5-Formyl- and 10-Formyl-5,6,7,8-tetrahydrofolate. Conformation of the Tetrahydropyrazine Ring and Formyl Group in Solution†

Martin Poe* and Stephen J. Benkovic

ABSTRACT: The chemical shifts and spin–spin coupling constants for the 300-MHz ^{1}H NMR spectra of (6*RS*)-10-formyl- and (6*RS*)-5-formyl-5,6,7,8-tetrahydro-L-folate at 25 °C in 0.1 M NaOD and at neutral pH are reported and analyzed. Assignments of most resonances are based on comparison to closely related compounds; the resonances of C(6)–H, C(7)–2H, and C(9)–2H are assigned by preparation of specifically deuterated formyltetrahydrofolates. From analysis of the C(7)–2H spin–spin coupling constants, it is proposed that the tetrahydropyrazine rings of both 10-formyl- and 5-formyl-H_4folate are in a half-chair conformation with C(6)–H equatorial. It is further proposed from model building that the 5-formyl group and C(9) of 5-formyl-H_4folate are trans. In the ^{1}H NMR spectrum of 5-CHO-H_4folate, there was a minor species which was present at a concentration roughly one-sixth that of the major species; this species did not correspond to oxidized 5-CHO-H_4folate and could not be separated from the major species by gel permeation or ion-exchange chromatography. It is proposed that this minor species corresponds to a conformer of 5-CHO-H_4folate in slow exchange with the major conformer; this slow exchange is attributed to slow rotation due to a partial double bond character of the N(5) to 5-formyl–carbon bond. During the preparation of 10-formyl-H_4folate from 5,10-methenyl-H_4folate in D_2O, ~60% of the formyl hydrogen was exchanged for deuterium. The equilibrium constant for the interconversion of 5,10-methenyl-H_4folate and 10-formyl-H_4folate, $K' = [5,10\text{-methenyl-}H_4\text{folate}]/([D^+][10\text{-formyl-}H_4\text{folate}])$, was measured to be $(1.4 \pm 0.6) \times 10^6$ M^{-1} in D_2O at 25 °C.

The formic acid adducts of 5,6,7,8-tetrahydrofolic acid of biochemical interest exist in the three interconvertible forms, 5,10-methenyl-, 5-formyl-, and 10-formyl-5,6,7,8-tetrahydrofolate. 5,10-Methenyl-5,6,7,8-tetrahydrofolate (5,10-CH^+-H_4folate)[1] and 10-formyl-5,6,7,8-tetrahydrofolate (10-CHO-H_4folate) are enzymatic cofactors essential in purine biosynthesis; it has been suggested (Hryniuk, 1972) that the effectiveness of antifolates like methotrexate in cancer chemotherapy may be due to "purineless death" accompanying deprivation of neoplastic cells of these formyltetrahydrofolates. 5-Formyl-5,6,7,8-tetrahydrofolate (5-CHO-H_4folate), which is also known as leucovorin and folinic acid (Roth et al., 1952), is used in cancer chemotherapy to "rescue" patients treated with high doses of methotrexate (Bertino, 1973). Thus, it appears that a better understanding of formyltetrahydrofolates might have useful applications in cancer chemotherapy.

Recent studies on model systems for formyltetrahydrofolates, reviewed by Benkovic (1978), and on the enzymes of purine biosynthesis that function in transformylation reactions, i.e., glycinamide ribonucleotide transformylase (Caparelli et al., 1979) and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (Baggott & Krumdieck, 1979; Mangum et al., 1979), have shown that useful studies of the enzymatic mechanisms can be done. We did the present studies of the conformational state in solution of formyltetrahydrofolates to provide insight into the structural basis of these mechanistic studies. These studies have allowed the determination of the conformation in solution of the tetrahydropyrazine rings of these compounds, have permitted an independent check of the equilibrium between 5,10-CH^+-H_4folate and 10-CHO-H_4folate, have demonstrated a conformational equilibrium for the formyl group of 5-CHO-H_4folate, and have demonstrated the exchangeability with the medium of the formyl hydrogen in the conversion of 5,10-CH^+-H_4folate to 10-CHO-H_4folate.

The crystal structures of both the natural and unnatural diastereomers of 5,10-CH^+-H_4folate have been recently solved by Fontecilla-Camps et al. (1979), and the ^{1}H NMR spectrum of its diastereomeric mixture has been fully analyzed to measure its conformation in solution by Khalifa et al. (1979). A partial ^{1}H NMR spectrum of 5-CHO-H_4folate has previously been reported by Pastore (1967) but not fully analyzed.

Materials and Methods

The calcium salt of (6*RS*)-5-formyl-5,6,7,8-tetrahydro-L-folate [(6*RS*)-5-CHO-H_4-L-folate], or calcium leucovorin, was a gift of Dr. E. W. Cantrall, Lederle, and was used without further purification. The diastereomeric mixture of C(6) stereoisomers of tetrahydrofolate, (6*RS*)-5,6,7,8-H_4-L-folate, was purchased from Sigma Chemical Co. as *dl*-L-tetrahydrofolate and used without further purification. The natural stereoisomer of tetrahydrofolate, (6*S*)-5,6,7,8-H_4-L-folate, was prepared as described by Poe et al. (1979a). The diastereomeric mixture of C(6) stereoisomers of tetrahydrofolate specifically deuterated at C(7), (6*RS*)-5,6,7,8-H_4-L-folate-7-*d* (50% *d*) was prepared as described before (Poe et al., 1979a). The diastereomeric mixture of C(6) stereoisomers of tetrahydrofolate specifically deuterated at C(6) and C(7), (6*RS*)-5,6,7,8-H_4-L-folate-6-*d*,7-*d* (50% *d*), was prepared as described earlier (Poe et al., 1979a). (6*S*)-5,6,7,8-H_4folate-6-*d*, the natural stereoisomer of tetrahydrofolate specifically deuterated at C(6), was prepared as described by Poe & Hoogsteen (1978).

The preparation of 5,10-CH^+-H_4folate from various tetrahydrofolate preparations described in the preceding paragraph was done essentially according to Rowe (1968). Tetrahydrofolic acid (100 mg) was dissolved in 98% formic acid (25

† From the Department of Biophysics, Merck Institute for Therapeutic Research, Rahway, New Jersey 07065 (M.P.), and the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802 (S.J.B.). *Received February 25, 1980.*

[1] Abbreviations used: 5,10-CH^+-H_4folate, 5,10-methenyl-5,6,7,8-tetrahydrofolate; 10-CHO-H_4folate, 10-formyl-5,6,7,8-tetrahydrofolate; 5-CHO-H_4folate, 5-formyl-5,6,7,8-tetrahydrofolate; 6*RS*, diastereomeric mixture of two C(6) stereoisomers of a tetrahydrofolate derivative; 5,6,7,8-H_4-L-folate, tetrahydrofolate diastereomer L or *S* at the α carbon of the glutamate moiety; pH*, direct meter reading of a pH electrode in D_2O solution.

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mL, Merck), containing 2% (v/v) 2-mercaptoethanol (Aldrich) in an aluminum foil covered flask equipped with a reflux condenser. The solution was maintained at 60 °C in a water bath for 3 h under a nitrogen atmosphere. After the solution was cooled to 0 °C in ice, the 5,10-CH^+-H_4folate was precipitated out of the formic acid solution by the addition of 6 volumes of anhydrous diethyl ether (Baker). The product was collected by centrifugation at 1000*g* for 1 min, washed 3 times with ether to minimize residual formic acid, and then lyophilized to dryness in an aluminum foil wrapped flask. The dry powder was stored at −20 °C under vacuum.

Both 5-CHO-H_4folate and 10-CHO-H_4folate can be made from 5,10-CH^+-H_4folate by nonenzymatic hydrolysis of the latter compound in neutral or slightly alkaline solutions (Robinson, 1971). The hydrolysis of 5,10-CH^+-H_4folate will first produce 10-CHO-H_4folate as the "product of kinetic control". The hydrolysis of 5,10-CH^+-H_4folate to 10-CHO-H_4folate can also be catalyzed enzymatically (Kay et al., 1960). The 10-CHO-H_4folate is metastable and will slowly convert to 5-CHO-H_4folate which is the thermodynamically stable product of hydrolysis, the "product of thermodynamic control". The nonenzymatic conversion of 10-CHO-H_4folate to 5-CHO-H_4folate is quicker at elevated temperature provided care is taken to protect the highly labile 10-CHO-H_4folate from light and oxygen (Robinson, 1971).

The preparations of 10-CHO-H_4folate were made from 5,10-CH^+-H_4folate, by dissolving the latter in a solution at neutral or alkaline pH which had been extensively purged with nitrogen. In several experiments, 0.14 M 2-mercaptoethanol was included to stabilize the rather unstable 10-CHO-H_4folate (Rabinowitz, 1971). The hydrolysis of 5,10-CH^+-H_4folate to form 10-CHO-H_4folate is a reasonably rapid reaction in neutral or slightly alkaline buffers (Robinson, 1971).

A solution of (6*RS*)-5-CHO-5,6,7,8-tetrahydro-L-folate-6-*d*,7-*d* (50% *d*) was prepared by dissolving (6*RS*)-5,10-CH^+-5,6,7,8-tetrahydro-L-folate-6-*d*,7-*d* (50% *d*) in 0.1 M sodium phosphate buffer at pH* 6.79 in D_2O, followed by heating the solution in the dark at 79 °C under nitrogen for 90 min. The conversion to the 5-CHO-H_4folate was followed by ^{1}H NMR. This procedure was adapted from Roth et al. (1952); the conversion goes more rapidly in slightly alkaline solutions (Zakrzewski & Sansone, 1971). Robinson (1971) has noted that the conversion is unsuccessful in 0.1 M NaOH, since high OH^- concentrations lead to production of H_4folate instead of 5-CHO-H_4folate.

Solutions of 5,10-CH^+-H_4folate and 5-CHO-H_4folate were standardized spectrophotometrically with ϵ(345 nm) = 26 000 cm^{-1} M^{-1} in 1 M HCl (Rabinowitz, 1963) and ϵ(282 nm) = 32 600 cm^{-1} M^{-1} in 0.1 M NaOH (Huennekens & Osborn, 1959), respectively.

The ^{1}H NMR spectra were measured at 300 MHz on a Varian SC-300 as described before (Poe & Hoogsteen, 1978), except that the sensitivity of the spectrometer was artificially enhanced by use of an exponential weighting of the free induction decays with a time constant of −1.0 s rather than artificial resolution enhancement. Six thousand data points were taken for each 3000-Hz spectrum. Pulse widths were chosen to give a 90° pulse, and a 1-s accumulation time was used. In some spectra, a pulse delay of up to 30 s between Fourier transform pulses was used. T_1 measurements were made by the standard two-pulse sequence 180°−τ−90° (Wüthrich, 1976) with a data accumulation time of 1 s and pulse delay of 10 s between the two-pulse sequences. T_1 was measured for each resonance by using the relation $\tau_0 = T_1 \ln 2$, where τ_0 was the interval in seconds between the 180 and 90° pulses that led to a null or zero amplitude signal for the resonance. The spectra were obtained in D_2O and at 25 °C unless otherwise noted; solutions of 10-CHO-H_4folate were usually purged with nitrogen. The spectra were referenced internally to the methyl resonance of sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 with downfield shifts positive. pH* is the direct electrode reading of a pH electrode in D_2O solution (Markley, 1975).

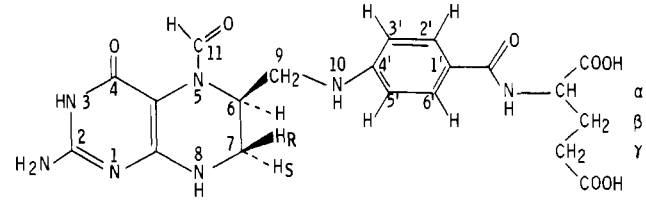

FIGURE 1: Structural formula for 5-formyl-5,6,7,8-tetrahydrofolic acid.

The numbering system used for 5-formyl-5,6,7,8-tetrahydrofolate is shown in Figure 1; the numbering is based on the tentative rules given for 5,6,7,8-tetrahydrofolate by the Subcommittee on Folates of the IUPAC–IUB Commission on Biochemical Nomenclature (1966). 10-Formyl-5,6,7,8-tetrahydrofolate has the same structure as 5-formyl-5,6,7,8-tetrahydrofolate, except that the hydrogen on N(10) and the formyl group on N(5) are interchanged. 5,10-Methenyl-5,6,7,8-tetrahydrofolate has the same structure as the 5-formyl compound except that the hydrogen on N(10) and the 5-formyl group are replaced by a bridging methylenylium, i.e., by —CH^+=, where it understood that in reality the positive charge is located on either N(5) or N(10). These three formyltetrahydrofolates have two chiral centers, one at the α carbon of the glutamate moiety and one at C(6). The stereoisomers at the α carbon are referred to as L or D; all studies herein are with the natural L stereoisomer, which has the absolute configuration *S* at the α carbon. The stereoisomers at C(6) are referred to as 6*R* or 6*S* and the diastereomeric mixture is referred to as 6*RS*. The natural stereoisomer for 5,10-CH^+-H_4folate is *R* at C(6) (Fontecilla-Camps et al., 1979) and the diastereomer of 10-CHO-H_4folate with the same configuration at C(6) is also *R* at C(6). However, the diastereomer of 5-CHO-H_4folate with the same configuration at C(6) as the natural stereoisomer of 5,10-CH^+-H_4folate has *S* configuration at C(6). The designation 7-*d* (50% *d*) means that in all formyltetrahydrofolates, one of the two hydrogens at C(7) is deuterium. Irrespective of the absolute configuration at C(6), the two prochiral hydrogens at C(7) can be designated as H_R up and H_S down in Figure 1, according to the Hanson system of notation (Bentley, 1969).

Results and Discussion

5-CHO-H_4folate. The 300-MHz ^{1}H NMR spectrum of a diastereomeric mixture of the two C(6) stereoisomers of 5-formyl-5,6,7,8-tetrahydrofolate at 3.65 mM in 0.1 M NaOD at 41 °C was replotted for Figure 2. The intense HDO resonance and its spinning sidebands were omitted in the replotted spectrum. There were several interesting features in this spectrum which were not discussed in the previous ^{1}H NMR report on 5-formyl-H_4folate (Pastore, 1967). First, there appeared to be two species present. This was most clearly seen in the two resonances at 8.75 and 7.90 ppm, which we propose to correspond to C(11)–H, the formyl hydrogen, but may also be seen in the C(2′,6′)–2H, C(3′,5′)–2H, C(9)–2H, and C(7)–2H resonances. Second, the 8.75-ppm resonance had a much longer spin–lattice relaxation time (T_1) than the other resonances. And, importantly for insight into the con-

FIGURE 2: Overall 300-MHz ^{1}H NMR spectrum of 5-CHO-H_4folate at 3.65 mM in 0.1 M NaOD at 41 °C. The region between 4.54 and 4.73 ppm is obscured by the HDO resonance and is omitted.

Table I: Chemical Shifts and Coupling Constants for the Major Form of (6*RS*)-5-Formyl-5,6,7,8-tetrahydro-L-folate at 41 °C and 3.65 mM in 0.1 M NaOD

resonance(s) of	chemical shift (ppm)	multiplicity[a]	|J| (Hz)
C(11)-H	8.747	s	s
C(2′,6′)-2H	7.71	d	8.7 ± 0.5
C(3′,5′)-2H	6.825	d	8.7
C(6)-H	4.87[b]	m	
α-CH	4.323	dd	4.4, 8.8
C(7)-H cis	3.50	dd	0.5, 12.0
C(7)-H trans	3.373	dd	5.0, 12.0
C(9)-H_A	3.357	dd	5.0, 13.5
C(9)-H_B	3.207	dd	8.2, 13.5
γ-CH_2	2.30	t	7.3
β-CH_A	2.143[b]	m	
β-CH_B	2.023[b]	m	

[a] s is singlet; d is doublet; t is triplet; m is multiplet; dd is doublet of doublets. [b] Position of the most intense resonance of the multiplet.

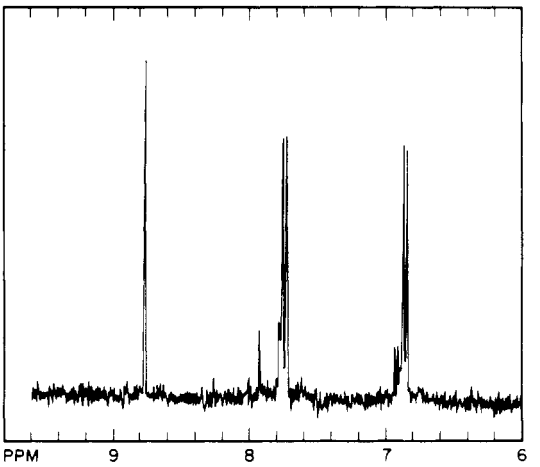

FIGURE 3: Aromatic region of the ^{1}H NMR spectrum of 5-CHO-H_4folate in 0.1 M NaOD at 23 °C. For this spectrum, a pulse delay of 30 s was used between Fourier transform pulses.

formation of 5-formyl-H_4folate in solution, the region from 2.8 to 4.0 ppm contained resolved resonances corresponding to the four hydrogens on C(9) and C(7) for which spin–spin coupling constants could be measured.

The chemical shifts and spin–spin coupling constants observed in the spectrum replotted for Figure 2 for the major species of 5-CHO-H_4folate are given in Table I. The assignments given in Table I are the same as those of tetrahydrofolate for the C(2′,6′)–2H, C(3′,5′)–2H, α-CH, γ-CH_2, β-CH_A, and β-CH_B resonances (Poe & Hoogsteen, 1978; Furrer et al., 1978).

The relatively low proportion of resonances corresponding to oxidized or degraded 5-CHO-H_4folate in the spectrum reproduced in Figure 2 demonstrated the relative stability of this compound. The calcium leucovorin was dissolved in nitrogen-purged 0.1 M NaOD, transferred to an NMR tube, and stored away from light for ~30 min until its ^{1}H NMR spectrum was run. 5-CHO-H_4folate had earlier been noted to be one of the most stable tetrahydrofolates (Roth et al., 1952).

When the aromatic region of the ^{1}H NMR spectrum of 5-CHO-H_4folate was observed in 0.1 M NaOD with long pulse delays between sample irradiation pulses, the area of the aromatic resonances changed relative to one another. For a pulse delay of 30 s between pulses as in the spectrum replotted for Figure 3, the 7.71- and 6.82-ppm resonances were equal in area, and the 8.75-ppm resonance had half this area and ~6 times the area of the 7.90-ppm resonance. The reason for this change in relative areas was that the 8.75- and 7.71-ppm resonances had significantly longer spin–lattice relaxation times, T_1, than did the other resonances (see Table II).

Above room temperature, the area of the resonances corresponding to the minor form of 5-CHO-H_4folate diminished relative to those of the major form. The minor form of 5-CHO-H_4folate could not be separated from the major form by gel permeation chromatography on Sephadex G-25 or by a salt gradient elution from an ion-exchange column packed with DEAE-Sephadex A-25. The minor form had different chemical shifts for its resonances than did the degradation products seen when 5-CHO-H_4folate was exposed to oxygen or light; the resonances of the minor form were also different from those of 10-CHO-H_4folate (see Table IV) and from 5,10-methenyl-5,6,7,8-tetrahydrofolate (Khalifa et al., 1979).

Table II: Spin–Lattice Relaxation Times (T_1) for Resonances of (6*RS*)-5-Formyl-5,6,7,8-tetrahydro-L-folate at 12.9 mM in 0.1 M NaOD at 23 °C

resonance(s) of	T_1 (s)
C(11)-H	6.1
C(11)-H, minor form	0.8
C(2′,6′)-2H	1.4
C(3′,5′)-2H	0.7
α-CH	1.2
C(7)-H cis	0.3
C(7)-H trans	0.3
C(9)-H_A	0.2
C(9)-H_B	0.2
γ-CH_2	0.5
β-CH_A	0.3
β-CH_B	0.3

Upon the compounds being heated to 79 °C, the C(11)–H resonances of both forms of 5-CHO-H_4folate in 0.1 M NaOD were slightly broadened. This slight broadening and the closeness of the 7.90-ppm resonance to the C(2′,6′)–2H resonances may explain the inability of Pastore (1967) to observe this resonance. When a space-filling model of 5-CHO-H_4folate was built with Corey–Pauling–Koltun models for the tetrahydropyrazine ring in our proposed conformation, there was little restraint on rotation of the 5-formyl group about the N(5) to C(11) bond. The probable explanation for the observation of the two C(11)–H resonances is restricted rotation due to

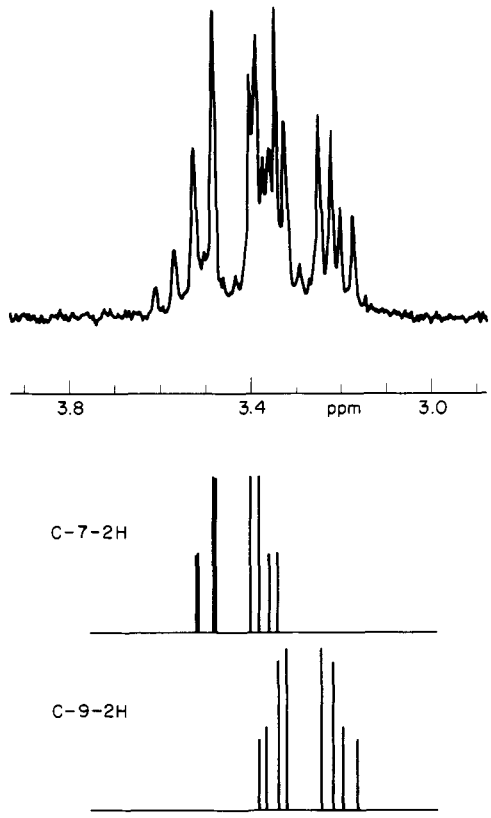

FIGURE 4: The 2.8–4.0-ppm region of the 300-MHz spectrum of 5-CHO-H_4folate. The upper trace is a portion of the spectrum shown in Figure 2, while the middle trace is the suggested position of the resonances in the spectrum that correspond to the two C(7) hydrogens and the bottom trace shows the suggested C(9) hydrogen resonances.

a partial double bond character of the nitrogen–carbon bond such as that which explains the two methyl resonances of the ^{1}H NMR spectrum of *N,N*-dimethylformamide (Phillips, 1955). The downfield resonance would plausibly be the form with the hydrogen nearer O(4); its downfield position would be explained by the diamagnetic shielding of the carbonyl double bond just as the downfield position of one C(11)–H of 5,10-methylenetetrahydrofolate is explained by similar diamagnetic shielding (Poe et al., 1979b). This assignment of the 8.75-ppm resonance as C(11)–H as the "rotomer" of 5-CHO-H_4folate with C(11)–H near O(4) can explain the C(11)–H relative intensity data and the T_1 data. Steric and electronic repulsion between O(4) and the formyl oxygen would favor the "rotomer" with these oxygens more separated, thereby explaining why the C(11)–H resonance at 8.75 ppm was larger than the 7.90-ppm resonance. In the "rotomer" with C(11)–H near O(4) for the tetrahydropyrazine ring conformation given in Figure 7, the hydrogens at C(6), C(7), and C(9) and on the rest of 5-CHO-H_4folate would be relatively remote from C(11)–H, which would explain the long T_1 value for the 8.75-ppm resonance. Despite the plausibility of the above assignment for the major and minor forms of 5-CHO-H_4folate, it is not impossible that the minor form could be a closely related impurity that is difficult to separate. The ^{1}H NMR spectrum of 5-methyl-5,6,7,8-tetrahydrofolate showed no evidence of a minor form (Poe et al., 1979a).

The resonances in Figure 2 with chemical shifts between 3.0 and 3.8 ppm correspond to the four hydrogens on C(9) and C(7) of 5-CHO-H_4folate; they were replotted for Figure 4. The small resonances at 3.29, 3.44, 3.51, 3.56, and 3.61 ppm appeared to be due to the minor form of 5-CHO-H_4folate since their intensity was appreciably diminished at high temperature. This region of the 300-MHz ^{1}H NMR spectrum

Table III: Chemical Shifts of C(6)–H in Six Tetrahydrofolates

compd	solvent	chemical shift (ppm)	ref
H_4folate	0.1 M NaOD	3.43	Poe & Hoogsteen (1978)
10-CHO-H_4folate	pH* 6.79[a]	3.60	this work
5-CHO-H_4folate	0.1 M NaOD	4.87	this work
5,10-CH^+-H_4folate	0.5 M HCl	5.30	Khalifa et al. (1979)
5,10-CH_2-H_4folate	0.1 M NaOD	3.57	Poe et al. (1979b)
5-CH_3-H_4folate	pH* 6.79[a]	3.08	Poe et al. (1979a)

[a] In 0.1 M sodium phosphate buffer, pH* 6.79, in D_2O.

of 5-CHO-H_4folate was essentially the same at pH* 4.60 and 6.79 as in 0.1 M NaOD. When (6*RS*)-5-CHO-5,6,7,8-tetrahydro-L-folate-6-*d*,7-*d* (50% *d*) was prepared as described under Materials and Methods, the 2.8–4.0-ppm region of its ^{1}H NMR spectrum was considerably simpler than the spectrum in Figure 2, and the resonance at 4.87 ppm was absent. The resonance at 4.87 ppm clearly corresponded to C(6)–H; the chemical shift was near that for C(6)–H in 5,10-methenyl-H_4folate but was 1.27–1.79 ppm downfield of the C(6)–H resonance position for H_4folate, 5-methyl-H_4folate, 5,10-methenyl-H_4folate, and 10-CHO-H_4folate (see Table III). The pair of resonances centered at 3.50 ppm in 5-CHO-H_4-folate were replaced by a singlet at 3.48 ppm with an intensity corresponding to a half-proton in the 6-*d*,7-*d* (50% *d*) compound; there was another half-proton singlet at 3.34 ppm. These are the C(7)–2H resonances. The one-proton doublet of doublets centered at 3.21 ppm in the protonated compound was a one-proton doublet centered at 3.22 ppm with a spin–spin splitting of 14 Hz for the 6-*d*,7-*d* (50% *d*) compound. The other one-proton doublet for the deuterated compound was centered at 3.36 ppm; these one-proton doublets must be the C(9)–2H resonances. Assuming that the chemical shifts of the C(7) and C(9) hydrogens are similar in normal and selectively deuterated 5-CHO-H_4folate, the assignments for C(7)–2H and C(9)–2H given in Table I were made. Two line spectra showing the resonances suggested to correspond to C(7)–2H and C(9)–2H resonances of 5-CHO-H_4folate are also given in Figure 4, in the middle and lower line spectra, respectively.

The narrowness of the resonances in Figures 2 and 3 showed that the chemical shifts of the resonances of the two C(6) diastereomers of 5-CHO-H_4folate were the same within ±0.001 ppm. This similarity of chemical shifts was also seen at pH* 4.60 and 6.79. This demonstrated that the two diastereomers formed similar mirror-image conformations in solution. The self-aggregation noted for oxidized folates (Poe, 1973) should be largely absent in the solution used for Figure 2, due to the low concentration of 5-CHO-H_4folate used.

10-CHO-H_4folate. The lability of 10-formyl-5,6,7,8-tetrahydrofolate is well-known (Rabinowitz, 1971) and formidable; the two 300-MHz ^{1}H NMR spectra of this compound replotted for Figure 5 were a clear example of this lability. The upper spectrum was of a diastereomeric mixture of the two C(6) stereoisomers of 10-CHO-H_4folate, (6*RS*)-10-CHO-5,6,7,8-H_4-L-folate, in 0.1 M NaOD at 25 °C with 0.14 M 2-mercaptoethanol present. The lower spectrum was of a saturated solution (1.9 mM) of a diastereomeric mixture of the two C(6) stereoisomers of 10-CHO-H_4folate in 0.1 M sodium phosphate, pH* 6.79, which had been continuously bubbled with nitrogen in the dark for 45 min at 23 °C before

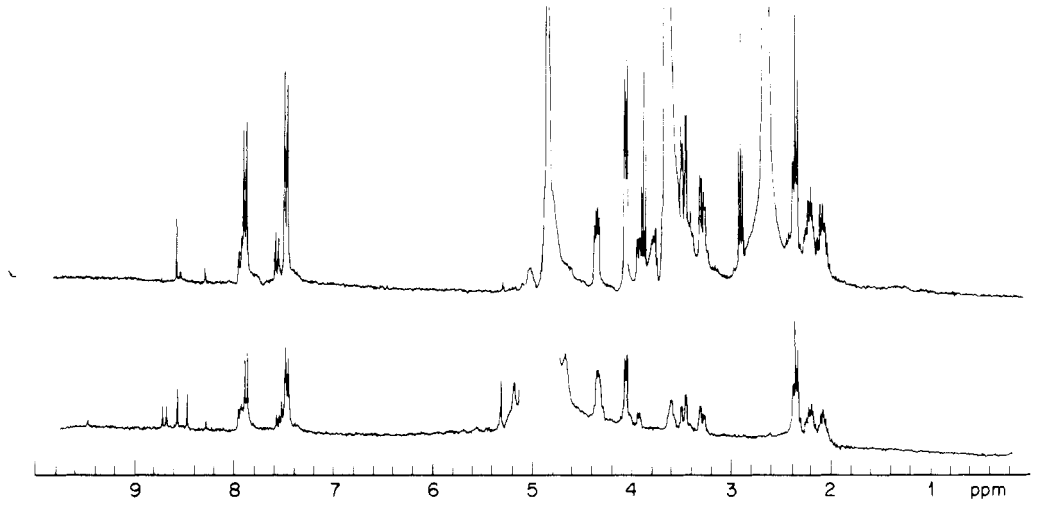

FIGURE 5: Overall 300-MHz ^{1}H NMR spectra of 10-CHO-H_4folate at 25 °C in 0.1 M NaOD and 0.14 M 2-mercaptoethanol (upper trace) and in 0.1 M sodium phosphate buffer, pH* 6.79 (lower trace). The 10-CHO-H_4folate was made from 5,10-methenyl-H_4folate initially at 10.7 mM for the upper spectrum and 1.9 mM for the lower spectrum.

Table IV: Chemical Shifts and Coupling Constants for (6*RS*)-10-Formyl-5,6,7,8-tetrahydro-L-folate at 25 °C in 0.1 M Sodium Phosphate, pH* 6.79

resonance(s) of	chemical shift (ppm)	multiplicity[a]	$\|J\|$ (Hz)
C(11)-H	8.570	s	
C(2′,6′)-2H	7.87	d	8.5 ± 0.5
C(3′,5′)-2H	7.465	d	8.5
α-CH	4.35	dd	4.5, 9.0
C(9)-2H	4.067	d	6.5
C(6)-H	3.60[a]	m	
C(7)-H cis	3.483	dd	3.4, 12.8
C(7)-H trans	3.297	dd	3.4, 12.8
γ-CH_2	2.35	t	8.0
β-CH_A	2.193[a]	m	
β-CH_B	2.08[a]	m	

[a] Symbols and positions are as described in Table I.

transfer to an NMR tube. The resonances assigned to the carbon-bound protons of 10-CHO-H_4folate are listed in Table IV.

The 10-CHO-H_4folate in the upper spectrum in Figure 5 was formed from a clean preparation of 5,10-CH^+-H_4folate, as judged by the latter's ^{1}H NMR spectrum, by dissolving the 5,10-CH^+-H_4folate in nitrogen-purged solvent; the NMR spectrum was run from 10 to 12 min after dissolving the 5,10-CH^+-H_4folate. Even with the precautions of promptly running the spectrum under nitrogen with 2-mercaptoethanol present, there was still some oxidized or degraded material obtained as can be seen directly in the doublet at 7.56 ppm which had almost 10% of the area of the C(3′,5′)–2H doublet of 10-CHO-H_4folate centered at 7.45 ppm. There was no unhydrolyzed 5,10-CH^+-H_4folate left in this solution, since the 9.48-ppm resonance of this compound was clearly absent. The resonances of 10-CHO-H_4folate were partially obscured in the upper spectrum of Figure 5 by the intense resonance of HDO at 4.82 ppm, by the intense resonances of 2-mercaptoethanol at 2.64 and 3.62 ppm, and by the triplets centered at 2.88 and 3.86 ppm corresponding to oxidized 2-mercaptoethanol. The broad resonances at 3.76 and 5.01 ppm were of unknown origin. The doublets at 3.92, 7.56, and 7.93 ppm and the small singlet at 8.54 ppm probably corresponded to oxidized or degraded 10-CHO-H_4folate; the small singlet at 8.19 ppm was of HCOOD. There was no 5-CHO-H_4folate present, since the 6.825-ppm resonance of 5-CHO-H_4folate was absent.

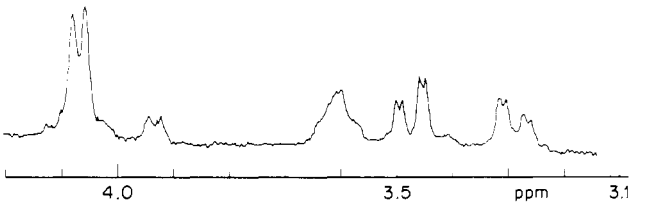

FIGURE 6: The 3.1–4.2-ppm region of the 300-MHz ^{1}H NMR spectrum of 10-CHO-H_4folate. This is an expanded portion of the lower spectrum in Figure 5.

The spectrum in the upper trace of Figure 5 did not have a resonance assignable to C(6)–H; this resonance was probably buried beneath the 3.62-ppm resonance of 2-mercaptoethanol. The resonance of C(6)–H in 10-CHO-H_4folate could be seen at 3.60 ppm in the lower spectrum replotted in Figure 5. The 3.2–4.2-ppm region of this solution containing 10-CHO-H_4folate at pH* 6.79 showed the C(6)–H, C(7)–2H, and C(9)–2H resonances of 10-CHO-H_4folate. However, the extra three resonances between 8.4 and 8.8 ppm showed that appreciable material besides 10-CHO-H_4folate was formed during hydrolysis of 5,10-CH^+-H_4folate when no oxygen scavenger was present; these three resonances may correspond to 10-CHO-H_2folate and 10-CHO-folate. There was also a small amount of 5,10-CH^+-H_4folate present, as was shown by the resonance at 9.48 ppm.

The chemical shift and spin–spin splittings of the resonances of 10-CHO-H_4folate in 0.1 M sodium phosphate, pH* 6.79, are given in Table IV. The resonances corresponding to C(2′,6′)–2H, C(3′,5′)–2H, α-CH, γ-CH_2, β-CH_A, and β-CH_B were assigned as in 5,10-CH^+-H_4folate (Khalifa et al., 1979) and 5-CHO-H_4folate (see Table I). The resonance of 8.57 ppm was assigned to the formyl hydrogen, C(11)–H, through the similarity of its chemical shift to those of model compounds (Burdick et al., 1977) and to the chemical shift of C(11)–H in 5-CHO-H_4folate (see Table I) and 5,10-CH^+-H_4folate (Khalifa et al., 1979). This resonance had an area corresponding to 0.4 ± 0.1 proton when standardized against the C(2′,6′)–2H and C(3,5′)–2H resonances. The relative areas of the resonances assigned to 10-CHO-H_4folate did not change when the ^{1}H NMR spectra were accumulated with a pulse delay of 10 s between pulses.

An expanded version of the 3.1–4.2-ppm region of 10-CHO-H_4folate at pH* 6.79 is given in Figure 6. When the 300-MHz ^{1}H NMR spectrum of (6*RS*)-10-CHO-H_4folate-

Table V: Chemical Shifts and Spin-Spin Couplings to C(6)-H for C(7) Hydrogens of Six Tetrahydrofolates[a]

compd	C(7)-H trans δ	C(7)-H trans |J| (Hz)	C(7)-H cis δ	C(7)-H cis |J| (Hz)
H_4folate	3.21	6.6	3.52	3.0
10-CHO-H_4folate	3.30	3.4	3.49	3.4
5-CHO-H_4folate	3.37	5.0	3.50	0.5
5,10-CH^+-H_4folate	4.12	10.6	4.64	4.2
5,10-CH_2-H_4folate	3.13	10.0	3.35	3.0
5-CH_3-H_4folate	3.25	1.0	3.49	2.2

[a] For references, see Table III.

6-*d*, prepared at 25 °C in nitrogen-purged 0.1 M sodium phosphate, pH* 6.79, from (6*RS*)-5,10-CH^+-H_4-L-folate-6-*d*, was examined, the resonance seen in Figure 6 at 3.60 ppm was absent, the two-proton resonance at 4.04 ppm for the specifically deuterated compound was a singlet, and the one-proton resonances at 3.29 and 3.47 ppm for the deuterated compound were doublets, each having a spin–spin coupling of 12.5 ± 0.5 Hz. When the 300-MHz ^{1}H NMR spectrum of (6*RS*)-10-CHO-H_4folate-6-*d*,7-*d* (50% *d*), prepared at 25 °C in nitrogen-purged 0.1 M sodium phosphate from (6*RS*)-5,10-CH^+-H_4folate-6-*d*,7-*d* (50% *d*), was measured as in Figure 6, there was a two-proton singlet at 4.02 ppm and two half-proton singlets at 3.24 and 3.43 ppm for the specifically deuterated compound. Thus, the resonance at 3.60 ppm clearly corresponded to C(6)–H, the resonances at 4.07 ppm corresponded to C(9)–2H, and the resonances at 3.48 and 3.30 ppm corresponded to the two C(7) hydrogens.

The two C(7)–H resonances of 10-CHO-H_4folate have equal spin–spin couplings to C(6)–H, so that their assignment as C(7)–H trans and C(7)–H cis cannot be made on the basis of the magnitude of this coupling constant alone. However, since for the other five tetrahydrofolates examined to date C(7)–H cis is 0.13–0.52 ppm downfield of C(7)–H trans (see Table V), it is suggested that the 3.48-ppm resonance corresponds to C(7)–H cis and the 3.30-ppm resonance corresponds to C(7)–H trans.

The two-proton doublet at 4.07 ppm in Figure 6 doubtless is due to the two C(9) hydrogens. The apparent equivalence of their chemical shifts is unexpected, since C(9) is immediately adjacent to the chiral center at C(6). It is possible the 10-formyl group makes the C(9) hydrogens equivalent, or perhaps the C(9) doublet is the AB portion of a "deceptively simple" ABX spectrum (Abraham & Bernstein, 1961) with $\nu_0\delta_{AB}/J_{AB}$ and the ratio $^1/_2(J_{AX} - J_{BX})/J_{AB}$ both small compared to 1.

The resonance assigned to C(11)–H of 10-CHO-H_4folate in the ^{1}H NMR spectra replotted for Figure 5 did not correspond in area to a full proton. The missing 0.6 ± 0.1 proton can be ascribed to exchange of this hydrogen for deuterium during the hydrolysis in D_2O of 5,10-CH^+-H_4folate to form 10-CHO-H_4folate. When the 10-CHO-H_4folate was made in H_2O from 5,10-CH^+-H_4folate and the resulting 10-CHO-H_4folate was lyophilized to dryness, followed by dissolution in D_2O for ^{1}H NMR spectroscopy, the C(11)–H resonance corresponded to 0.8 ± 0.3 proton. The apparent exchange of the formyl hydrogen during formation of 10-CHO-H_4folate from 5,10-CH^+-H_4folate in D_2O can probably be explained by exchange of the bridgehead carbon's hydrogen by means of an ylide intermediate (Kohn et al., 1972; Olofson et al., 1966). The hydroxide- or deuteroxide-catalyzed formation of the ylide, which would have a positively charged N(5) or N(10) and a negatively charged C(11) with no hydrogen bound to it, would compete with the hydrolysis of 5,10-CH^+-H_4folate to 10-CHO-H_4folate (Robinson & Jencks, 1967). The ylide would re-form the methenyl compound with D at C(11) if the hydrolysis were carried out in D_2O.

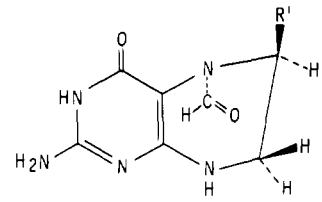

FIGURE 7: Schematic representation of the tetrahydropyrazine ring conformation of 5-formyl-5,6,7,8-tetrahydrofolic acid in solution. The ring is in a half-chair conformation with C(6)–H equatorial; the 5-formyl group and R′ are trans. R′ is $-CH_2NH$-*p*-$C_6H_4C(O)$-$NHCH(COOH)CH_2CH_2COOH$. The molecule here is the natural C(6) stereoisomer with C(6) as *S*.

Kay et al. (1960) have measured the equilibrium constant *K* for the reaction in eq 1 to be (0.9 ± 0.2) × 10^6 M^{-1}, where

$$5,10\text{-}CH^+\text{-}H_4\text{folate} + H_2O \rightleftharpoons 10\text{-}CHO\text{-}H_4\text{folate} + H^+ \quad (1)$$

K equals [5,10-CH^+-H_4folate]/([H^+][10-CHO-H_4folate]). *K* was measured from the UV absorbance of equilibrium mixtures of the two tetrahydrofolates. The ^{1}H NMR data presented in the lower spectrum of Figure 5 can also be used to determine this constant, since both 5,10-CH^+-H_4folate and 10-CHO-H_4folate were present at pH* 6.79. The relative areas of the C(11)–H resonances in a solution containing both 5,10-CH^+-H_4folate and 10-CHO-H_4folate were measured, and the area of the C(11)–H resonance of 10-CHO-H_4folate was multiplied by 2.5 to correct for exchange. The exchange-corrected area ratio was multiplied by the deuterium ion concentration, which was determined by using pD = pH* + 0.4 (Glashoe & Long, 1960). For K' = [5,10-CH^+-H_4folate]/([10-CHO-H_4folate][D^+]), K' was calculated to be (1.4 ± 0.6) × 10^6 M^{-1} in five determinations, four at pH* 6.79 and one at pH* 4.60. Only the C(11)–H resonances were suitable for this determination, since the other resonances of the two tetrahydrofolates were either insufficiently resolved or were obscured by 2-mercaptoethanol, which was necessary to stabilize 10-CHO-H_4folate during attainment of equilibrium. The attainment of equilibrium between the two tetrahydrofolates took 20 min for 1.9 mM 5,10-CH^+-H_4folate at pH* 6.79 at 25 °C in D_2O; the equilibrium was checked by measurement for 50 min after apparent equilibrium had been reached.

Conformations in Solution. The conformation of the tetrahydropyrazine ring of the major form of 5-CHO-H_4folate in solution must be a half-chair form, since the two half-boat forms would have at least one spin–spin coupling constant near 10 Hz between C(6)–H and one of the C(7) hydrogens. In the half-chair form, C(6)–H is equatorial, since if it were axial the coupling constant to C(7)–H trans would be near 10 Hz. For the half-chair form with C(6)–H equatorial, we propose that N(5) is such that C(9) is trans to C(11), since when C(9) and C(11) are cis there is substantial strain in a space-filling, Corey–Pauling–Koltun model. A schematic representation of our proposed conformation for the tetrahydropyrazine ring of 5-CHO-H_4folate is given in Figure 7; this is the natural C(6) stereoisomer. We suggest that this conformation is found in both neutral and alkaline solutions. In this model, C(7)–H cis is at a dihedral angle to C(6)–H [C(6)–H to C(6) to C(7) to C(7)–H cis] near −80° and thus would have a small spin–spin coupling to C(6)–H; for this natural stereoisomer this is the *pro-R* hydrogen. The other C(7)–H would be at a dihedral

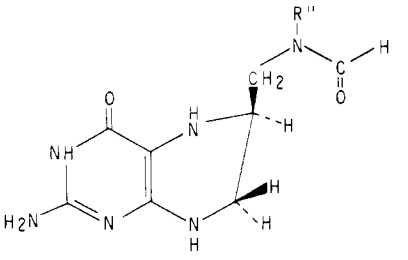

FIGURE 8: Schematic representation of the tetrahydropyrazine ring conformation of 10-formyl-5,6,7,8-tetrahydrofolic acid in solution. The ring is in a half-chair conformation with C(6)–H equatorial. R″ is -*p*-$C_6H_4C(O)NHCH(COOH)CH_2CH_2COOH$. This is the natural C(6) stereoisomer with C(6) as *R*.

angle to C(6)–H [C(6)–H to C(6) to C(7) to C(7)–H trans] near +40° which would explain its middle-sized coupling constant to C(6)–H. In this model, C(11) is "pseudoaxial" which puts C(11) in a position where there is essentially free rotation about the N(5) to C(11) bond. There is also a great deal of rotational freedom for C(9). The nonequivalence of the two C(9) hydrogens is probably due to the adjacent chiral center at C(6).

The conformation of the tetrahydropyrazine ring of 10-CHO-H_4folate in solution must be a half-chair conformation because the half-boat forms would have one coupling constant near 10 Hz for C(6)–H to one of the C(7) hydrogens. Also, C(6)–H must be equatorial, for the coupling constant C(6)–H to C(7)–H trans would be near 10 Hz if C(6)–H were axial. In the half-chair form of the tetrahydropyrazine ring of 10-CHO-H_4folate with C(6)–H equatorial, the fact that the two coupling constants for the C(7)–H protons to C(6)–H are moderately small (3.4 Hz) suggests they are symmetrically disposed about C(6)–H. The *pro-R* hydrogen C(7)–H cis would have a dihedral angle [C(6)–H to C(6) to C(7) to C(7)–H cis] near +50° while the *pro-S* hydrogen C(7)–H trans would have its dihedral angle [C(6)–H to C(6) to C(7) to C(7)–H trans] near −70°. The fact that C(7)–H trans is trans and coplanar to N(8) would explain why its coupling constant to C(6)–H would be the same as for C(7)–H cis to C(6)–H, despite having its dihedral angle to C(6)–H nearer 90° [see Poe et al. (1979a)]. Our proposed conformation for the tetrahydropyrazine ring of 10-CHO-H_4folate is schematically represented in Figure 8. In this conformation, there is very little conformational restriction upon the 10-CHO group. It is unclear why the 10-CHO group does not exhibit the restricted rotation that we have proposed for the 5-CHO group.

Added in Proof

During the review of this manuscript, Feeney et al. (1980) published proof by saturation transfer experiments that 5-formyl-5,6,7,8-tetrahydrofolate in solution was a mixture of two slowly interconverting conformations of unequal population, in agreement with our proposal.

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