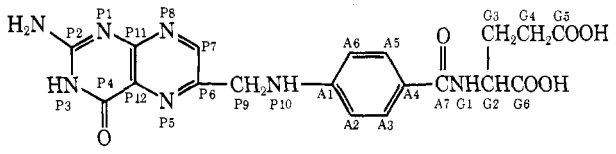
Assignment of the Carbon-13 Nuclear Magnetic Resonance Spectrum of Folic Acid†

Jeffrey A. Lyon, Paul D. Ellis,* and R. Bruce Dunlap*

ABSTRACT: Both the proton-decoupled and coupled ^{13}C nmr spectra of folic acid in D_2O at pD 7.9 are presented and analyzed. To aid in the assignment of its ^{13}C resonances, folic acid was considered to be composed of three components: L-glutamic acid, *p*-aminobenzamide, and the pteridine ring system. Assignment of the ^{13}C nmr spectrum of *N*-*p*-aminobenzoyl-L-glutamate was accomplished by examination of both the ^{13}C nmr spectrum of L-glutamic acid and the chemical shifts predicted for *p*-aminobenzamide on the basis of additivity for aniline and benzamide. The pteridine ring portion of folic acid was analyzed by analogy with ^{13}C nmr data for certain purine nucleosides, quinazoline, quinoxaline, and 4-hydroxypteridine.

The nutritional importance and clinical usages of the vitamin, folic acid (**1**) (pteroylglutamic acid), are well established. Folate analogs, such as amethopterin and aminopterin, have attracted a good deal of attention in recent years, especially in view of their anticancer chemotherapeutic properties (Blakley, 1969). A reduced form of folic acid, namely tetrahydrofolate, participates as the main biochemical carrier and transfer agent for reactions involving single carbon units at various levels of oxidation (Huennekens, 1968). To date, the solution dynamics of the folates and the folate antagonists have received relatively little attention although some preliminary work performed with proton magnetic resonance spectroscopy was interpreted to indicate that folic acid readily undergoes self-association while many of its relatives do not (Pastore, 1971). We are currently employing high-resolution ^{13}C nmr spectroscopy in an attempt to characterize the conformational aspects of the folates in solution and the nature of their binding interactions with various enzymatic systems. In this report we present ^{13}C spectra and data for folic acid and certain of its components, and we propose a rationale for the assignment of the ^{13}C nuclear magnetic resonance (nmr) spectrum of folic acid.



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Experimental Section

Chemicals were of reagent grade and were obtained as follows: folic acid (Calbiochem, La Jolla, Calif.), L-glutamic acid (Matheson, Coleman & Bell), *N*-*p*-aminobenzoyl-L-glutamic acid (Sigma), 4-hydroxypteridine (Aldrich), deuterium oxide (D_2O) (Columbia Organic Chemicals), and dioxane (Allied Chemicals) and were used without further purification. Carbon-13 spectra were obtained on a Varian XL-100 (nmr) spectrometer locked to deuterium and operating at 25.2 MHz in the Fourier transform mode. The ^1H noise decoupling was accomplished with a Varian Gyrocode decoupler. Spectra were run at ambient temperature using 3-ml samples in 12-mm tubes of folate (0.2 M), L-glutamate (1 M), or *N*-*p*-aminobenzoyl-L-glutamate (1 M), each of which was dissolved in 0.5 M potassium phosphate–D_2O buffer and adjusted to pD 7.9 (Glasoe and Long, 1960). Similarly, ^{13}C spectra of 4-hydroxypteridine (0.1 M) were recorded in 0.5 M potassium phosphate–D_2O buffer (pD 9.9).

All chemical shifts were referenced to the chemical shift of dioxane in a 1 M solution in D_2O contained within a 5-mm nmr tube, which was held in the 12-mm tube by Teflon spacers. The same 5-mm tube was used for all experiments. Both proton coupled and decoupled ^{13}C spectra were recorded.

Results and Discussion

Both the decoupled and coupled ^{13}C spectra of folic acid are presented in Figure 1. In order to identify the individual carbons clearly in folic acid, this compound can be conveniently considered to be composed of three chemical moieties: L-glutamic acid (G), *p*-aminobenzamide (A), and the substituted pteridine ring system (P). As shown in **1**, each carbon is clearly identified by a number together with the letter corresponding to its particular region in folic acid, *i.e.*, P2, A5, G3. These labels are used to delineate the assignment of the peaks in the decoupled spectrum of folic acid (Figure 1) and are used henceforth to identify individual atoms. The chemical shift data obtained for folic acid and its components are compiled in Tables I–IV. All chemical shifts are referenced to the resonance of dioxane in an external capillary. In the cases of glutamic acid and aniline, whose ^{13}C chemical shifts were already known relative to benzene (Horsley *et al.*, 1970; Maciel 1965), a conversion factor of −61.8 ppm was used to convert the chemical shifts to a dioxane scale.

As is apparent from Figure 1, the ^{13}C resonances of folic acid can be grouped into three main regions which correspond with few exceptions to the three chemical regions of the

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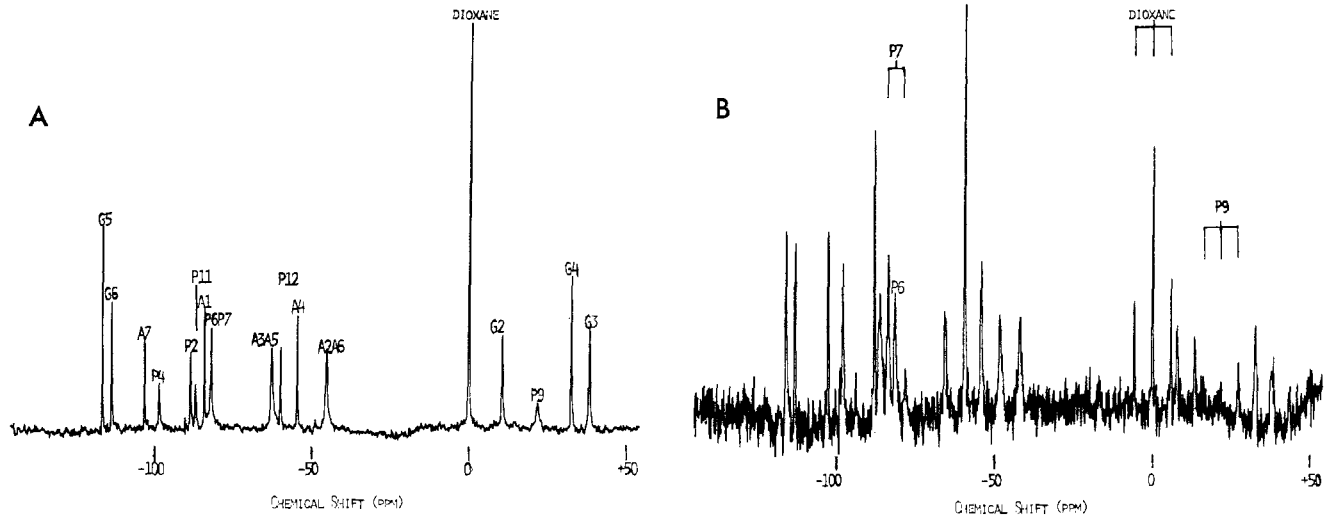


FIGURE 1: The ^1H decoupled ^{13}C Fourier transform spectrum. (A) Of a 0.2 M folic acid solution at pD 7.9 after 295,000 transients. The experimental parameters were: pulse width 65 μsec, aquisition time 0.4 sec, pulse delay 0.4 sec, and sweep width 5000 Hz. The D_2O solvent provided the ^2H lock signal. (B) Of a 0.2 M folic acid solution at pD 7.9 after 194,000 transients. The experimental parameters were: pulse width 65 μsec, aquisition time 0.4 sec, pulse delay 0.4 sec, and sweep width 5000 Hz. The D_2O solvent provided the ^2H lock signal.

molecule alluded to above. The assignments of the glutamic acid, *p*-aminobenzamide and the pteridine ring system regions of folic acid will now be considered in turn.

L-Glutamic Acid. The assignments of the carbons in the L-glutamic acid region of folic acid were made by comparing our data for L-glutamic acid (Table I) to that of Horsley *et al.* (1970). The small differences in chemical shifts between our data and theirs can be attributed to the different experimental conditions employed. For the sake of consistency, we will use our data for L-glutamic acid for further work in this paper.

N-p-Aminobenzoyl-L-glutamic Acid and p-Aminobenzamide. The next step in the overall assignment of the peaks in the ^{13}C nmr spectrum of folic acid is the assignment of the ^{13}C resonances of *N-p*-aminobenzoyl-L-glutamic acid. This task is greatly simplified by assuming that the chemical shifts of the L-glutamic acid portion of the latter molecule are not appreciably different from those of L-glutamic acid itself (Table I). Only the resonances attributable to the *p*-aminobenzamide moiety remain unassigned. One would expect that only five ^{13}C nmr peaks would result from this molecule since carbons A2 and A6 are chemically equivalent as are carbons A3 and A5. The assignments of A1, A2–A6, A3–A5, and A4, and A7 are made on the basis of additivity predictions as described previously (Savitsky and Namikawa, 1964; Paul and Grant, 1963). Additivity was applied to the known chemical shift assignments of aniline (Maciel, 1965) and benzamide (P. D. Ellis, R. R. Fisher, R. B, Dunlap, A. P. Zens, and T. A. Bryson, submitted for publication) to calculate the ^{13}C nmr spectrum of *p*-aminobenzamide. The additivity predicted chemical shifts for the *p*-aminobenzamide portion of *N-p*-aminobenzoyl-L-glutamic acid are in excellent agreement with the experimentally determined values obtained with the parent molecule (Table I). For our purposes, the additivity calculations employed herein are derived from eq 1, since all of the chemical shifts reported in this paper are given with respect to dioxane.

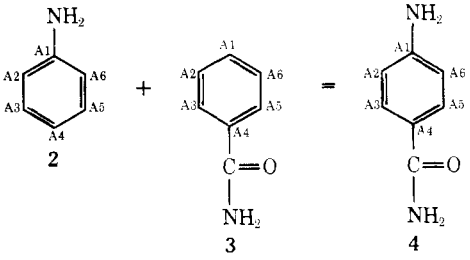
$$\delta x_{\text{calcd}} = \Delta\delta x_a + \Delta\delta x_b + (-61.8 \text{ ppm}) \quad (1)$$

where

$$\Delta\delta x_a = \delta x_a - (-61.8 \text{ ppm}) \quad (2)$$

$$\Delta\delta x_b = \delta x_b - (-61.8 \text{ ppm}) \quad (3)$$

δx_a and δx_b represent the chemical shifts of carbon atoms in substituted benzenes while each of the values given by $\Delta\delta x_a$ and $\Delta\delta x_b$ are actual chemical shift differences between the carbon atoms of a substituted benzene and benzene itself. Thus, if the chemical shifts are known for the individual carbons in aniline and benzamide with respect to benzene, they may be manipulated by eq 1 to arrive at a predicted chemical shift value for each carbon in a corresponding disubstituted benzene, such as *p*-aminobenzamide. For example, consider carbon A1 in *p*-aminobenzamide (**4**). To predict the chemical



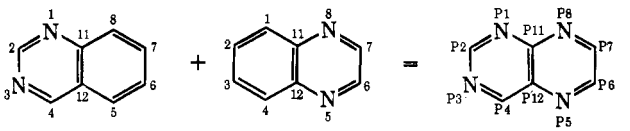
shift for this carbon atom, let δx_a equal the chemical shift of carbon A1 in aniline and δx_b equal the chemical shift of carbon A1 in benzamide. Equation 2 provides an expression for calculating the chemical shift difference ($\Delta\delta$A1-aniline) between carbon A1 in aniline and the carbons in benzene. Similarly, eq 3 is used to express the chemical shift difference ($\Delta\delta$A1-benzamide) between carbon A1 in benzamide and the carbons in benzene. The chemical shift differences are then substituted into eq 1, to give eq 4 where the appropriate conversion factor (−61.8 ppm) is applied to permit calculation of $\delta A1_{\text{calcd}}$ *p*-aminobenzamide with respect to dioxane.

$$\delta A1_{\text{calcd}}\ p\text{-aminobenzamide} = \Delta\delta\text{A1-aniline} + \Delta\delta\text{A1-benzamide} + (-61.8 \text{ ppm}) \quad (4)$$

A sample calculation is given below for carbon A1. From Table I, δA1-aniline equals −79.8 ppm and δA1-benzamide equals −64.9 ppm. Thus, by using equations 2 and 3, $\Delta\delta$A1-

aniline and $\Delta\delta$A1-benzamide are calculated to be -18.0 and -3.1 ppm, respectively. Substitution of these values into eq 4 results in a calculation of a value of -82.9 ppm for the additivity predicted chemical shift of carbon A1 in *p*-aminobenzamide. Chemical shift predictions for the remaining carbons in *p*-aminobenzamide were calculated in a similar fashion. The basis for these and other additivity-based predictions of ^{13}C chemical shifts is described in detail by Stothers (1972) and by Levy and Nelson (1972).

Pteridine Ring System. The assignments of the carbons in the pteridine ring system were accomplished by combining the results of two analytical procedures. By applying additivity relationships to the ^{13}C chemical shifts of quinazoline (**5**) and quinoxaline (**6**) (Pugmire *et al.*, 1969) the chemical shifts of the corresponding carbons in pteridine (**7**) can be approximated



quinazoline **5** + quinoxaline **6** = pteridine **7**

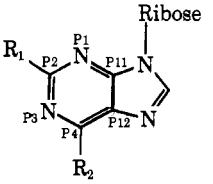
(see Table II). For example, the ^{13}C chemical shift value of carbon P2 in pteridine (**7**) can be approximated from the chemical shifts of P2 in quinazoline (**5**) and quinoxaline (**6**) by using eq 1–3 and 5.

$$\delta P2_{calcd}\ \text{pteridine} =$$

$$\Delta\delta\text{P2-quinazoline} + \Delta\delta\text{P2-quinoxaline} + (-61.8) \quad (5)$$

Thus, from Table II, δP2-quinazoline is -93.8 ppm and δP2-quinoxaline is -63.2 ppm. By using eq 2 and 3, one arrives at values of -32.0 and 1.4 ppm for $\Delta\delta$P2-quinazoline and $\Delta\delta$P2-quinoxaline, respectively. Substitution of these values into eq 5 yields a calculated chemical shift of -95.2 ppm for the P2 carbon atom. An experimental check of this approximation is obviated by the instability of pteridine itself. Furthermore, the application of this method for predicting chemical shifts cannot be employed with substituted pteridines since the required analogs of **5** and **6** have not as yet been examined. To circumvent this problem, it is worthwhile to note that pteridine bears structural similarity to purine, and a rather complete study of a series of substituted purine ribonucleosides has been published by Jones *et al.* (1970). In the discussion which follows we employ these data to predict the effect of substituents on the ^{13}C chemical shifts at the P2, P4, P11, and P12 positions of various pteridines. We will use nebularine, 2-aminopurine ribonucleoside, inosine, and guanosine to this end. The pertinent ^{13}C chemical shifts for these compounds are provided in Table III.

Folic acid can be considered as a 2-amino-4-hydroxy-6-sub-



nebularine, $R_1 = R_2 = H$
2-aminopurine ribonucleoside, $R_1 = NH_2$; $R_2 = H$
inosine, $R_1 = H$; $R_2 = OH$
guanosine, $R_1 = NH_2$; $R_2 = OH$

TABLE I: ^{13}C Chemical Shifts for the *N*-*p*-Aminobenzoyl-L-glutamic Acid Portion of Folic Acid.[a]

Compound	A1	A2–A6	A3–A5	A4	A7	G2	G3	G4	G5	G6
L-Glutamic acid						+11.8	+39.4	+33.0	−114.0	−107.3
Benzamide[b]	−64.9	−61.8	−61.1	−68.0	−101.7					
Aniline[c]	−79.8	−48.5	−62.7	−52.0						
p-Aminobenzamide[d]	(−82.9)	(−48.5)	(−62.0)	(−58.2)	(−101.7)					
N-*p*-Aminobenzoyl-L-glutamic acid	−84.0	−48.5	−62.0	−56.3	−102.9	+11.1	+37.9	+32.3	−115.5	−112.5
	(−82.9)	(−48.5)	(−62.0)	(−58.2)	(−101.7)	(+11.8)	(+39.4)	(+33.0)	(−114.0)	(−107.3)

[a] All chemical shifts are given in ppm with respect to dioxane. [b] P. D. Ellis, R. R. Fisher, R. B. Dunlap, A. P. Zens, and T. A. Bryson (submitted for publication). [c] Maciel (1965). [d] Values in parentheses are those predicted on the basis of simple additivity; see the main text for details.

TABLE II: ^{13}C Chemical Shifts of Pteridine and Related Compounds.[a]

Compound	P2	P4	P6	P7	P11	P12
Quinazoline[b]	−93.8	−89.0	−61.2	−67.4	−83.4	−58.5
Quinoxaline[b]	−63.2	−63.1	−78.8	−78.8	−76.5	−76.5
Pteridine[c]	(−95.2)	(−90.3)	(−78.2)	(−84.4)	(−98.1)	(−73.2)
4-Hydroxypteridine	−93.5	−105.6	−76.9	−82.9	−87.8	−65.7
	(−92.5)	(−99.0)	(−78.2)	(−84.4)	(−92.0)	(−63.4)
6-Alkyl-2-amino-4-hydroxypteridine	(−97.9)	(−99.1)	(−86.0)	(−84.4)	(−97.4)	(−55.6)

[a] All chemical shifts are given in ppm with respect to dioxane. [b] Pugmire *et al.* (1969). [c] Values in parentheses are those predicted by simple additivity relationships.

stituted pteridine. Guanosine, which can be viewed as a 2-amino-4-hydroxy-substituted purine ribonucleoside, serves as an excellect model for the combined effect of the P2 amino and the P4 hydroxy substituents on carbons P2, P4, P11, and P12 in folic acid. The ^{13}C chemical shifts of carbons P2, P4, P11, and P12 in guanosine with respect to the analogous carbons in nebularine are −2.7, −8.8, −0.7, and 17.6 ppm, respectively. Accordingly, the ^{13}C chemical shifts for carbons P2, P4, P11, and P12 in 2-amino-4-hydroxypteridine (see Table II) may be predicted by adding the above chemical shifts differences to those estimated for the analogous carbons in pteridine by the additivity procedure employed above. These calculations are summarized in Table II. The effect of a substituent at P6 on the ^{13}C chemical shifts of the carbons within a substituted pteridine ring system remains to be discussed.

If the approach used here is valid, the effect of individual substituents on the ^{13}C chemical shifts of carbons in the pteridine ring can be evaluated by comparing the effects of individual substituents in the purine ribonucleoside series, *i.e.*, 2-aminopurine ribonucleoside relative to nebularine, and similarly for inosine (see Table III). Note that in comparing the resonances of nebularine with those of the 2-amino compound, a deshielding of 8.69 ppm is observed at the substituted position. In comparison, however, the amino-substituted carbon in aniline is deshielded by 18.0 ppm relative to benzene (Maciel, 1965). Similarly, a deshielding of 8.6 ppm is observed for the hydroxy-substituted carbon (position 4) in inosine relative to carbon atom 4 in nebularine. Again, a much larger deshielding of 26.9 ppm is observed for the substituted carbon in phenol relative to benzene. Therefore, in order to be consistent with the trends observed above, one would predict that an alkyl substituent at P6 of pteridine would elicit a muted substituent effect on the ^{13}C chemical shift of P6 when compared to the ^{13}C chemical shift of the substituted carbon in ethylbenzene relative to benzene. If the trends found above are at all typical, it is not unreasonable to assume that the chemical shift due to an alkyl group at P6 would be reduced by at least a factor of 2 compared to the corresponding change in an alkyl-substituted benzene. Since the ^{13}C chemical shift of the substituted carbon in ethylbenzene relative to benzene is deshielded by 15.6 ppm (Lauer *et al.*, 1972), one would expect the ^{13}C chemical shift of P6 in 6-ethylpteridine to be deshielded by approximately 8 ppm with respect to P6 in pteridine. Furthermore, the para carbon in ethylbenzene is shielded by 2.6 ppm relative to benzene, leading one to believe that a similar effect would occur at P11 (para to P6) in 6-alkylpteridine. At this point, one can combine the predicted chemical shifts for 2-amino-4-hydroxypteridine with the predicted effects on the chemical shifts estimated for a P6 alkyl-substituted pteridine to construct a hypothetical ^{13}C nmr spectrum of the pterin portion of 2-amino-4-hydroxy-6-alkylpteridine (see Tables II and III).

TABLE III: ^{13}C Chemical Shift of Various Purine Nucleosides.[a]

Compound	P2	P4	P11	P12
Nebularine	−85.2	−82.3	−86.3	−68.4
2-Amino-9-(β-D-ribofuranosyl)-purine	−93.8	−84.0	−88.1	−58.0
Δ chemical shift[b]	−8.6	−1.7	−1.8	+10.4
Inosine	−82.5	−91.0	−80.2	−58.6
Δ chemical shift[b]	+2.7	−8.7	+6.1	+9.8
Guanosine	−87.9	−91.1	−85.6	−50.8
Δ chemical shift[b]	−2.7	−8.8	+0.7	+17.6

[a] Data were obtained from a paper by Jones *et al.* (1970). [b] Δ chemical shifts are with respect to nebularine.

Before considering the overall assignment of the ^{13}C spectrum of folic acid, we substantiate some of the assumptions made in the preceding analysis by briefly examining the ^{13}C nmr spectrum of 4-hydroxypteridine. By using a procedure analogous to that applied to guanosine for the prediction of ^{13}C chemical shifts in the corresponding pteridine, one can also use inosine to arrive at a predicted spectrum of 4-hydroxypteridine (see Table II). This model predicts that the ^{13}C resonances resulting from 4-hydroxypteridine should occur in the following order of increasing shielding: P4, P2, P11, P7, P6, and P12 where carbons P4, P11, and P12 are tertiary carbons. Under the experimental conditions employed, tertiary carbons will give rise to lower intensity signals than those representing proton-bearing carbons (Allerhand *et al.*, 1971). The decoupled ^{13}C nmr spectrum of 4-hydroxypteridine is characterized by six lines: three high-intensity peaks and three low-intensity peaks. The experimental spectrum is consistent with the intensity pattern predicted above and, therefore, the assignment of P4, P11, and P12 follows directly. This assignment is further supported by chemical shift data for guanosine presented in Table III. That is, as in guanosine, P4 and P12 in 4-hydroxypteridine are the most deshielded and shielded peaks, respectively, and therefore, P11 is assigned by default. By comparing the predicted and experimental chemical shifts for 4-hydroxypteridine, it is obvious that P2 is the most deshielded proton-bearing carbon. If we assume that the predicted order of chemical shifts is completely valid, then the assignment of P6 and P7 follows.

Overall Assignment of Folic Acid. We now move to the

TABLE IV: ^{13}C Chemical Shifts of Folic Acid.[a]

Compound	P2	P4	P6	P7	P9	P11	P12	A1	A2-A6
Folic acid[b]	−87.6	−97.5	−81.0	−81.0	+21.6	−86.1	−59.3	−83.3	−45.0
	(−97.9)	(−99.1)	(−86.2)	(−84.9)		(−97.4)	(−55.6)	(−84.0)	(−48.4)
	A3-A5	A4	A7	G2	G4	G3	G5	G6	
	−62.2	−54.1	−102.2	+10.5	+32.2	+38.0	−115.6	−112.7	
	(−62.3)	(−56.3)	(−102.9)	(+11.1)	(+32.3)	(+37.9)	(−115.6)	(−112.5)	

[a] Chemical shifts are given in ppm with respect to dioxane. [b] Values in parentheses are those predicted by simple additivity relationships.

assignment of the peaks in the ^{13}C nmr spectrum of folic acid (see Table IV). This procedure is greatly simplified by assuming that the ^{13}C resonances previously measured for *N-p*-aminobenzoyl-L-glutamic acid will not be significantly changed by substitution of a hydrogen on the *p*-amino group with the methylene carbon attached to P6 of 2-amino-4-hydroxypteridine (pterin) (see **1**). Examination of the data for *N-p*-aminobenzoyl-L-glutamic acid portion of folic acid itself (Table IV) demonstrates the validity of this assumption. Thus, only the six carbons of the 2-amino-4-hydroxypteridine moiety and the methylene bridge carbon (P9) remain to be assigned in the folic acid. Since P9 is a methylene carbon, its resonance is expected to occur in the "glutamic acid region" of the folic acid spectrum. The assignment of P9 to the resonance at +21.6 ppm is consistent with that fact.

At this point we reexamine the predicted chemical shifts for 2-amino-4-hydroxy-6-substituted pteridine (in Table IV). Because of the assumptions inherent in this model, we would expect that the model would only predict the order of chemical shifts within the pteridine ring system of folic acid. If this model is valid, the signals appearing at −97.5, −87.6, −86.1, −81.0 (2), and −59.3 are assigned to P4, P2, P11, P6–P7, and P12, respectively. Some aspects of this assignment deserve further comments. Inherent in this assignment is the fact that the resonance appearing at −81.0 ppm is actually the superpositioning of a resonance due to the tertiary carbon P6 and the proton-bearing carbon P7. This unique feature of the decoupled ^{13}C spectrum of folic acid was verified by examination of the ^{1}H-coupled spectrum of folic acid (Figure 1). Studies of both the pH dependence of the ^{13}C nmr of folic acid and dihydrofolate corroborate that the peak at −81.0 ppm is due to carbons P6 and P7 (J. A. Lyon, P. D. Ellis, and R. B. Dunlap, in preparation). The resonance for P12 was found to lie within 3 ppm of the peak assigned to the equivalent carbons A3 and A5. Any ambiguity in the identification of these resonances was resolved by considering both the peak intensities in the decoupled spectrum and the splitting patterns in the coupled spectrum of folic acid. In further defense of this assignment, P12, which lies ortho to the 4-hydroxy substituent, in folic acid exhibits a pH dependence which is not dissimilar to that observed for the ortho carbons in phenol (Nakashima and Maciel, 1972). In a forthcoming paper we will discuss the concentration dependence and pH dependence of the ^{13}C chemical shifts of folic acid and dihydrofolate in addition to presenting the analysis of the ^{13}C nmr spectra of a number of folate analogs.

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