Hz under these conditions. The existence of the geminal coupling in the two C_{11} resonances shows they are not due to a 50:50 mixture of two slowly interconverting conformations.

When a model of CH_2-H_4folate is constructed, the conformations with C_6 and C_7 both on the same side of the pyrimidine plane are not possible because of the imidazolidine ring, and the forms with the N_5 to C_{11} bond pseudoequatorial are not possible for a tetrahedral nitrogen. The two half-chair conformations can have the C_6 hydrogen either equatorial or axial. The spin–spin couplings between C_6–H and the two C_7 protons show that the conformation with C_6–H axial is strongly preferred. If C_6–H is equatorial, the dihedral angles C_6–H to C_6 to C_7 to C_7–H are +60 and −60° for the two C_7 hydrogens, and the spin–spin couplings from the C_7 hydrogens to C_6 would be small and equal. The observed coupling constants of 10 and 3 Hz agree quite nicely with coupling constants expected for a frozen conformation with C_6–H axial. We propose that the tetrahydropyrazine ring of CH_2-H_4folate in solution is in a half-chair conformation with C_6 and C_7 on opposite sides of the pyrimidine plane and with the C_6 proton axial.

Our proposed conformation for (6*R*)-CH_2-H_4-L-folate in solution may be seen in Figure 3 in a stereo ORTEP plot (Johnson, 1965). The *p*-aminobenzoyl-L-glutamate moiety in Figure 3 is in a configuration similar to that of the *p*-aminobenzoyl-L-glutamate moiety of methotrexate when bound to *E. coli* dihydrofolate reductase (Matthews et al., 1977). The *p*-aminobenzoyl ring of CH_2-H_4folate cannot rotate freely about the $C_{1'}$ to $C_{4'}$ axis in a space-filling model, due to the hydrogens on C_9 and C_{11}. The lack of free rotation suggests that the *p*-aminobenzoyl ring is bonded to N_{10} so as to go away from the pteridine rings, rather than "fold back" toward the pteridine rings, at least for CH_2-H_4folate in 0.1 M NaOD. This is suggested because the hydrogens on the *p*-aminobenzoyl ring show no evidence of the ring-current field of the pyrimidine; i.e., the hydrogens on $C_{2'}$ and $C_{6'}$ in CH_2-H_4folate are equivalent within ±0.001 ppm as are the hydrogens on $C_{3'}$ and $C_{5'}$ [for a fuller discussion of ring-current fields, see Emsley et al. (1965) and Wüthrich (1976)]. The equivalence would not be expected to be the case for the "folded-back" conformation, unless there was free rotation about $C_{1'}$ to $C_{4'}$, but would be expected for the extended conformation. However, for the neutral form of CH_2-H_4folate it is possible that the diamagnetic anisotropy of the carbonyl double bond (C_4 to O_4) might cancel out the effect of the pyrimidine ring-current field on the hydrogens of the *p*-aminobenzoyl ring, since the diamagnetic anisotropy cannot be rigorously calculated (Jackman & Sternhell, 1969). In the extended conformation the negative charge at O_4 of the pyrimidine ring (for CH_2-H_4folate in 0.1 M NaOD) would be further away from the negative charges on the glutamate carboxyls than in the folded-back conformation.

Summarized in Table I are the coupling constants and chemical shifts of the various resonances of (6*R*,*S*)-5,10-methylene-5,6,7,8-tetrahydro-L-folate in 0.1 M NaOD at 17 mM in D_2O at 25 °C, with the assignments as assigned above. For the natural stereoisomer, (6*R*)-CH_2-H_4-L-folate, C_9–H cis is H_R and C_9–H trans is H_S, while C_7–H cis is H_S and C–H trans is H_R. It is probably true that the hydrogen on C_{11} nearer the C_4 oxygen, H_R in Figure 1, corresponds to the downfield C_{11} resonance at 5.05 ppm in Table I. The downfield field shift of C_{11}–H_R relative to C_{11}–H_S would be due to the carbonyl oxygen on the pyrimidine (Jackman & Sternhell, 1969). This assignment has been confirmed by experiments on the acetaldehyde adduct of tetrahydrofolate in work to be published.

The reason for the strong preference of CH_2-H_4folate for having its tetrahydropyrazine ring in a half-chair conformation with C_6–H axial is clear in both space-filling and skeletal models, since this conformation partially alleviates the strain in the imidazolidine ring. Like CH_2-H_4folate, 6-methyl-

5,6,7,8-tetrahydropterin (Frick, 1975) and 5,10-methylene-(6*R*,*S*)-5,6,7,8-tetrahydro-L-folate (Khalifa et al., 1979) prefer to have their C_6 protons axial. However, 5-methyl-5,6,7,8-tetrahydrofolate has C_6–H in an equatorial position (Poe et al., 1979) and 5,6,7,8-tetrahydrofolate has C_6–H in rapid exchange between an axial position and equatorial position (Poe & Hoogsteen, 1978; Furrer et al., 1978). These four tetrahydrofolate cofactors have the same half-chair conformations for their tetrahydropyrazine rings but with different proportions of the two half-chair forms.

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