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Carbon-13 Nuclear Magnetic Resonance Study of Protonation of Methotrexate and Aminopterin Bound to Dihydrofolate Reductase†

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ABSTRACT: Methotrexate, aminopterin, and folate have been synthesized with 90% enrichment of C-2 with ^{13}C. ^{13}C nuclear magnetic resonance has been used to examine the state of protonation of the pteridine ring of these compounds under various conditions and gives much more clear-cut results than most other methods. For the free compounds the following pK values were obtained: methotrexate, 5.73 ± 0.02 (N-1); aminopterin, 5.70 ± 0.03 (N-1); folic acid, 2.40 (N-1) and 8.25 ± 0.05 (N-3, O-4 amide group). The state of protonation of these compounds when complexed to dihydrofolate reductase (isoenzyme 2 from *Streptococcus faecium*) was also studied over the pH range 6–10. The resonance from bound methotrexate showed a constant chemical shift over the whole pH range studied, and it is inferred that in the complex the pteridine ring remains protonated to at least pH 10. The same result was obtained for the binary complex of aminopterin with the reductase and for either methotrexate or aminopterin in ternary complex with reductase and $NADPH_4$. The latter is an inhibitor of the reductase competitive with NADPH. However, folate bound to the reductase in either the binary or the ternary complex shows the same protonation behavior as in the free state. The data indicate that the association constant for binding of methotrexate is increased enough when protonation of N-1 occurs to account for the enhanced binding of methotrexate as compared with folate.

More than 20 years ago methotrexate (4-amino-4-deoxy-10-methylfolic acid, MTX), aminopterin, and related 2,4-diamino heterocycles were shown to be potent inhibitors of dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3, DHFR). Typically MTX binds to the enzyme with an association constant considerably greater than that for combination with the substrate, dihydrofolate (DHF) or folate. When MTX combines with the enzyme–NADPH complex, the equilibrium favors the ternary complex so greatly that the association constant frequently cannot be measured with accuracy. In the case of DHFR from *Streptococcus faecium*, for example, this association constant is $>10^9$ (Williams et al., 1979). The clinical usefulness of inhibitors such as MTX and trimethoprim give added significance to this interaction of the inhibitors with DHFR, their primary target.

Almost as soon as it was discovered, Baker (1959) suggested that the tight binding of aminopterin to DHFR is due to the increased basicity of the pteridine ring in this inhibitor, which is a consequence of the replacement of the 4-oxo substituent of folate by a 4-amino group. It was postulated that the increased basicity permits a strong interaction between the protonated species of the inhibitor with an acidic group in the catalytic site of the enzyme. This interaction would not occur in the case of folate or DHF. Subsequently Baker and his colleagues obtained considerable evidence in support of this view [reviewed by Baker (1967)]. They compared the association constants for the binding to DHFR of homologous inhibitors which are protonated, unprotonated, or partially protonated at neutral pH and found that binding increased with degree of protonation. Similarly, Montgomery et al. (1971) found that the more basic 3-deazamethotrexate binds more tightly than 1-deazamethotrexate.

Spectroscopic evidence consistent with this view was first obtained by Erickson & Mathews (1972), who examined the electronic absorption spectrum of MTX and aminopterin. The difference spectrum obtained by comparing the free inhibitor with the complex of inhibitor with DHFR of T4 phage (both at pH 7.0) approximated the difference spectrum obtained by comparing inhibitor at low pH (4.8 or 2.8) vs. inhibitor at pH 7.0. This suggested that enzyme-bound methotrexate is protonated, although, as might be expected, the two difference spectra showed significant deviations. Similar results were obtained by Poe et al. (1974) for DHFR from *Escherichia coli*, by Gupta et al. (1977) for the enzyme from the L1210 murine lymphoma, by Hood & Roberts (1978) for DHFR from *Lactobacillus casei*, and by Subramanian & Kaufman (1978) for chicken liver DHFR. Saperstein et al. (1978) found that Raman spectra were also indicative of protonation of the pteridine ring of MTX bound to *E. coli* DHFR. Subramanian & Kaufman (1978) obtained calorimetric evidence that when MTX binds to the chicken liver enzyme, a proton is abstracted

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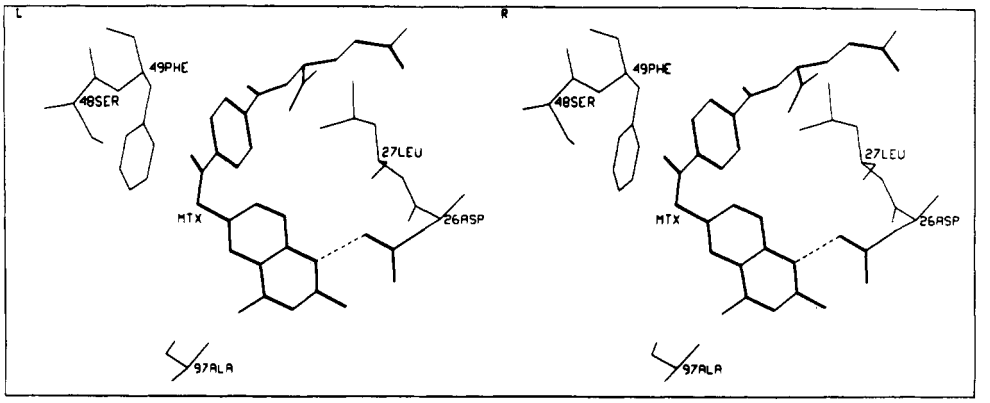


FIGURE 1: Stereodiagram of methotrexate bound in the active-site cavity of *L. casei* dihydrofolate reductase. The stereo figure was generated from coordinates kindly provided by Dr. D. A. Matthews and the use of a graphics program written by Patrick Briley. The broken line indicates the putative electrostatic interaction between N-1 of MTX and the aspartate carboxyl.

from the solvent, whereas this does not occur in the binding of folate, DHF, or reduced nicotinamide adenine dinucleotide phosphate (NADPH).

The X-ray crystallographic results of Matthews et al. (1978) for the structure of the ternary complex of *L. casei* DHFR with NADPH and MTX clearly indicated the existence of interaction between N-1 of MTX with the side-chain carboxyl of Asp-26. The close approach of these groups is shown in Figure 1. Exactly the same interaction was also observed in the binary complex of *E. coli* reductase with MTX (Matthews et al., 1977). These results strongly suggest interaction of the protonated N-1 of MTX with the carboxylate group of Asp-26 (Asp-27 for the *E. coli* enzyme). Since this Asp is invariant in bacterial DHFR and there is a corresponding Glu carboxyl in vertebrate DHFR (Blakley, 1981; Hitchings & Smith, 1980), the interaction explains the tight binding of MTX to DHFR from all these sources.

To obtain additional information about this interaction of MTX with DHFR, we have synthesized MTX, aminopterin, folate, and DHF with 90% enrichment of ^{13}C in position 2 of the pteridine ring, next to the nitrogen which is believed to protonate as MTX binds to DHFR. The interaction of these isotopically labeled pteridines with DHFR from *S. faecium* has been studied by ^{13}C NMR.

Previous investigations of the protonation of bound MTX have been concerned with the binary complex, but it is of interest to compare ternary complexes also, since MTX is more tightly bound in these and they are probably important in vivo. For this reason, tetrahydro-NADP ($NADPH_4$), an inactive analogue of NADPH, has been prepared and used to form ternary complexes with MTX–DHFR.

Experimental Procedures

Materials. Dihydrofolate reductase was isolated and purified from *S. faecium* var. *durans* strain A as described by Blakley et al. (1978). $NADPH_4$ was prepared by catalytic hydrogenation of NADPH (Biellmann & Jung, 1971). The reduction was stopped when absorbance at 340 nm was no longer detectable, but before significant decrease in absorbance at 260 nm had occurred. The solution was quickly filtered through Celite and then through a millipore filter to remove residual catalyst, lyophilized, and stored at −20 °C in a desiccator. Solutions in water initially had an absorbance ratio A_{288}/A_{263} of 0.85–0.90. Biellmann & Jung (1971) report a value of 0.837 for this ratio. However, since upon allowing the solution to stand this ratio changed, even at 0 °C, freshly prepared solutions were always used. [^{13}C]Guanidine hydrochloride was obtained from Kor Isotopes. All other materials were of the highest grade commercially available and used without further purification.

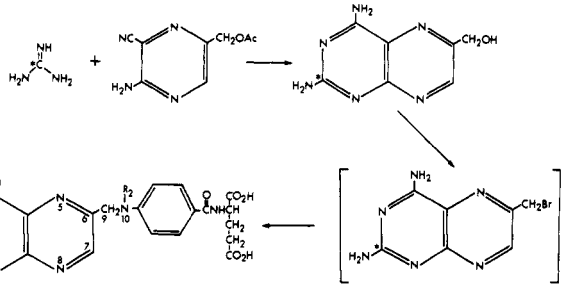


FIGURE 2: Outline of method for synthesis of ^{13}C-labeled pteridines. [2-^{13}C]Aminopterin: $R_1 = NH_2$, $R_2 = H$. [2-^{13}C]Methotrexate: $R_1 = NH_2$, $R_2 = CH_3$. [2-^{13}C]Folic acid: $R_1 = OH$, $R_2 = H$. (*) indicates 90% enrichment with ^{13}C.

Methods. Synthesis of ^{13}C-Labeled Pteridines. The general synthetic scheme followed is shown in Figure 2.

(*a*) *2,4-Diamino[2-^{13}C]pteridine-6-methanol.* The procedure was similar to that reported by Taylor et al. (1975). Solid sodium methoxide (1.88 g, 34.8 mmol) was added with stirring to a solution of [2-^{13}C]guanidine hydrochloride (1.65 g, 17.1 mmol) in anhydrous methanol (60 mL) under nitrogen. The resulting mixture was stirred for 10 min, and the insoluble sodium chloride was removed by filtration. To the filtrate was added 2-amino-3-cyano-5-(acetoxymethyl)pyrazine (Taylor et al., 1975; 2.88 g, 15.0 mmol), and the solution was refluxed for 21 h. The resulting mixture was cooled (5 °C) for 3 h, and the product was collected by filtration and dried in vacuo over P_2O_5: yield, 2.45 g (85%). The high-performance liquid chromatography (HPLC) chromatogram [0.1 M (pH 6.9) NH_4OAc–MeCN (9:1)] showed trace impurities at 5, 8, 10.5, and 13 min and a major peak at 5.5 min, which was identical with the retention time of an authentic sample of 2,4-diaminopteridine-6-methanol hydrobromide (Piper & Montgomery, 1977).

(*b*) *[2-^{13}C]Aminopterin.* Bromine (1.92 mL, 5.99 g, 37.4 mmol) was added dropwise with stirring over 35 min to a solution of triphenylphosphine (9.84 g, 37.6 mmol) in anhydrous *N,N*-dimethylacetamide (72 mL) while the temperature was maintained between 4 and 7 °C with an ice bath. The resulting mixture was stirred for an additional 15 min followed by the removal of the ice bath and the addition of 2,4-diamino[2-^{13}C]pteridine-6-methanol (2.30 g, 11.9 mmol). The latter dissolved within 1 h, and the whole mixture was stirred at room temperature under N_2 for 22 h to form the intermediate 6-(bromomethyl)pteridine. Solid *p*-aminobenzoyl-L-glutamic acid (10.0 g, 37.6 mmol) was added to the reaction mixture, and the whole mixture was stirred at room

temperature under N_2 with protection from light for 24 h.

The reaction mixture was added with stirring to cool (5 °C), deoxygenated H_2O (1200 mL), which had been adjusted to pH 1.4 (meter) with concentrated HCl. After being stirred for 30 min most of the insoluble material was removed by centrifugation. This residue and that obtained by filtration of the cloudy supernatant through Celite were washed with H_2O (150 mL, pH 1.4), and the combined filtrate and wash were adjusted to pH 4 (meter) with 1 N NaOH. After being cooled in the ice bath the solid that deposited was collected by filtration, washed with H_2O, and dried in vacuo over P_2O_5: yield, 1.97 g. An additional amount of product was obtained by washing the residues from the centrifugation and Celite pads with deoxygenated 0.1 N NaOH (600 mL). The wash was acidified to pH 4 (meter) with 1 N HCl, and the resulting mixture was heated to 80 °C, cooled to room temperature, and then refrigerated for 18 h. The solid that deposited was collected by filtration, washed with H_2O, and dried in vacuo over P_2O_5: yield, 1.02 g.

The combined crops were dissolved by warming in anhydrous *N,N*-dimethylacetamide (44 mL), and the cloudy solution was treated with charcoal (100 mg). After filtration through Celite and washing of the residue with dimethylacetamide (6 mL), the combined filtrate and wash were added to deoxygenated H_2O (260 mL) at 80 °C. The resulting mixture was allowed to cool to room temperature and then refrigerated for 18 h. The solid that deposited was collected by filtration, washed successively with H_2O (75 mL), acetone (75 mL), and ether (3 × 180 mL), and dried in vacuo over P_2O_5: yield 2.19 g (40%). The HPLC chromatogram [0.1 M (pH 3.6) NH_4OAc–MeCN (85:15), chart speed 1 cm/min] showed aminopterin at 5.8 min and unidentified impurity peaks at 5.1, 8.5, and 11.5 min: λ_{max} ($\epsilon \times 10^{-3}$) (0.1 N HCl) 243 nm (17.6), 288 (20.8), 334 (11.4), 347 (sh, 9.87); (pH 7) 261 (25.6), 282 (24.5), 367 (7.86); (0.1 N NaOH) 260 (25.8), 281 (24.7), 368 (8.62). Anal. Calcd for $C_{19}H_{20}N_8O_5 \cdot H_2O$: C, 49.88; H, 4.83; N, 24.39. Found: C, 49.71; H, 5.13; N, 24.02.

(*c*) [*2-^{13}C*]*Methotrexate*. The procedure used was similar to that reported by Ellard (1978) and Piper & Montgomery (1977) for the conversion of 2,4-diaminopteridine-6-methanol to methotrexate. The bromodehydroxylation of 2,4-diamino[2-^{13}C]pteridine-6-methanol (1.92 g, 10.0 mmol) with dibromotriphenylphosphorane in dimethylacetamide to give the 6-(bromomethyl)pteridine intermediate was carried out over 24 h. A convenient test for the formation of 6-(bromomethyl)pteridine involves the addition of a drop of the reaction mixture to dimethylacetamide containing thiophenol. After the mixture was allowed to stand at room temperature for several hours, the HPLC chromatogram [H_2O–MeCN (60:40)] of a portion was compared with that of an authentic sample of 6-[(phenylthio)methyl]-2,4-diaminopteridine (Montgomery et al., 1979). The final reaction mixture containing the 6-(bromomethyl)pteridine was treated with diethyl *p*-(methylamino)benzoylglutamate (3.40 g, 10.0 mmol) and the whole mixture was heated at 45 °C for 24 h followed by treatment with additional side-chain ester (0.50 g) and continued heating at 45 °C for 16 h to give diethyl [2-^{13}C]methotrexate: yield, 3.03 g (~59%). The HPLC chromatogram [H_2O–MeCN–HOAc (60:40:1)] showed the product (96%) at 9.44 min and an unidentified peak at 5.1 min. The ester groups of this sample were hydrolyzed in ethanol (90 mL) containing 1 N NaOH (18 mL) over 19 h to deposit the sodium salt (2.28 g), which was collected by filtration, dissolved in H_2O (150 mL), and acidified to pH 4 with 1 N HCl. The solid that deposited was collected by filtration, washed with H_2O, and dried in vacuo over P_2O_5: yield, 2.07 g (42%). The HPLC chromatogram [0.1 M (pH 3.6) NH_4OAc–MeCN (85:15)] showed MTX at 9.01 min: λ_{max} ($\epsilon \times 10^{-3}$) (0.1 N HCl) 243 nm (18.1), 307 (22.4), 335 (sl sh, 13.2), 349 (sh, 9.65); (pH 7) 221 (22.1), 258 (24.1), 303 (24.7), 371 (8.11); (0.1 N NaOH) 222 (22.5), 258 (24.7), 302 (24.9), 370 (8.25). Anal. Calcd for $C_{20}H_{22}N_8O_5 \cdot 2H_2O$: C, 49.08; H, 5.33; N, 22.80. Found: C, 48.78, H, 5.36; N, 23.09.

(*d*) [*2-^{13}C*]*Folic Acid*. The procedure used was similar to that reported by Seeger et al. (1949) for the conversion of aminopterin to folic acid. A solution of [2-^{13}C]aminopterin (305 mg, 0.665 mmol) in deoxygenated 1 N NaOH (10 mL) was heated in an oil bath at 102 °C for 5 h under deoxygenated N_2 [10% $Na_2S_2O_4$ (w/v)] to give crude product: yield, 264 mg. This material was purified by the method of Waller et al. (1948): yield, 120 mg (38%). The HPLC chromatogram [0.1 M (pH 5) NH_4OAc–MeCN (9:1)] showed folic acid (92%) at 5.32 min and unidentified impurity peaks at 6.63, 13.94, and 17.6 min: λ_{max} ($\epsilon \times 10^{-3}$) (0.1 N HCl) 246 nm (sh, 14.4), 296 (20.3); (pH 7) 280 (28.0), 345 (9.41); (0.1 N NaOH) 256 (25.6), 282 (24.6), 365 (9.55). Anal. Calcd for $C_{19}H_{19}N_7O_6 \cdot 2H_2O$: C, 47.91; H, 4.85; N, 20.49. Found: C, 47.66; H, 4.87; N, 20.71.

NMR Methods. Most spectra were obtained with a Nicolet Technology Corp. NT-200 spectrometer operating at 50.30 MHz. Typical recording conditions were as follows: pulse width, 15 μs; acquisition time, 0.541 s; cycle time, 1.041 s; spectral width, ±7575 Hz, collected in 16 K. Spectra were exponentially multiplied to give a 3-Hz broadening, and between 2000 and 15000 transients were collected. Other spectra from samples in aqueous solution were obtained with an NT-360 instrument operating at 90.52 MHz. Typical recording conditions were as follows: pulse width, 15 μs; acquisition time, 0.369 s; cycle time, 0.969 s; spectral width, ±11 111 Hz, collected in 16 K; collection of between 12000 and 30000 transients. Spectra in Me_2SO-d_6 were determined on a Varian XL-100-15 spectrometer with tetramethylsilane as an internal reference.

For NMR of uncomplexed ligands, saturated solutions of the ligands in 0.05 M KH_2PO_4 (in 20% D_2O) at pH ~4 were used, the concentrations being as follows: folic acid, 0.13 mM; aminopterin, 0.11 mM; MTX, 0.75 mM. Recording was performed on samples (10 mL) in a 20-mm flat-bottomed tube fitted with a vortex suppressor and external reference capillary. The pH* (uncorrected meter reading) was measured before and after the spectrum was collected and was found to be unchanged within 0.05 pH unit. When spectra were to be recorded at a variety of pH values, the pH was adjusted by addition of 1 N KOH or 1 N HCl. Any precipitate which formed when the pH was lowered was removed by centrifugation before the spectrum was collected. At lower pH values longer accumulation times were used to compensate for the decreased concentration.

For recording of spectra in the presence of DHFR, the enzyme was initially present at 0.54 mM in 50 mM potassium phosphate buffer and 300 mM KCl at pH* 6.0 (in 20% D_2O). The pteridine ligands were at either 1.0 or 1.5 mM and $NADPH_4$ at 1.0 mM. Sample volumes were 10 mL for the 20-mm tube or, when the NT-360 was used, 2.0 mL in a 10-mm tube. The pH was adjusted by addition of 1 N KOH. In all cases, after the titration of the binary complex was completed, the pH of the solution was readjusted to 6.0 by addition of 1 N HCl, $NADPH_4$ was added, and the pH dependence of the chemical shift of the ternary complex was then determined over the accessible pH range.

Computer analysis was used to extract the pK from the pH titration data, by least-squares fitting to either of the following equations (Browne et al., 1976) after appropriate rearrangement

$$\frac{[H^+]}{K_a} = \frac{\Delta_0 - \Delta_1}{\Delta_1} \quad (1)$$

$$\frac{K_a}{[H^+]} = \frac{\Delta_0 - \Delta_2}{\Delta_2} \quad (2)$$

where $\Delta_0 = \delta_B - \delta_{HB}$, $\Delta_1 = \delta - \delta_{HB}$, $\Delta_2 = \delta_B - \delta$, δ = the chemical shift at a particular pH, δ_{HB} = the chemical shift of the protonated species, and δ_B = the chemical shift of the unprotonated species. The program was developed by the general method used by Cleland (1967) and uses Δ_0 as an adjustable parameter, so that only an estimate is needed. The best-fit value of Δ_0 is calculated by the program. Curves shown in Figures 4 and 5 were calculated from the estimated pK and the chemical shift of the protonated species.

The external standard, which also served as a temperature probe (Vidrine & Peterson, 1976), contained CH_2I_2 and cyclooctane. Temperatures were maintained at 21 ± 2 °C for the free ligand studies and at 23 ± 2 °C for the enzyme studies.

Dissociation Constant for NADPH$_4$ Complex with Dihydrofolate Reductase. An estimate of the dissociation constant for the $NADPH_4$ complex was obtained by measuring inhibition of enzyme activity. Activity was determined spectrophotometrically in a Cary 14 recording spectrophotometer at 37 °C with 5 cm path length cells and a 0–0.1 absorbance scale. Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.0), 50 μM DHF, and 1–5 μM NADPH. With NADPH as the varied substrate, the concentration of $NADPH_4$ was found to be linearly competitive with respect to NADPH. Data were analyzed by Cleland's COMP program (Cleland, 1967), which was modified to run on a Vax 11/780 (Digital Equipment Corp.). The value obtained for K_i was 13 ± 2 μM. This may be compared with $K_{i,NADPH}$ which was previously found to be 2.3 ± 0.3 μM (Gleisner & Blakley, 1975).

HPLC of Pteridines. High-performance liquid chromatography was performed on a Waters Associates ALC-242 liquid chromatograph equipped with a reversed-phase μBondpak C_{18} column, an ultraviolet detector (254 nm), and an M-6000 pump. All chromatograms were recorded at a flow rate of 1 mL/min, a chart speed of 0.5 cm/min (unless otherwise noted), and an attenuation of 16 absorbance units full scale at ambient temperature.

Results

Chemical Shifts for C-2 of Labeled Pteridines. The chemical shifts of C-2 in MTX and aminopterin were determined in Me_2SO-d_6, and for all the pteridines chemical shifts in water were determined at various pH values. Typical spectra for folate and methotrexate are shown in Figure 3. In Table I the chemical shifts obtained are compared with others in the literature. It may be seen that although there is general agreement, in some cases significant discrepancies are evident. Some of these are due to concentration differences. Poe (1973) showed that with increasing concentration in aqueous solution the chemical shifts of most resolvable proton resonances from folate and MTX move upfield. He interpreted this to indicate stacking of pteridines in solution and proposed that dimerization is the predominant process. Similar upfield shifts of ^{13}C resonances with increased concentration may explain differences between our values for MTX and folate and those of Lyon et al. (1973, 1975). However, there are also small discrepancies between our data for MTX and those of Ewers et al. (1973).

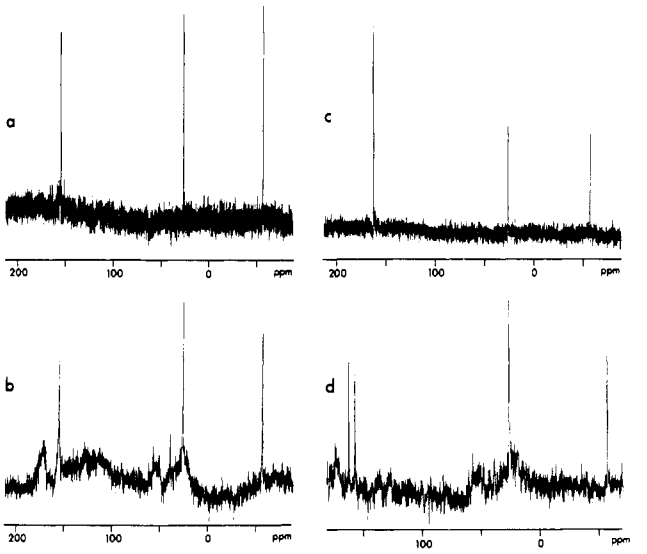


FIGURE 3: ^{13}C NMR spectra of [2-^{13}C]pteridines at 50.31 MHz. (a) Spectrum of [2-^{13}C]folic acid at pH* 5.35 (2170 transients). (b) Spectrum of [2-^{13}C]folic acid in the presence of dihydrofolate reductase at pH* 6.00 (15 800 transients). (c) Spectrum of [2-^{13}C]MTX at pH* 7.45 (2140 transients). (d) Spectrum of [2-^{13}C]MTX in the presence of dihydrofolate reductase at pH* 7.65 (10 360 transients). In each spectrum the two upfield peaks are from the external standards CH_2I_2 (furthest upfield) and cyclooctane. Other details are given under Experimental Procedures.

Table I: Chemical Shifts for C-2 in [2-^{13}C]Pteridines[a]

MTX	aminopterin	folate	condition[b]
162.50	162.50	156.38	phosphate buffer, pH* ~7.6 (<1 mM)[c]
162.57	162.59	164.32	phosphate buffer, pH* ~10 (<1 mM)[c]
162.56	162.72		Me_2SO-d_6 (44 mM)[c]
162.9		164.5	3 N NaOH (0.1–0.3 M)[d]
163.5		157.4	Me_2SO-d_6 (0.1–0.3 M)[d]
		154.8	phosphate buffer, pD 7.9 (0.1 M)[e]
161.83		154.85	phosphate buffer, pD 8.0 (0.1 M)[f]

[a] Chemical shift values are given in ppm with reference to tetramethylsilane. Literature values reported with respect to another reference material have been recalculated. [b] Solvent, concentration in parentheses, reference. [c] This work. [d] Ewers et al. (1973). [e] Lyon et al. (1973). [f] Lyon et al. (1975).

pH Dependence of Chemical Shifts of MTX and Aminopterin. The pH dependence of the chemical shift for C-2 of MTX and aminopterin is shown in Figure 4. In the pH range studied (1.5–10.5) one dissociation is observed corresponding to a pK of 5.73 ± 0.02 for MTX and 5.70 ± 0.03 for aminopterin. Poe (1973) obtained a value of 5.71 ± 0.02 for the pK of MTX and assigned it to protonation of N-1 in the pteridine ring. It is evident from Figure 4 that, as might be expected, the presence of a methyl group at N-10 does not influence the chemical shift of C-2 or the pK of N-1. Since the behaviors of MTX and aminopterin were identical in this study, attention will be directed to MTX in what follows, but similar remarks apply to aminopterin also.

The downfield movement of the C-2 resonance of aminopterin and MTX that occurs when these pteridines are deprotonated is closely analogous to the behavior of C adjacent to N in other heterocycles (Pugmire & Grant, 1968).

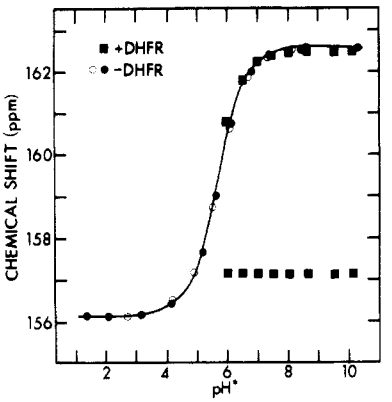


FIGURE 4: Dependence on pH* of ^{13}C chemical shifts of 2-^{13}C-labeled inhibitors. The chemical shift of the C-2 resonance (at various pH* values) for MTX (●), aminopterin (○), and MTX in the presence of dihydrofolate reductase and $NADPH_4$ (□). The pK values were computed as indicated under Experimental Procedures.

Downfield movements (in parts per million) of the ^{13}C resonance on deprotonation of an adjacent nitrogen are as follows: MTX, 6.31; pyridine, 7.78; pyrimidine (C-2), 7.28.

When NMR spectra were recorded for [2-^{13}C]MTX or [2-^{13}C]aminopterin in the presence of DHFR (mole ratio of MTX to DHFR, 2:1), two resonances were observed (Figure 3). The background in these spectra contains other peaks, many of them very broad, due to natural abundance ^{13}C resonances of the protein. Such spectra were recorded at pH values between 6 and 10. Observations could not be extended below pH 6 because of the greatly decreased solubility of the enzyme as the pH decreased below 6. Of the two MTX resonances seen in all these spectra, the more downfield corresponds to unbound MTX since, at all pH values employed, it has a chemical shift identical with that recorded for MTX in the absence of enzyme (Figure 4). This identification was confirmed by the observation that when MTX and DHFR were present in a 3:1 ratio, additional intensity appeared in the downfield peak. Furthermore, as the pH increased from 7 to 10 the intensity of the downfield peak increased and that of the upfield peak decreased due to increasing dissociation of the MTX–DHFR complex with increasing pH. The upfield MTX resonance, which did not shift with pH (Figure 4), is assigned to enzyme-bound MTX. Addition of 2 equiv of $NADPH_4$ (relative to DHFR) to give the ternary enzyme–MTX–$NADPH_4$ complex did not cause any change in the NMR spectrum at any pH value.

pH Dependence of Chemical Shift of Folate. NMR spectra obtained for folate are shown in Figure 3. In the absence of enzyme two dissociations were detected (Figure 5), one with pK = 2.40 ± 0.02 and the other with pK = 8.25 ± 0.05. These correspond to values of 2.35 given for the pK of N-1 (Poe, 1977) and 8.38 for the N_3–C_4O amide group (Poe, 1973).

When the chemical shift of the folate was measured at various pH values in the range 6.0–9.3 in the presence of DHFR, exactly the same spectrum was obtained as in the absence of DHFR (Figure 5), but the line width was greater in the presence of the enzyme. Furthermore, the formation of the ternary complex by the addition of $NADPH_4$ was also without influence on the chemical shift of folate.

Discussion

Enrichment of ^{13}C at the 2 position of the pteridine ring provided a very sensitive method of detecting dissociation of protons from either N-1 or the amide (N_3–C_4) group. Changes in chemical shift with dissociation of a proton ranged from 2 to almost 10 ppm. Since the chemical shift can be estimated

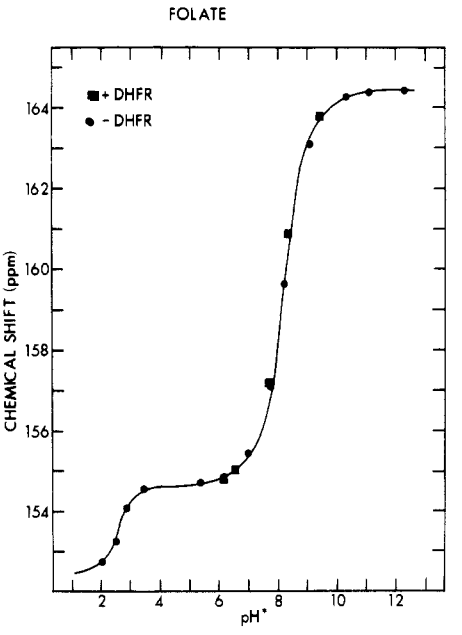


FIGURE 5: Dependence on pH* of ^{13}C chemical shift of folate. The chemical shift of the C-2 resonance (at various pH values) of free [2-^{13}C]folate (●) and [2-^{13}C]folate in the presence of dihydrofolate reductase and $NADPH_4$ (■).

and is reproducible to <0.05 ppm, dissociation curves can be plotted with great accuracy. In general the pK values obtained agreed well with estimates obtained by other methods (Poe, 1973, 1977).

Behavior of MTX–DHFR Complex. The ^{13}C NMR spectrum for enzyme-bound MTX showed two resonances, one of which had a chemical shift varying with pH in the same way as the chemical shift of free MTX. This resonance therefore arises from free MTX in slow exchange with the MTX–DHFR complex. Since the dissociation constant for the MTX–DHFR binary complex is very low (Gleisner & Blakley, 1975; Williams et al., 1979), exchange slow enough to produce a distinct, sharp resonance for free MTX is to be expected.

The second resonance, which does not have a chemical shift at the same position as free MTX, evidently corresponds to MTX bound to the enzyme. The remarkable and entirely unanticipated characteristic of this resonance is that its chemical shift does not change over the accessible pH range, pH 6–10. Given the sensitivity of the chemical shift of C-2 to proton dissociation from the pteridine ring, this clearly indicates that over the pH range 6–10, there is no change in the state of protonation of the pteridine ring of MTX when the latter is bound to *S. faecium* DHFR.

This observation can be explained in two ways. (1) The enzyme-complexed MTX remains protonated throughout the pH range examined, and the small chemical shift difference (0.93 ppm) relative to the free, protonated MTX reflects either contributions of other nearby groups on the enzyme or the hydrogen-bonding interaction between the N-1 proton and the carboxyl of Asp-27, or both. The latter interaction would decrease the deshielding effect at C-2 produced by the charge at N-1. (2) Alternatively, the enzyme-complexed MTX remains unprotonated throughout the pH range studied. It is then necessary to explain first the lower pK of bound MTX (compared with free MTX) and also the large upfield shift difference (−5.44 ppm) relative to the free, unprotonated MTX. Such shifts can, in principle, be interpreted on the basis of ring current contributions resulting from nearby aromatic residues or on the basis of electric field contributions arising

from nearby charged residues. The magnitude of such an effect could not be explained on the basis of ring currents alone [cf. Perkins & Dwek (1980)]. Charged groups can produce shifts of comparable magnitude in ethylenic and acetylenic unsaturated systems (Seidman & Maciel, 1977), and, presumably, they might occur in systems such as the pteridine ring of MTX. In this case an electric field contribution would presumably arise from the negatively charged carboxyl of Asp-27. However, it is impossible to predict accurately the result of this electric field contribution at C-2. Appropriate theoretical calculations have not been developed for effects of electric fields on nuclei in aromatic or heterocyclic systems, and, in addition, the exact location of the active-site carboxyl of *S. faecium* DHFR with respect to bound MTX is unknown. It is therefore doubtful whether a large upfield shift would be caused by the effect of the electric field from the active-site carboxyl on C-2 in bound, unprotonated MTX. Finally, in view of the evidence from difference spectroscopy, Raman spectroscopy, and calorimetry indicating that at neutral pH MTX is protonated when bound to DHFR from various sources, the second alternative can be conclusively eliminated.

If the preceding argument is accepted, the absence of any change in the chemical shift over the pH range 6–10 indicates that the pK for N-1 must be at least 10.7, that is, increased 5 pH units above the value for the MTX. Changes of pK for protein side chains by 5 pH units are highly unusual but not unknown (Lipscomb, 1978). Typically, when such changes occur, they are brought about by the presence of a nearby oppositely charged group, especially in a hydrophobic environment from which water is partially excluded. This is exactly the situation in the hydrophobic cavity of DHFR, from which water is largely excluded, especially when NADPH is also bound in the cavity (Matthews et al., 1978).

This result is in contrast to the results of the spectroscopic study by Hood & Roberts (1978), from which the pK for MTX bound to DHFR of *L. casei* was estimated as 8.55 ± 0.01. It is possible that the apparent discrepancy between our results and those of Hood and Roberts arises from differences in the structure of DHFR from the two bacteria, but this seems unlikely because of the similarity in the binding of MTX to DHFR from *E. coli* and from *L. casei* (Matthews et al., 1978). The homology between DHFR from *L. casei* and *S. faecium* is at least as great as that between *L. casei* and *E. coli* (Blakley, 1981). At present the discrepancy remains unresolved, therefore, and is being further investigated.

Behavior of MTX–NADPH$_4$–DHFR Complex. Since it was desirable to compare ternary complexes of inhibitors with those of substrates, NADPH could not be used, and NADPH$_4$ was therefore selected as a suitable coenzyme analogue. NADH$_4$ has been shown to act as an inhibitor of several dehydrogenases and is competitive with NADH (Biellman & Jung, 1971; Dunn et al., 1975). Our results indicated that NADPH$_4$ is a linear competitive inhibitor of *S. faecium* DHFR with respect to NADPH. The K_i of 13 ± 2 μM is indicative of rather tight binding.

The chemical shift of the aminopterin and MTX C-2 in the ternary complex was found to be the same as that for the binary MTX complex. This is not surprising since Matthews et al. (1978) found evidence from their crystallographic studies that MTX is bound almost identically in the binary complex with *E. coli* DHFR and in the ternary complex with NADPH and *L. casei* DHFR.

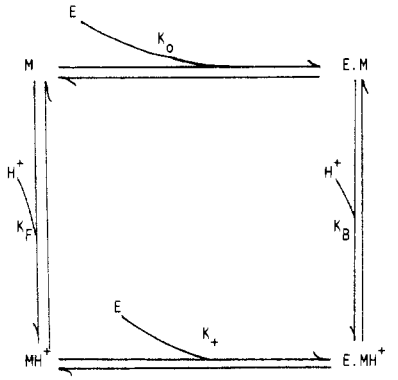
Behavior of Folate–DHFR Complex. In the presence of DHFR there is no change in the chemical shift of C-2 of folate. This is not due to the failure of folate to form a complex with the enzyme. Complex formation is indicated both by the broadening of the resonance in the presence of the enzyme and by the demonstration that the dissociation constant of the complex is ~5 μM over the pH range 6–7.5 (L. Cocco and R. L. Blakley, unpublished results). From this value and the concentration of folate (1 mM) and of DHFR (0.54 mM), it can be calculated that 54% of the total folate present was bound to DHFR in the NMR experiments, and at higher pH the percent bound was probably still sufficient to detect any change in chemical shift. The absence of any change in chemical shift for C-2 of folate in the presence of DHFR is therefore a clear indication that N-1 of folate does not undergo an interaction with the carboxyl of Asp-27 like that occurring in the case of MTX. Whether the results also exclude the possibility that N-5 of bound folate is protonated and interacts with the carboxyl of Asp-27 is less certain. Such a conclusion is apparently consistent with calorimetric data for the binding of folate and dihydrofolate to chicken liver DHFR (Subramanian & Kaufman, 1978), which indicate that the substrates bind at neutral pH in an unprotonated form. Results of spectroscopic studies on the binding of folate to DHFR (Poe et al., 1974; Gupta et al., 1977; Hood & Roberts, 1978) and on the binding of dihydrofolate to DHFR (Erickson & Mathews, 1972; Poe et al., 1974; Gupta et al., 1977) are also consistent with lack of protonation of bound substrates. However, it is more difficult to interpret the difference spectra because absorbance is due to a mixture of bound pteridine and an excess of free pteridine and was compared with that of a fixed amount of free pteridine. Furthermore, the proportion of bound and free pteridine varies with pH.

A substantial difference in the mode of binding of folate and dihydrofolate to DHFR from that revealed by X-ray crystallography for MTX has been indicated by another line of evidence. Results of Charlton et al. (1979) taken together with the crystallographic data of Fonticella-Camps et al. (1979) have determined that in the reaction catalyzed by DHFR, hydrogen is added to the *si* face of the pteridine ring at C-7 of folate and to the same face of the ring at C-6 of dihydrofolate. A similar conclusion has been reached by Armarego et al. (1980) concerning addition of hydrogen at C-6 of 6-methyl-7,8-dihydropterin. This is the face of the ring opposite to that presented by MTX to NADPH in the crystalline complexes studied by Matthews et al. (1978).

Binding of folate to DHFR in the unprotonated form is not unexpected in view of the low pK for protonation of N-1 in free folate. The value for this pK obtained in this study was 2.40, so that at the pH of the experiments the fraction of free folate in the protonated form would be $\sim 10^{-5}$.

Contribution of Protonation of MTX to Enhanced Binding. Hood & Roberts (1978) considered binding of MTX to DHFR according to Scheme I. If this scheme is correct, they calculated from their spectroscopic data that the protonation of MTX, and the enhanced binding of the protonated species to DHFR, could only account for about one-third of the increase in the association constant for MTX binding to DHFR over that for folate. In this case the major part of the enhanced binding of MTX is difficult to explain. Most of the binding interactions between MTX and protein side chains are hydrophobic in nature, and none appears to be specific for the diaminopteridine structure, with the single exception of the N-1 interaction with the side-chain carboxyl (Matthews et al., 1978). Why then does folate bind so much less tightly, since it should be capable of the same interactions with the protein as MTX? If, however, the pK for protonated MTX is >10, as indicated by our results, so that the ratio K_F/K_B in Scheme

Scheme I: Equilibria Describing Binding of Neutral and Protonated Methotrexate to Enzyme[a]



[a] Hood & Roberts, 1978. $K_F = [M][H^+]/[MH^+]$, $K_B = [E{\cdot}M][H^+]/[E{\cdot}MH^+]$, $K_0 = [E{\cdot}M]/([E][M])$, and $K_+ = [E{\cdot}MH^+]/([E][MH^+])$ where MH^+ represents protonated methotrexate and M represents neutral methotrexate.

I is 10^5 rather than 1600 as estimated by Hood and Roberts, then K_+/K_0 is also 10^5. This means that the protonated species of MTX binds with an association constant 10^5 times greater than that for the unprotonated species. Charge interaction between the protonated pteridine ring of MTX and the aspartate carboxyl is therefore completely adequate to explain the enhanced binding of MTX as compared with folate. Our data therefore appear to substantiate Baker's original hypothesis.

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