Conformational Studies on 5-Formyl-5,6,7,8-tetrahydrofolic Acid (Folinic Acid) using ^1H and ^{13}C Nuclear Magnetic Resonance Measurements: Two Interconverting Conformations

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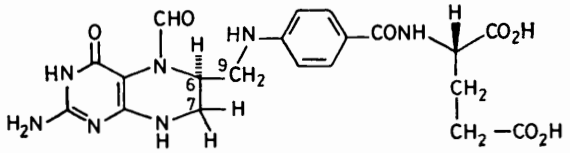
The ^1H and ^{13}C n.m.r. spectra of the (±)-L-diastereoisomers of folinic acid have been recorded. No spectral differences between the two diastereoisomers were detected but a mixture of two slowly interconverting conformations of unequal populations was shown to be present. The relative populations of the two forms changed with temperature and the thermodynamic parameters for the equilibrium have been obtained. Transfer of saturation experiments have been used to show that the two forms are slowly interconverting. The most likely explanation for the difference between the two forms is deduced as due to hindered rotation of the formyl group about the C–N bond. The 9-methylene side-chain exists in a well defined conformation with the methylene protons both in *gauche*-positions with respect to the 6-H proton.

ANTIFOLATE drug molecules such as methotrexate and related folate analogues act by strongly inhibiting the enzyme dihydrofolate reductase.[1-3] Because of the non-selectivity of some of these drugs they often have serious toxic effects by inhibiting dihydrofolate reductase in normal cells. Folinic acid is a useful rescue drug which reverses this toxicity by providing a source of reduced folate to supply the metabolic requirements of the cell in the absence of tetrahydrofolate produced *via* dihydrofolate reductase action on dihydrofolate.[3]

In our studies of complexes of dihydrofolate reductase with its substrate analogues [4-6] we have included folinic acid because it provides a convenient model for studying tetrahydrofolate-type binding to the enzyme. In contrast to tetrahydrofolic acid itself, which is unstable under non-anaerobic conditions, folinic acid is very stable to oxygen in neutral and mildly alkaline conditions.[3] A necessary first step when studying the n.m.r. spectra of complexes of dihydrofolate reductase with substrate analogues is to characterise the spectra of the free ligands. This is usually a trivial part of the analysis but in the case of folinic acid the spectrum is found to be complicated due to the presence of two slowly interconverting conformations with unequal populations at



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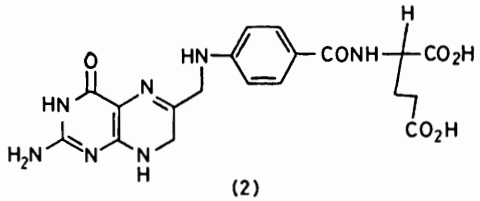
room temperature. In this paper we report on the n.m.r. spectral analysis of the two conformations and indicate the origin of the non-equivalent forms of folinic acid.

Folinic acid is the 5-formyl derivative of tetrahydrofolic acid and has structure (1). It has two asymmetric carbons, one at C-6 and one at the α-carbon of the glutamic acid moiety. Naturally occurring folinic acid is the (−)-L-diastereoisomer in which C-6 has the *S*-configuration and the glutamate also has the *S*-configuration.[7] [The active isomer is referred to as the (−)-L-isomer in the biochemical literature, the inactive compound being the (+)-L-isomer. (±) refers to the stereochemistry at C-6 while L refers to the stereochemistry of the glutamate moiety.]

EXPERIMENTAL

Materials—Commercial folinic acid (Sigma Chemicals) is a diastereoisomeric mixture, with C-6 in both *R* and *S* configurations and the α-carbon of the glutamate moiety in the *S* configuration. This is commonly referred to as (±)-L-folinic acid. The (+)-L- and (−)-L-diastereoisomers were separated by chromatography and crystallisation.[8]



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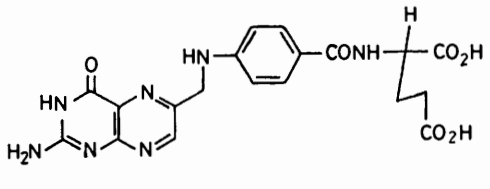

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The active isomer, (−)-L-folinic acid,[9] has been directly inter-related with the active isomer (+)-5,10-methenyltetrahydrofolate.[7] (±)-L-Folinic acid, partially and non-stereospecifically deuteriated at C-7 was prepared from [7-^{2}H]-dihydrofolic acid (2). The latter compound was prepared from folic acid (3) by a modification of the method of Zakrzewski.[10] To prevent exchange of deuterium into other positions, the reaction was quenched with dilute hydrochloric acid after 5 min, precipitating the dihydro-

folate. The dihydrofolate was further reduced to tetrahydrofolate using a modification of the method of Scrimgeour and Vitols.[11] Formylation[8] then gave the desired (7*RS*)-[7-$^2H_1$]folinic acid.

^1H N.m.r. spectroscopy was carried out using Varian XL-100 (100 MHz) and Bruker WH270 (270 MHz) spectrometers operating in the Fourier-transform mode. Typically, 400—4000 transients were collected using a spectral width of 4200 Hz in 16 K data points. The ^{13}C spectrum was obtained on a Nicolet NT-150 spectrometer at 37.7 MHz under conditions of proton noise-decoupling and using the Fourier-transform mode of operation (8 000 Hz spectral width and 16 K data points).

^1H Chemical shifts were measured from a dioxan internal reference and ^{13}C shifts from an external tetramethylsilane reference.

For the ^1H n.m.r. studies the folinic acid samples (1—8mM solutions) were examined in D_2O buffer solutions containing 50mM-potassium phosphate and 500mM-potassium chloride at pH 6.5 (meter reading uncorrected for deuterium isotope effects).

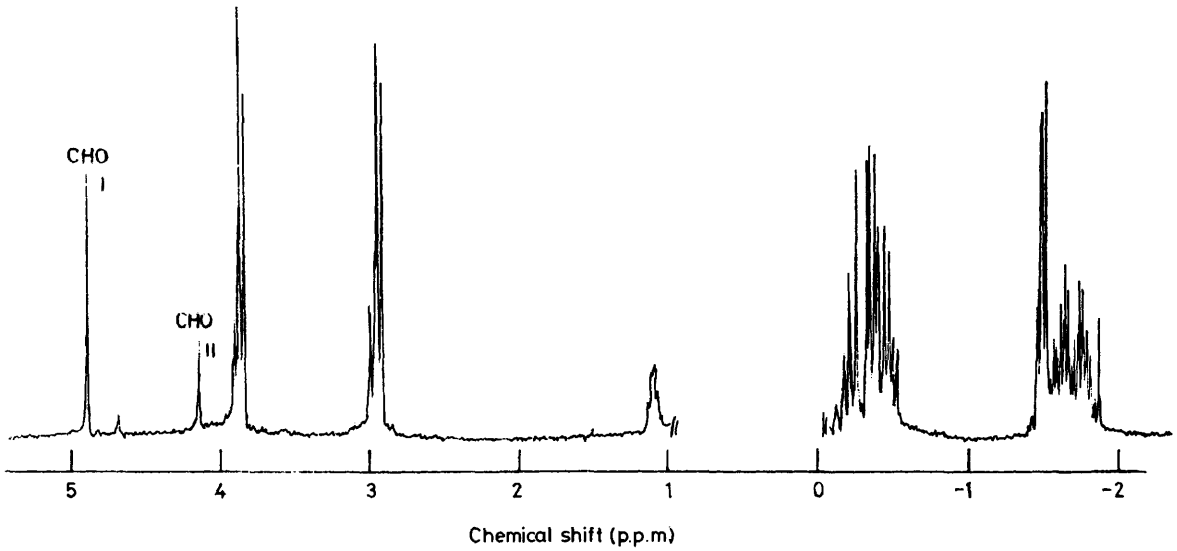


FIGURE 1 The 270-MHz ^1H resonance spectrum of 2mM-(±)-L-folinic acid in D_2O solution containing 50mM-potassium phosphate and 500mM-potassium chloride at 60 °C. The chemical shift scale is referenced to dioxan. [The dioxan signal is 3.71 p.p.m. downfield from that of sodium dimethylsilapentane-5-sulphonate (DSS)]

RESULTS

^1H *N.M.R. Spectrum of Folinic Acid.*—The ^1H n.m.r. spectra of the (+)-L- and (−)-L-forms of folinic acid in aqueous solution are identical and the spectrum of the commercial sample (±)-L-folinic acid is identical with those of the two diastereoisomers. The two forms should in principle have different n.m.r. spectra but clearly the differences are too small to be detected even at 270 MHz. There are no substantial changes in the ^1H n.m.r. spectrum of (±)-L-folinic acid when the pH is varied over the range 4—8. However, in each case the spectrum showed the presence of two separate species with unequal populations as illustrated in Figure 1 which shows the ^1H n.m.r. spectrum of (±)-L-folinic acid. In the low-field region of the spectrum there are two singlets (intensity ratio 3 : 1) with chemical shifts characteristic of formyl protons (4.88 and 4.16 p.p.m. from dioxan). There are also two overlapping pairs of doublets (J 8.5 Hz) with the same 3 : 1 intensity ratio at 3.87 and 2.96 p.p.m. corresponding to the aromatic protons of the benzoyl ring. There appears to be only one set of complex signals from the glutamate moiety (α-CH 0.53; β-CH_2 — 1.62, — 1.74; γ-CH_2 — 1.45 p.p.m.) and thus there is no evidence of the two forms of folinic acid in the glutamate spectrum. The remaining multiplets in the spectrum at 1.1 and — 0.27 p.p.m. have been connected by spin-decoupling and can be assigned to 6-H and 7- and 9-H_2, respectively. A detailed analysis of the multiplet at — 0.27 p.p.m. shows it to be a superimposition of two patterns of multiplets with an overall intensity ratio of 3 : 1. Each of these patterns is made up of two AB parts of ABX spectra corresponding to the methylene protons at C-7 and -9 coupling with 6-H. This is not immediately obvious from an inspection of the multiplets shown in Figure 2a but becomes clear when the spectra are simplified by decoupling at the 6-H frequency (Figure 2b). There are in fact two separate 6-H resonances corresponding to the two forms. The 6-H signal of the more abundant form is clearly visible in the spectrum (1.1 p.p.m.) but the 6-H resonance of the less abundant form is under the water peak and its chemical shift (0.61 p.p.m.) could only be found by using decoupling experiments. Irradiation at 1.1 p.p.m. causes one set of lines in the methylene multiplet to simplify to two AB quartets (see Figure 2b) while irradiation at 0.61 p.p.m. causes collapse of the other set of methylene lines corresponding to the minor component.

When the assignments had been made by use of the decoupling experiment it was then relatively easy to analyse the original spectrum as two overlapping pairs of ABX spectra and the results for the chemical shifts and coupling constants are summarised in Tables 1 and 2. To assign the multiplets to 7- or 9-H_2 respectively it was necessary to examine the ^1H n.m.r. spectrum of a sample of (±)-L-folinic acid selectively deuteriated at C-7. Loss of intensity of the multiplets at — 0.35 and — 0.46 p.p.m. identified these as the 7-H signals.

The sets of coupling constants for the two different forms of folinic acid are seen to be very similar, indicating that they have similar conformations in their C-7–C-6–C-9 frag-

TABLE 1

The ^1H chemical shifts for the two conformations of 2mM-folinic acid in phosphate buffer at 30 °C

Proton	Chemical shift (p.p.m.) * Form (1A) (major)	Form (1B) (minor)
CHO	4.88	4.16
6-H	1.11	0.61
7A-H	−0.35	−0.31
7B-H	−0.46	−0.42
9A-H	−0.22	−0.14
9B-H	−0.34	−0.22
2′-, 6′-H	3.87	3.90
3′-, 5′-H	2.96	2.99
Glu-α-CH	0.53	0.53
β-CH–A †	−1.74	−1.74
β-CH–B †	−1.62	−1.62
γ-CH_2	−1.45	−1.45

* Shifts measured from dioxan internal reference: downfield positive. The dioxan signal is 3.71 p.p.m. downfield from that of DSS. † Shifts measured to centres of multiplets.

ments. Thus the origin of the difference between the two forms cannot arise from a conformational difference in the reduced pyrazine ring.

The protons in the 9-methylene group have small coupling

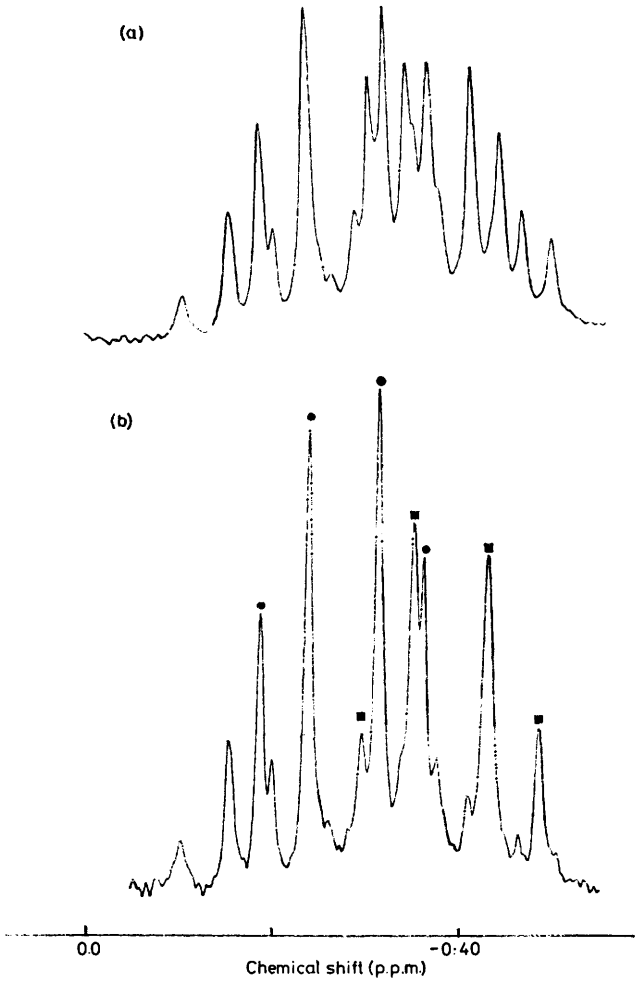


FIGURE 2 The multiplets from the 7- and 9-methylene protons in the 270-MHz ^1H resonance spectrum of (±)-L-folinic acid at 30 °C: (a) without irradiation; (b) with irradiation at 1.1 p.p.m., the position of the 6-H resonance in the major form (I); ■ 7-H signals; ●9-H signals in the major form (IA)

constants to 6-H (4.3 and *ca.* 1 Hz) indicating a conformation with 6-H *gauche* to both its vicinal protons. The 7-methylene protons have larger coupling constants to 6-H (8.5 and 5.5 Hz). These values are similar to those observed by Storm and Chung [12] in the tetrahydropyrazine ring of 6-phenyltetrahydropterin (8.6 and 5.4 Hz). These coupling constants indicate that the proton at position 6 is in a pseudo-axial position and the reduced pyrazine ring is in a half-chair conformation.[12]

Poe and Hoogsteen [13] have measured the vicinal coupling constants for the reduced pyrazine ring protons in 5,6,7,8-tetrahydrofolic acid ($J_{6,7}$ 3.0 and 6.6 Hz): these are seen to be substantially different from those in folinic acid indicating

TABLE 2

The ^1H–^1H coupling constants in the two conformations of folinic acid

^1H–^1H coupling constants (Hz)	Form (1A) (major)	Form (1B) (minor)
$J_{6.7A}$	5.5	6
$J_{6.7B}$	8.5	8.8
$J_{6.9A}$	<1	~1
$J_{6.9B}$	4.3	4
$J_{7A.7B}$	14.5	14.4
$J_{9A.9B}$	13.2	13.2
$J_{2'.3'}$	8.5	8.5

Errors: ±0.3 Hz.

a clear conformational difference. They explained the coupling constants in terms of two interconverting half-chair conformations on the tetrahydropyrazine ring. More recently, Furrer and his co-workers [14] have reported similar coupling constants for 5,6,7,8-tetrahydrofolic acid and interpreted them in terms of a single half-chair conformation with 6-H in a pseudo-axial position.

^{13}C *N.M.R. Spectrum of Folinic Acid.*—The ^{13}C n.m.r. spectrum of (±)-L-folinic acid has also been measured and the chemical shifts are given in Table 3. The ^{13}C spectrum

TABLE 3

The ^{13}C chemical shifts for the two conformations of folinic acid (20mM in D_2O solution)

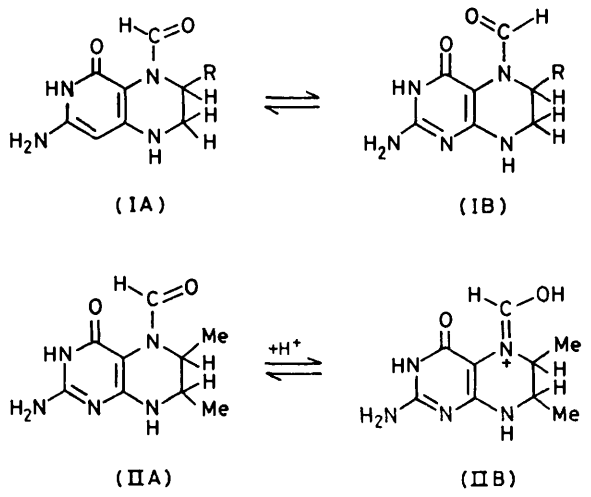
Carbon	Chemical shift (p.p.m.) † Form (1A) (major)	Form (1B) (minor)
CHO	165.2	163.2
3′,5′	129.6	129.7
2′,6′	112.9	113.1
7	{43.7}	{(44.0)}
9	{42.3}	{(42.8)}
6	(50.1)	{(42.6)}
Glu-α	56.6	56.6
Glu-β	29.2	29.2
Glu-γ	35.0	35.0

† Shifts reported with respect to tetramethylsilane reference (external). The assignments in parentheses may be reversed. Chemical shifts of the non-protonated carbons were not measured.

provides evidence for two non-equivalent forms. Thus there are two separate formyl ^{13}C signals of unequal intensities and with chemical shifts (163.2 and 165.2 p.p.m.) in the range expected for amide carbon resonances,[15] two separate signals for C-6 with a chemical shift difference of at least 6.1 p.p.m. and two separate signals for each type of aromatic carbon and for the C-7 and C-9.

Interconversion between the Two Conformations.—When the formyl proton resonance of the more abundant form (IA) is irradiated at temperatures above 50 °C in a double-resonance experiment, the formyl proton signal of the less

abundant form (1B) is removed by transfer of saturation.[16] This indicates that there is exchange between the two forms (exchange rate estimated to be $<2\ s^{-1}$ based on observation of separate spectra for protons (2'- and 6'-H and 3'- and 5'-H) with shift difference of 3 Hz at 100 MHz in the two forms). Further evidence that we are dealing with a pair of interconverting molecular conformations is obtained from variable temperature studies. At 25 °C the ratio of the two forms in the equilibria is 2.35 : 1 (the form with the formyl proton signal at lower field being the more abundant).



Increasing the temperature caused the major form to become more populated and at 77 °C the ratio of the two forms is 4.66 : 1. The thermodynamic parameters obtained from the study of the equilibrium constants as a function of temperature are ΔG^0 −2.12 kJ mol^{-1}, ΔH^0 11.39 kJ mol^{-1}, and ΔS^0 0.046 J mol^{-1} K^{-1}.

DISCUSSION

Structures of the Two Forms of Folinic Acid.—Bieri and Viscontini[17] have measured the ^1H n.m.r. spectrum of 5-formyl-6,7-dimethyl-5,6,7,8-tetrahydropterin and reported two forms in CF_3CO_2H solution but only one form in DMSO or NaOD solution. They attribute the two forms in acid solution to a mixture of protonated and non-protonated molecules as shown [(IIB and A)]. However, in view of the absence of spectral changes for folinic acid over the pH range 4—8, we can exclude the possibility that such an equilibrium gives rise to the two forms we observe for folinic acid at pH 6.5. Furthermore, the similar ^{13}C shifts (163.2 and 165.2 p.p.m.) of the formyl carbons in folinic acid do not indicate a large electronic difference between the formyl groups in the two forms.

A comparison of the ^1H shifts in the two forms of folinic acid reveals that the largest differences are for the formyl and 6-H protons. Because the coupling constants between 6-H and the 7- and 9-methylene protons are similar in the two forms it seems unlikely that there could be a major conformational difference involving 6-H, and thus we can exclude the possibility that the two forms arise from different conformations in the tetrahydropyrazine rings. It seems much more likely that there is a difference between the formyl groups in the two forms. This difference is probably due to the presence of two conformations of the formyl group of the types shown in (1A and B) arising from hindered rotation about the C–N bond. Conformations of this type are well known in related molecules such as benzoyl- and ethyoxycarbonyl-substituted piperidines.[18] One would expect conformations (IA and B) to interconvert at a rate which is detectable by transfer of saturation experiments as observed for folinic acid.

In the two forms (IA and B) the formyl group cannot take up a planar conformation with respect to the ring system because of steric interactions with neighbouring groups. The out-of-plane distortion probably results in the formyl carbonyl being on the same side of the molecule as 6-H where there would be least steric interaction. [Such a configuration with the formyl group oriented as in form (IA) has been reported by Bieri and Viscontini[17] from crystal structure studies on 5-formyl-5,6,7,8-tetrahydropterin.] The measured ^1H chemical shifts of the formyl and 6-H protons in folinic acid can readily be explained on the basis of the above conformations. In form (IA) the formyl proton is approximately in the plane of the CO group at C-4 and would thus be deshielded as is seen in the more abundant form. 6-H in this form would also be deshielded because it is oriented towards the formyl CO group. In the less abundant form (IB), the formyl and 6-H protons would be much less influenced by these interactions. The large difference in ^{13}C chemical shifts for C-6 (>6 p.p.m.) in the two forms can also be explained readily: C-6 in form (IA) would experience an upfield shift of *ca.* 5 p.p.m. due to steric interactions between the oxygen atom of the formyl group and 6-H.[15]

One cannot rule out the possibility that the hindered rotation about the C–N bond involves two conformations with the formyl carbonyl group being above and below the plane of the ring. However, it would be very difficult to get interconversion between these forms at the rates observed here.

Conclusions.—There are two slowly interconverting forms (IA and B) of folinic acid corresponding to two conformations of the formyl group arising from hindered rotation about the C–N bond. The more populated conformation (IA) has the formyl carbonyl oriented towards 6-H in the tetrahydropyrazine ring. In both conformations, (IA and B), the 9-methylene protons are both in *gauche* positions with respect to 6-H.

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