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PREPARATION OF FOLIC ACID SPECIFICALLY LABELED WITH DEUTERIUM AT THE 3',5'-POSITIONS

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SUMMARY

A method was devised for the synthesis of 3',5'-[$^2H_2$]folic acid (d_2-folic acid) for use in studies of folate metabolism in human beings. Labeling was accomplished by catalytic dehalogenation of 3',5'-dibromofolate with deuterium gas and palladium/carbon catalyst. d_2-Folic acid was separated from reduced forms and residual 3'-monobromofolate by chromatography on DEAE-Sephadex. Analysis by proton NMR and mass spectrometry indicated 70-75% deuteration of the 3',5'-positions and lack of deuteration at other carbons.

KEY WORDS: Folate, folic acid, deuterium

INTRODUCTION

As an alternative to the use of radiochemicals in nutritional studies involving human subjects, specific labeling with stable isotopes has attracted considerable interest. We have recently developed a GC/MS method (1) suitable for evaluating urinary excretion of deuterated folates following administration of a dose of [$^2H_4$]glutamate-labeled folic acid monoglutamate (2).

Several procedures have been reported for the synthesis of stable isotopically labeled folates. Pastore (3) prepared 7-[^{2}H]dihydrofolate by dithionite reduction in deuterium oxide. Subsequent oxidation with iodine was used to form 7-[^{2}H]folate. Deuteration at carbon-6 was also achieved by enzymatic reduction (4). Because of the potential for metabolic losses of deuterium from these pteridine-labeled folates, these approaches would not be readily suitable for use in many in vivo studies. Plante et al. (5) reported the synthesis of folate labeled with carbon-13 at the benzoyl carbonyl group,

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although this singly-labeled compound would not be well suited for mass spectral quantitation of in vivo studies.

The predominant method for the labeling of folate with tritium is the catalytic dehalogenation of 3',5'-dibromofolate with tritium gas (6), which yields a product with 35-42% of the tritium at the 3',5'-positions and the remainder at other sites (i.e. carbons 7 and 9). A similar procedure has been employed for the tritiation of pteroic acid (7). The applicability of catalytic dehalogenation to the deuterium-labeling of folate has not been previously reported.

In this paper, we report the synthesis of 3',5'-[$^2H_2$]folic acid by catalytic dehalogenation using the general approach employed for tritium labeling (6,7). NMR and mass spectral data concerning the labeling of the product are also presented.

MATERIALS AND METHODS

Reagents. Folic acid, p-aminobenzoylglutamic acid and DEAE-Sephadex A-25 were obtained from Sigma Chemical Company, St. Louis, MO. Deuterium gas (99.5 atom %, Grade 2.5) was purchased from Airco Industrial Gases, Inc. (Riverton, NJ). Dihydrofolic acid (8) and 3'-monobromofolic acid (9) were prepared from folic acid for use as chromatographic standards.

Synthesis of d2-Folic Acid. 3',5'-Dibromofolic acid was prepared using the procedure of Cosulich et al. (10). The homogeneous orange solid was dried in a vacuum desiccator at ambient temperature. HPLC analysis showed a single peak with no evidence of unreacted folic acid or 3'-monobromofolic acid. In several preparations, the yield was consistently about 75%.

In a typical preparation, 750 mg of 3',5'-dibromofolic acid was dissolved in 60 ml of 0.1 M NaOH to which 75 mg 10% palladium on carbon (Kodak; Rochester, NY) was mixed. Following flushing with nitrogen, the mixture was reacted with deuterium gas (35 psi initial pressure) at ambient temperature for periods up to 66 hr. After filtration, the mixture was adjusted to pH 2 with HCl to precipitate the folate.

Aliquots were analyzed by HPLC to monitor the reaction progress in preliminary trials. The product was recovered by filtration through a sintered glass funnel and was washed with several 10-ml portions of cold water, then dried in a vacuum desiccator. Typically 220-250 mg of dry orange solid was obtained.

The solid product (typically 200 mg) was suspended in 10 ml of water and dissolved by dropwise addition of 1.0 M NaOH, followed by purification using ion exchange chromatography. The (DEAE-Sephadex A-25) column was eluted with a linear gradient of 0.05 M Na_2HPO_4 with 0.05 M NaCl (pH 8.5) and 0.05 M Na_2HPO_4 with 1.0 M NaCl (pH 8.5), followed by continued isocratic elution.

Fractions containing pure d_2-folic acid as monitored by HPLC and UV spectra were pooled and adjusted to pH 2 with HCl. The product was recovered by filtration through a fine mesh sintered glass funnel, washed with water, and dried under vacuum at ambient temperature. Typically 90-100 mg (0.20-0.23 mmol) of pure d_2-folic acid was obtained from 200 mg of the crude product.

HPLC Procedures. The concentration of individual folates in the reaction mixtures was determined by reverse phase HPLC (11) with 280 nm absorption detection.

NMR and Mass Spectral Analysis. Proton NMR analysis (300 MHz) was conducted on d_2- and unlabeled (d_0-) folic acid using 0.1 M sodium deuteroxide in deuterium oxide as a solvent (12). Three mg of each folate was dissolved in 0.6 ml of 0.1 M NaOD. One mg of sodium 3-trimethylsilyl-2,2,3,3-$^2H_4$-propionate (TSP; Wilmad Glass, Buena, NJ) was added to each sample as an internal standard.

Mass spectral analysis was performed after C9-N10 bond cleavage as described previously (1). Derivatization for GC/MS involved the formation of a pentafluoropropionyl-trifluoroethyl-lactam derivative of pABG by reaction with trifluoroethanol and pentafluoropropionic anhydride, which was a minor modification of the previously described method (1). All GC/MS data were obtained on a Finnigan 4500 GC/MS system in the electron impact ionization mode. The ion source temperature was 150°C with an electron energy of 120 eV.

RESULTS

This synthesis involved the use of catalytic debromination with deuterium gas as outlined in Figure 1. Initial studies were conducted to determine the appropriate reaction time to balance the formation of d_2-folate against the subsequent reduction to dihydrofolate. In preliminary trials involving

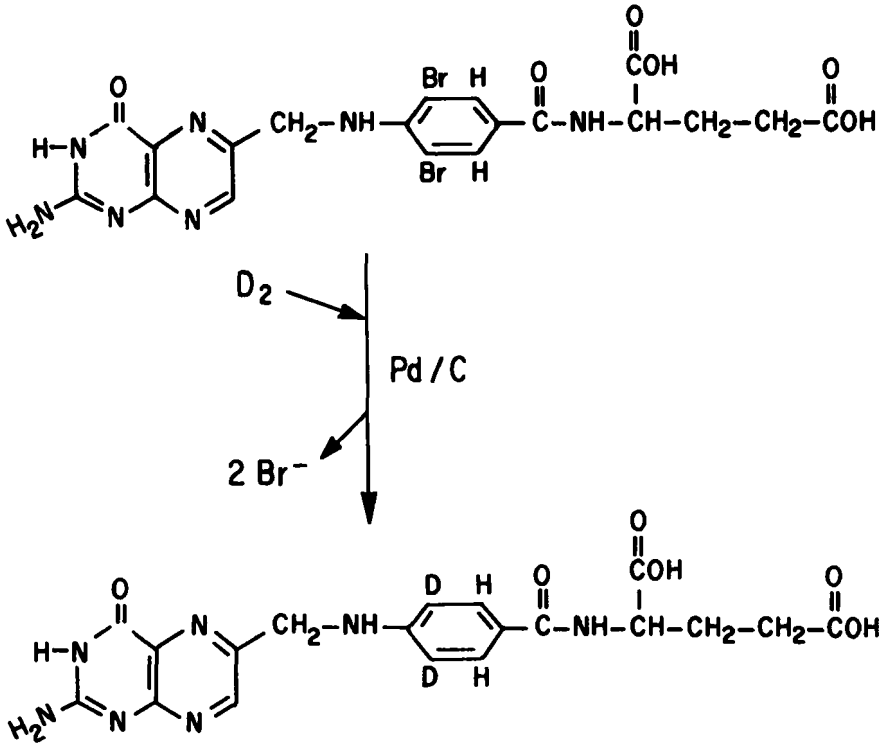


FIGURE 1. Synthesis of d_2-folic acid by catalytic debromination of 3',5'-dibromofolic acid.

catalytic debromination using hydrogen gas, complete debromination was obtained with little formation of reduced folates after only 2 hr at 35 psi gas pressure and ambient temperature. Under the same conditions except with deuterium gas, debromination was not complete after 66 hr as evidenced by remaining 3'-monobromofolate (Figure 2). A 66-hr reaction time with deuterium gas was a reasonable compromise between the debromination process and reduction to dihydrofolate.

The identity of the purified d_2-folic acid was established by HPLC, NMR, and mass spectrometry. UV spectra of d_0- and d_2-folic acid were identical

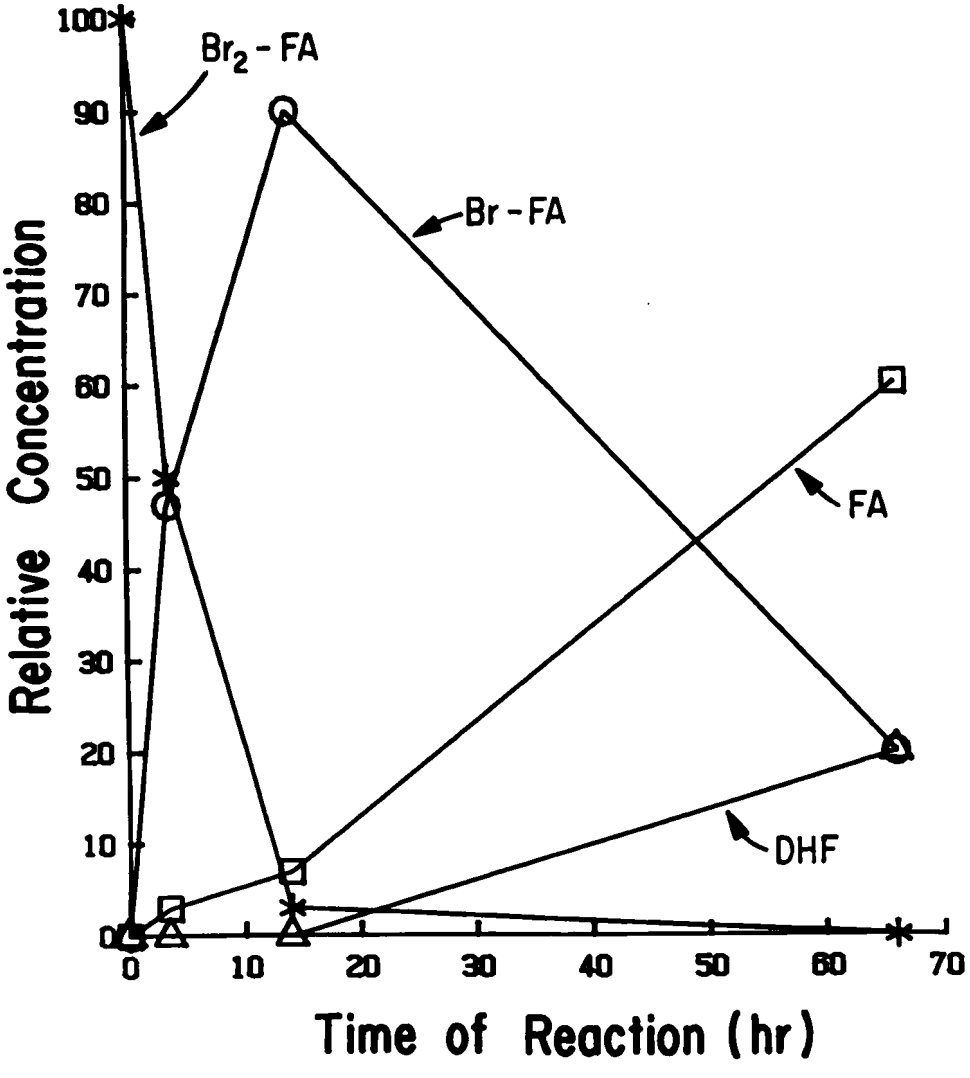


FIGURE 2. Relative concentration of folates during the catalytic reduction of 3',5'-dibromofolic acid with deuterium gas. Abbreviations: Br_2-FA, 3',5'-dibromofolate; Br-FA, 3'-monobromofolate; FA, folate; DHF, dihydrofolate.

at pH 1, 7, and 13 (not shown), with wavelength maxima and absorptivities consistent with published values (13). No impurities were found by HPLC.

Electron impact mass spectra confirmed the bideutero nature of the pABG moiety of the d_2-folic acid. A molecular ion of m/z 478 was observed for

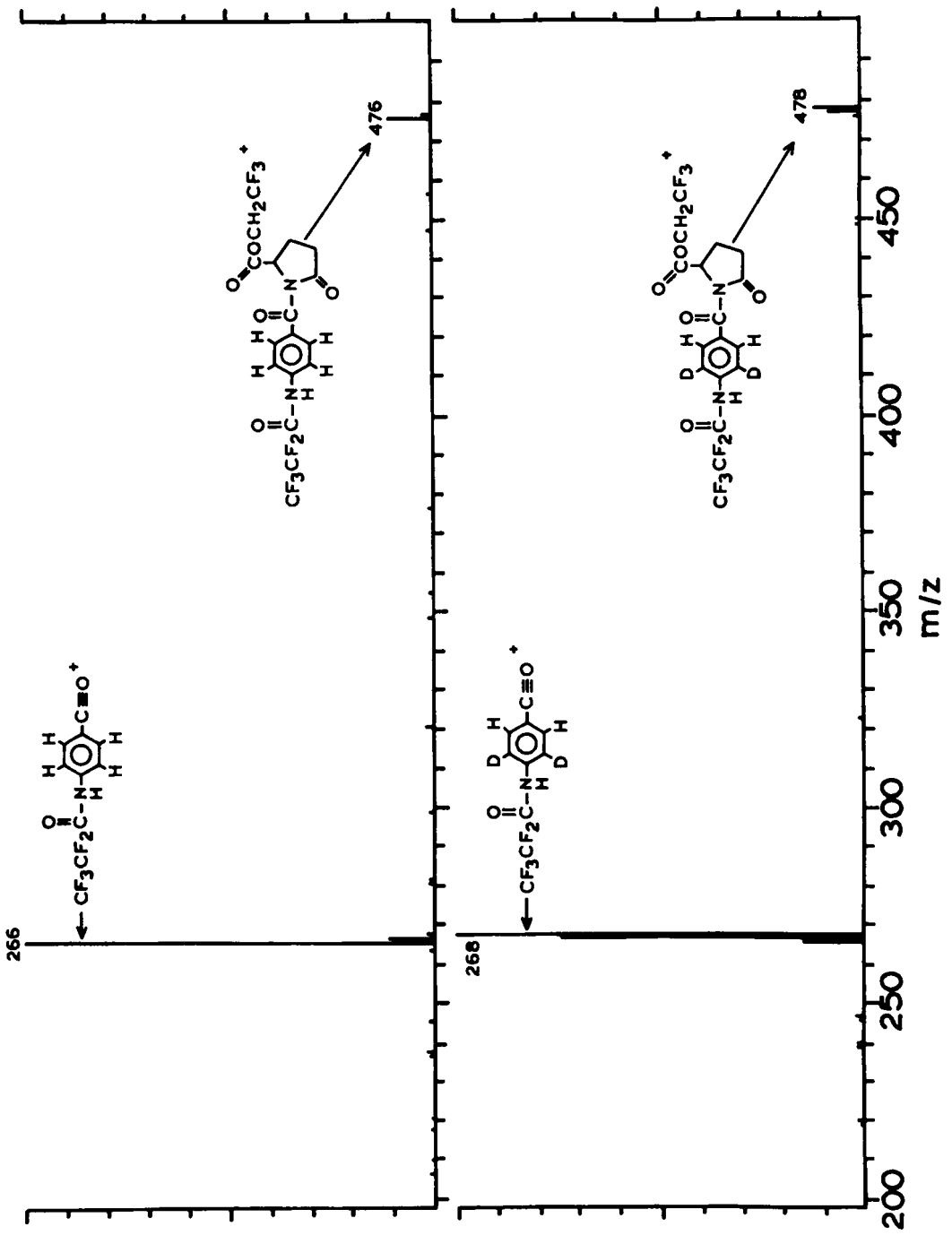


FIGURE 3. Electron impact mass spectra of: (upper) d_0-pABG derived from d_0-folic acid and (lower) d_2-pABG derived from d_2-folic acid.

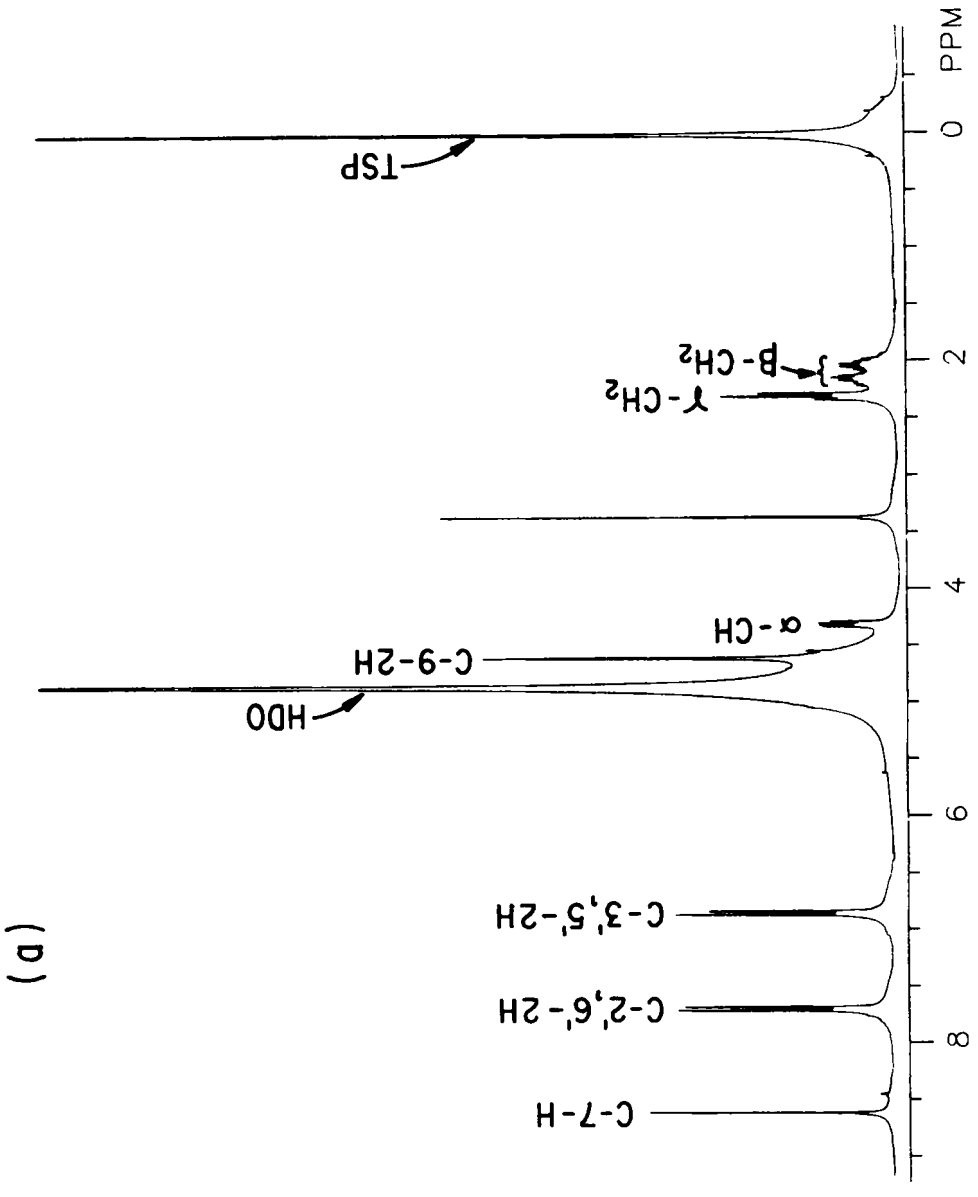
the d_2-pABG derivative in contrast to m/z 476 for the d_0-pABG derivative (Figure 3). The 2 amu difference was also seen in the signals at m/z 268 and 266 derived from the loss of the trifluoroethyllactam moiety of the derivatized pABG samples. The presence of monodeuterated folate was also apparent from the substantial signals at m/z 267 and 477. The actual composition of the d_2-pABG sample calculated these spectra was approximately 50% d_2, 41% d_1, and 9% d_0. This indicated 1.4 deuterium atoms per average d_2-folate molecule, or 70% deuteration of the 3',5'-positions.

Comparison of the proton NMR spectra of d_0- and d_2-folic acid confirmed the deuterium-labeling of the 3',5'-positions of the benzene ring (Figure 4). The observed 75% reduction in the 3',5'-proton signal of d_2-folate relative to d_0-folate is consistent with the 70% labeling determined by GC/MS. The loss of splitting of the 2',6'-proton signal of d_2-folic acid is further evidence of the deuteration of the adjacent 3',5'-position. No other qualitative or quantitative differences were observed, which indicated that deuteration of carbons at positions 7, 9, or the glutamate alpha, beta, or gamma carbons did not occur. The extraneous signal at 3.36 ppm of each spectrum was due to decomposition of the internal standard in alkaline medium, as it was detected in blank spectra run with TSP in 0.1 N NaOD but not TSP dissolved in deuterium oxide.

DISCUSSION

The synthesis of d_2-folic acid described here is a convenient procedure for the preparation of a stable-isotopically labeled folate suitable for use in metabolic studies with human subjects. Although the yield of the synthesis relative to the initial folic acid is low (ca. 15%), that is of little consequence in view of the low cost of folic acid. While the cost of deuterium gas is high, relatively small quantities are used in these reactions. The low yield of the synthesis is due mainly to incomplete debromination and partial reduction of the d_2-folate (mainly to dihydrofolate), in addition to minor losses due to incomplete resolution from dihydrofolate during purification.

Significant labeling of carbons 7 and 9 occurs during the tritium labeling of folate by catalytic dehalogenation (6), as typified by commercial


(a)
C-7-H
C-2',6'-2H
C-3',5'-2H
HDO
C-9-2H
α-CH
γ-CH2
β-CH2
TSP
8
6
4
2
0 PPM

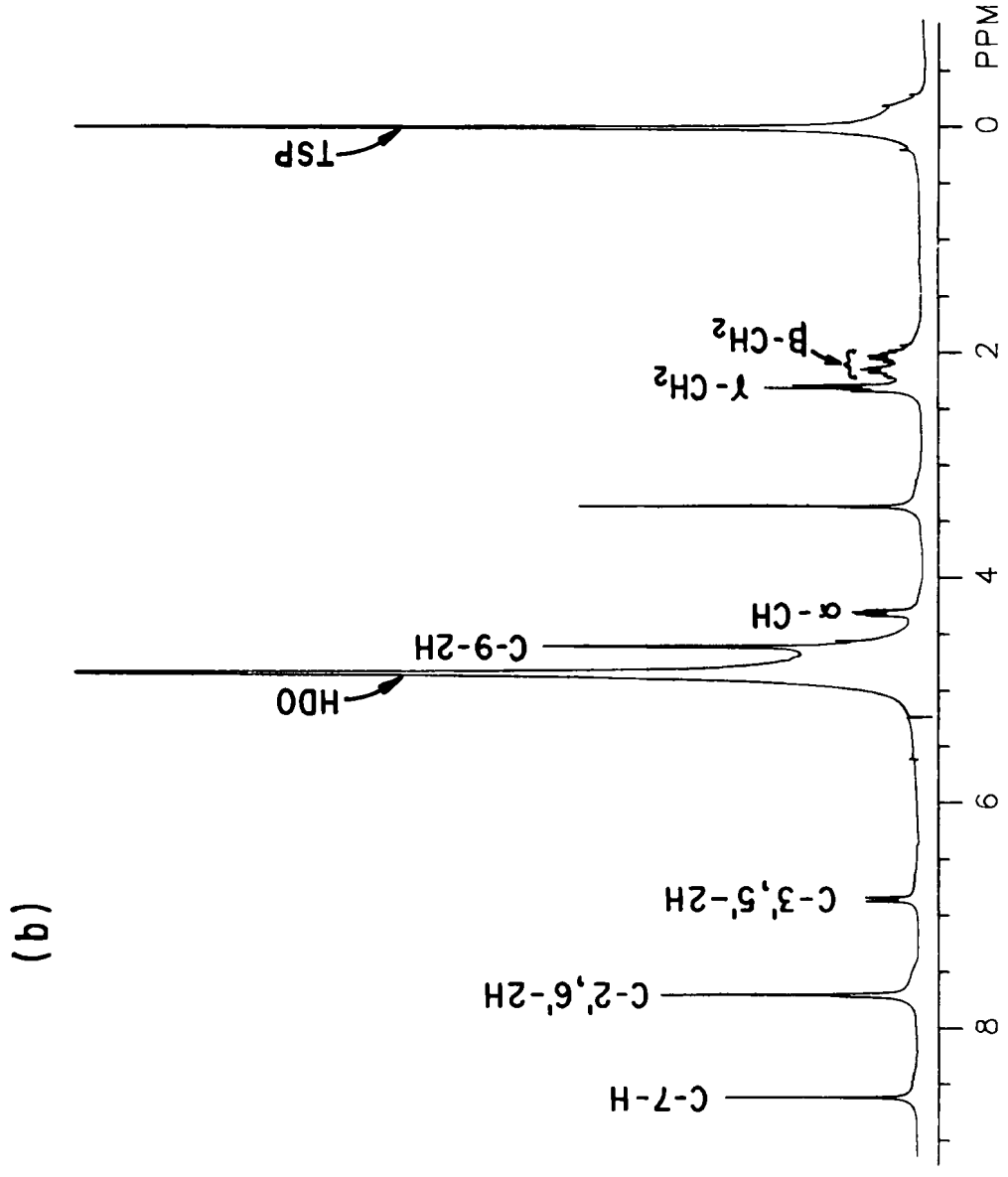


FIGURE 4. Proton NMR spectra of (a) d_0-folic acid and (b) d_2-folic acid in 0.1 M NaOD.

preparations of [^{3}H]folate exhibiting over 50% of the tritium at these positions. Unexpectedly, the deuterated folate prepared in this study exhibited no detectable labeling at positions other than 3' and 5'. Although differences in the selectivity of the catalyst may explain differences in the labeling pattern, isotopic differences in the relative rates of labeling the various positions probably also exist. The procedure described here is highly specific for the deuteration of the 3',5'-positions, in contrast to analogous tritiation procedures. While the deuteration of the 3',5'-positions was not complete, the 70-75% level of deuteration is sufficient for use of this compound for in vivo studies involving of the quantitation of excreted folates with selected-ion monitoring GC/MS (1). A trial conducted in an aprotic solvent (0.1 M NaOD in D_2O)) recently yielded essentially complete labelling.

In conclusion, this synthesis of d_2-folate provides a product that is suitable for use in in vivo studies of folate absorption and metabolism. The site of labeling at the 3',5'-position is metabolically and chemically stable, as is the glutamate labeling of the previously reported d_4-folate (2). The simultaneous use of these compounds will facilitate in vivo studies by permitting a dual-label approach that can be quantified by GC/MS analysis of urinary folates. It is also anticipated that this procedure for 3',5'-labeling will be suitable for the preparation of 3',5'-[$^2H_2$]pteroic acid as performed in a similar tritiation reaction (7), which will facilitate the synthesis of deuterated polyglutamyl folates.

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REFERENCES

1. Toth, J.P. and Gregory, J.F. Biomed. Environ. Mass Spectrom. (1988, in press).
2. Gregory, J.F. and Toth, J.P. Analyt. Biochem. (1988, in press).
3. Pastore, E.J. Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds.), Vol. 66, p. 538, Academic Press, New York, 1980.
4. Pastore, E.J. Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds.), Vol. 66, p. 541, Academic Press, New York, 1980.
5. Plante, L.T., Williamson, K.L. and Pastore, E.J. Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds.), Vol. 66, p. 533, Academic Press, New York, 1980.
6. Zakrzewski, S.F., Evans, E.A. and Phillips, R.F. Analyt. Biochem. 36, 197 (1970).
7. Godwin, H.A., Rosenberg, I.H., Ferenz, C.R., Jacobs, P.M. and Meinhofer, J. J. Biol. Chem. 247, 2266 (1972).
8. Futterman, S. J. Biol. Chem. 228, 1031 (1957).
9. Angier, R.B. and Curran, W.V. J. Am. Chem. Soc. 81, 2814 (1959).
10. Cosulich, D.B., Seeger, D.R., Fahrenbach, M.J., Roth, B., Mowat, J.H., Smith, J.M. and Hultquist, M.E. J. Am. Chem. Soc. 73, 2554 (1951).
11. Gregory, J.F., Sartain, D.B. and Day, B.P.F. J. Nutr. 114, 341 (1984).
12. Poe, M. Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds.), Vol. 66, p. 483, Academic Press, New York, 1980.
13. Blakley, R.L. The Biochemistry of Folic Acid and Related Pteridines, pp. 219-235, North-Holand, Amsterdam, 1969.