ratings were taken 4 weeks after application. Plants were kept at good growing conditions at a temperature regime of 24 °C/18 °C (day/night).

RESULTS

As results of preemergence applications indicate (Table I), compound **1a** very effectively controlled grassy weeds at a rate of 150 g of AI/ha. **1a** is very well tolerated by broadleaf crops like rapeseed, beets, soybean, or cotton at high rates of 600 g of AI/ha. Broadleaf crops were not controlled at all. Graminaceous crops proved to be very susceptible even at low dosage rates of 150 g of AI/ha. Herbicidal activity of compound **1a** is superior to that of sethoxydim and comparable to that of fluazifop-butyl.

Following postemergence application (Table II) compound **1a** showed excellent activity against annual and perennial grassy weeds at a rate of 150 g of AI/ha. Broadleaf crops demonstrated high tolerance to the herbicide, while graminaceous crops were rather susceptible. Broadleaf weeds were not controlled. In comparison with commercial herbicides **7** and **8**, compound **1a** showed superior herbicidal activity particularly with respect to perennial grassy weeds.

In summary, compound **1a** may be considered a very effective herbicide that can be used to selectively control a broad range of annual and perennial grassy weeds in broadleaf crops, such as sugarbeet, soybean, or cotton.

LITERATURE CITED

Handte, R.; Bieringer, H.; Hoerlein, G.; Schwerdtle, F. *Proc. Brit. Crop Protect. Conf.* **1982a**, 19.

Handte, R.; Bieringer, H.; Hoerlein, G.; Langelueddeke, P. *Proc. Brit. Crop Protect. Conf.* **1982b**, 11.

Johnston, H.; Troxell, L. (Dow Chemical Co.). EP 483, 1977; *Chem. Abstr.* **1979**, *90*, 203882v.

Kenyon, J.; Phillips, H.; Turley, H. *J. Chem. Soc.* **1924**, 399.

Koch, V.; Fuss, A. (Hoechst AG). EP-A 227047, 1985; *Chem. Abstr.* **1987**, *107*, 175893y.

Koch, V.; Fuss, A. *Synthesis* **1990**, in press.

Koch, V.; Willms, L.; Fuss, A.; Bauer, K.; Bieringer, H.; Buerstell, H. (Hoechst AG). EP-A 227045, 1985; *Chem. Abstr.* **1987**, *107*, 175892x.

Nestler, H., Langelueddeke, P., Schoenowsky, H., Schwerdtle, F., Geissbuehler, H., Eds. *Advances in Pesticide Science 2*; Pergamon Press: Oxford, 1978; p 248.

Nishiyama, R.; Haga, T.; Sakashita, N. (Ishihara Sangyo Kaisha, Ltd.). DE 2812571, 1977; *Chem. Abstr.* **1979**, *90*, 152017g.

Plowman, R.; Stonebridge, W.; Hawtree, J. *Proc. Brit. Crop Protect. Conf.* **1980**, 29.

Sakata, G.; Makino, K.; Kawamura, Y.; Ura, Y. *Proc. 10th Int. Congr. Plant Protect.* **1983**, 315.

Ura, Y.; Sakata, G.; Makino, K.; Kawamura, Y. (Nissan Chemical Industries, Ltd.). DE 3004770, 1979; *Chem. Abstr.* **1981**, *94*, 103421h.

Received for review May 23, 1989. Revised manuscript received November 17, 1989. Accepted November 29, 1989.

Improved Synthesis of [3′,5′-$^2H_2$]Folic Acid: Extent and Specificity of Deuterium Labeling†

Jesse F. Gregory III

Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida 32611-0163

As an extension of research concerning stable-isotopic labeling of folates, this report describes a modification of our previous procedure for the preparation of folic acid labeled with deuterium at the 3′,5′-positions. The major procedural changes were (a) preliminary removal of exchangeable protons from the substrate, 3′,5′-dibromofolate, and (b) the catalytic dehalogenation reaction conducted in an aprotic solvent of NaOD in D_2O. Proton NMR examination of the resulting [3′,5′-$^2H_2$]folic acid indicated complete labeling of the 3′,5′-positions. Labeling of other positions of the folate molecule was not detected.

The development of stable-isotopic methods has greatly facilitated research concerning the absorption, metabolism, and turnover of many nutrients in humans. Application of stable isotopes permits in vivo isotopic studies with no radiation hazard to human subjects and provides useful options in experimental design and analysis (Hachey et al., 1987; Bier, 1987). We have recently developed methods for the synthesis of deuterium-labeled forms of folic acid and related folates and have devised mass spectral methods for analysis of urinary folates (Gregory and Toth, 1988a,b, 1989).

To permit studies of dual-label design, methods were developed for synthesizing folates in either [3′,5′-$^2H_2$]-

† Florida Agricultural Experiment Stations Journal Series No. R-00089.

0021-8561/90/1438-1073$02.50/0

(d_2) or [$^2H_4$]glutamyl (d_4) form (Gregory and Toth, 1988a,b). While these compounds have been shown to be suitable for simultaneous in vivo use (Gregory et al., 1990), a limitation of the original preparation of [3′,5′-$^2H_2$]folate was incomplete labeling of the 3′,5′-positions. The originally published procedure, in which 3′,5′-dibromofolate was catalytically dehalogenated in 0.1 M NaOH, yielded approximately 75% labeling of the 3′,5′-positions (Gregory and Toth, 1988a). Although we observed that at least 90% labeling could be achieved with this reaction when using 0.1 M NaOD in D_2O rather than 0.1 M NaOH as the solvent (Gregory and Toth, 1989), complete labeling of the 3′,5′-positions has not been previously achieved. Alternatively, Hachey et al. (1978) reported a procedure for the preparation of [3′,5′-$^2H_2$]folic acid by trifluoroacetic acid catalyzed exchange in D_2O. This procedure yielded approximately 90% labeling of the 3′,5′-sites and, due to the slow rate of isotopic exchange, required several weeks to conduct.

Contradictory results have been obtained in research concerning the specificity of isotopic labeling obtained using catalytic dehalogenation of 3′,5′-dibromofolate. In studies of tritium labeling, Zakrzewski et al. (1970) reported that only 35–42% of labeling was at the 3′,5′-positions with the remainder of the isotope incorporated at C-7 and C-9. In later research, Evans et al. (1979) observed similar isotopic distributions although 3′,5′-labeling ranged from 42 to 73% of the total tritium. Gregory and Toth (1988a, 1989) reported that the preparation of [3′,5′-$^2H_2$]folic acid by catalytic dehalogenation yields little or no incorporation of the isotope at C-7 or C-9.

This paper describes modifications of our previous catalytic dehalogenation procedure that permits complete deuterium labeling of folic acid at the 3′,5′-positions. In addition, studies are presented concerning specificity of deuterium labeling by this method.

MATERIALS AND METHODS

Materials. Unlabeled folic acid (Sigma Chemical Co., St. Louis, MO) was converted to 3′,5′-dibromofolic acid by the method of Cosulich et al. (1951). Complete bromination was verified by reversed-phase high-performance liquid chromatography (HPLC) and UV spectrophotometry. Sodium deuteroxide (NaOD), deuterium chloride (DCl), and deuterium oxide were also obtained from Sigma; their respective isotopic enrichments were reported by the manufacturer to be 99+, 99+, and 99.8 atom %.

Synthesis of [3′,5′-$^2H_2$]Folic Acid. [$^2H_2$]Folic acid was prepared as previously described, with the following modification involving (a) preliminary treatment to remove exchangeable protons (i.e., of OH, COOH, NH, and NH_2 groups) from 3′,5′-dibromofolic acid, (b) catalytic dehalogenation of 3′,5′-dibromofolate in NaOD in D_2O, to provide an aprotic reaction medium, and (c) shorter reaction time during catalytic dehalogenation.

A 2-g portion of dry 3′,5′-dibromofolic acid was suspended in 60 mL of D_2O and then dissolved by mixing with 2.0 mL of 40% (w/w) NaOD. After approximately 5 min, the 3′,5′-dibromofolic acid was precipitated by addition of 1.6 mL of 35% (w/w) DCl, which yielded an approximately pH 2 solution by indicator paper, followed by centrifugation for 15 min at 5000g (4 °C). The pellet was suspended in 200 mL of D_2O and dissolved by addition of 2.5 mL of 40% (w/w) NaOD. The catalyst (0.25 g of 10% Pd on carbon; Kodak, Rochester, NY) was added without prior hydration, followed by bubbling with nitrogen gas to exclude dissolved oxygen. Catalytic dehalogenation was performed at room temperature (ca. 22 °C) with use of a Parr hydrogenation apparatus (Model 3911; Moline, IL) with a 500-mL reaction bottle agitated at 175 cycles/min. Deuterium gas (99.5 atom % deuterium, grade 2.5; Airco Industrial Gases, Riverton, NJ) was employed at an initial pressure of 42 psig. Deuterium gas pressure was monitored throughout the reaction period of 5–6 h.

At the end of the reaction period, the mixture was filtered under vacuum (Whatman No. 1 paper) to remove the catalyst. Crude [$^2H_2$]folic acid was recovered from the filtrate by acidification to pH 2 with HCl followed by centrifugation. [$^2H_2$]Folic acid was purified by ion-exchange chromatography on a column containing DEAE-Sephadex A-25 (35 × 2.5 cm (i.d.); Pharmacia Fine Chemicals, Piscataway, NJ) with a 2-L gradient of 0.1–0.8 M NaCl in 0.05 M sodium phosphate buffer (pH 8.5). Fractions containing pure [$^2H_2$]folic acid, as determined by HPLC, were pooled. The orange product was precipitated by acidification with HCl, recovered by centrifugation, and desiccated over P_2O_5.

This product was analyzed by HPLC, proton NMR, and microbiological assays as described below.

Analytical Procedures. HPLC was used to determine the purity of folic acid preparations essentially as described by Gregory et al. (1984) monitoring absorbance at 280 nm. Microbiological evaluation of the relative folate activity of unlabeled folic acid and [$^2H_2$]folic acid was performed with *Lactobacillus casei* as the assay organism (Phillips and Wright, 1982). The growth medium was Folate Casei Medium (Difco, Detroit, MI) adjusted to an initial pH of 6.2 (Phillips and Wright, 1982).

Proton NMR spectra were determined at 25 °C with 0.1 M NaOD in D_2O as solvent (Poe, 1980) on a 300-MHz instrument (Model NT-300; Nicolet Instrument Corp., Madison, WI). The concentration of each folate sample was 3 mg/mL.

Investigation of Nonspecific Labeling of Folic Acid. To investigate further the potential for deuterium labeling at positions other than the 3′,5′-carbons, folic acid rather than 3′,5′-dibromofolate was also carried through the above synthetic procedures. After purificiation, this product (catalytic exchange folate) was analyzed by proton NMR.

RESULTS

This research was conducted to extend methods for the preparation of deuterium-labeled folates for in vivo examination of folate absorption, metabolism, and turnover. Preliminary removal of exchangeable protons from the substrate (3′,5′-dibromofolate), along with the use of an aprotic solvent for catalytic debromination, provided [$^2H_2$]folate in 35% yield after purification. HPLC analysis of the crude product of catalytic dehalogenation indicated that the main components were folic acid and its reduction product, 7,8-dihydrofolic acid. As shown previously, optimization of the yield of [$^2H_2$]folate involves balancing the debromination process against subsequent reduction to dihydrofolate (Gregory and Toth, 1988a). The 5–6-h reaction period employed in this method represents a reasonable compromise. Dehalogenation occurred much more rapidly under the conditions of this reaction (5–6 h in NaOD) than the previous 66-h reaction in NaOH.

As with [$^2H_4$]folate and other preparations of [$^2H_2$]-folate, this form of [$^2H_2$]folate exhibited UV spectra, microbiological assay response, and HPLC behavior identical with those of unlabeled folic acid (data not shown). Microbiological dose–response curves for deuterium-labeled and unlabeled folic acid were superimposable, which indicated full biological activity of the labeled compound.

NMR analysis provided a direct indication of the extent of deuterium labeling of the 3′,5′-positions and other sites within the molecule (Figure 1). The spectrum of [$^2H_2$]-folic acid, in which no signal was detected for 3′,5′-protons, indicates complete deuterium labeling of these positions. Replotting the [$^2H_2$]folate spectrum at approximately 10-fold greater sensitivity also yielded no significant 3′,5′-proton signal. Further evidence of 3′,5′-labeling of [$^2H_2$]folate is found in the 2′,6′-proton signal, which exists as a doublet for unlabeled folate but collapses to a singlet for [$^2H_2$]folate in the absence of 3′,5′-protons.

The relative intensity of the various proton signals was evaluated to determine whether proton–deuterium

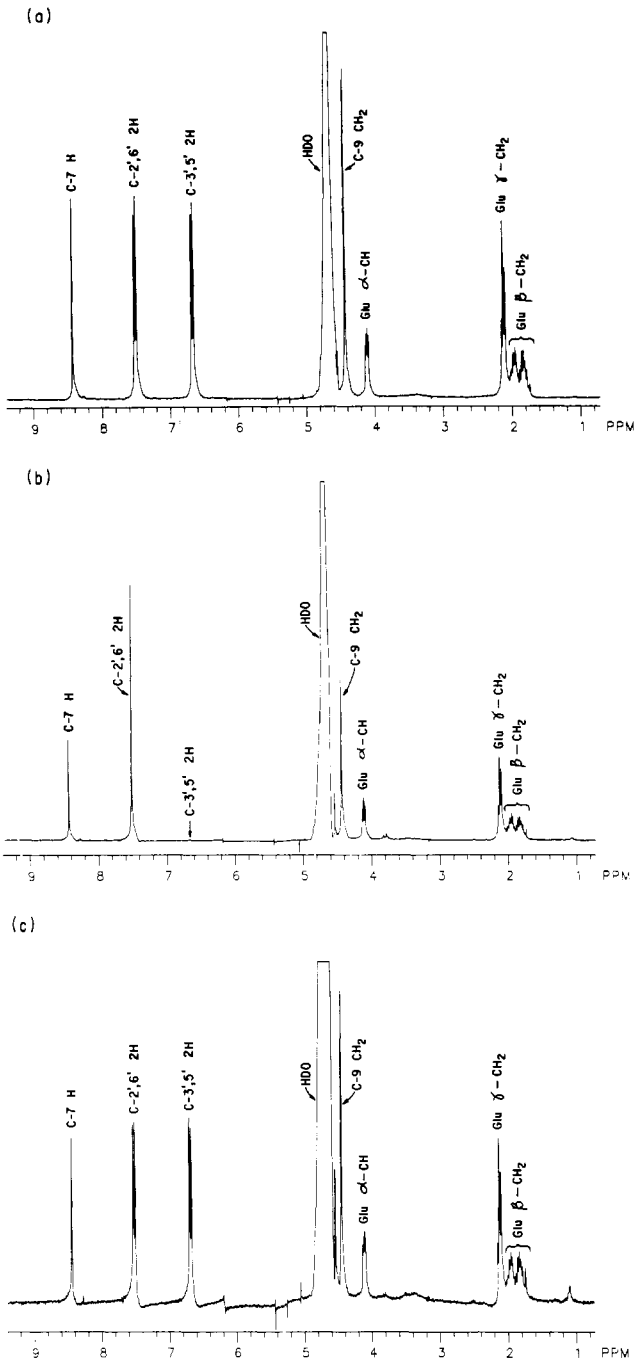

Figure 1. Proton NMR spectra of (a) unlabeled folic acid, (b) [$^2H_2$]folic acid, and (c) folic acid subjected to catalytic exchange (catalytic exchange folate).

exchange had occurred. When compared to unlabeled folic acid, [$^2H_2$]folate and the catalytic exchange folate preparations exhibited no quantitative spectral differences with respect to signals other than 3′,5′- and 2′,6′-positions. It should be recognized that minor labeling may not be readily detectable by examination of the disappearance of proton signals. However, these results clearly indicate the absence of significant labeling by proton–deuterium exchange under the conditions of the catalytic dehalogenation reaction. In addition, the intensity of 3′,5′-signal of the catalytic exchange folate preparation indicates that deuterium labeling of this position does not occur by simple isotopic exchange under the conditions of this synthesis.

The time course of the catalytic dehalogenation reaction was examined by monitoring changes in deuterium

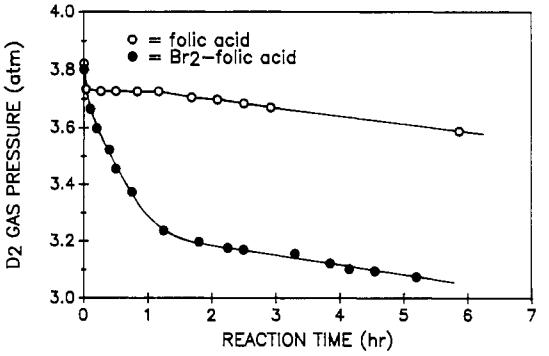

Figure 2. Consumption of deuterium gas during catalytic exchange of folic acid (catalytic exchange folate preparation) and catalytic dehalogenation of 3′,5′-dibromofolic acid.

gas pressure (Figure 2). When folic acid was employed in the catalytic exchange folate preparation, slow consumption of deuterium occurred over the reaction period. This presumably corresponded to formation of dihydrofolic acid, which was the major byproduct detected by HPLC. In contrast, 3′,5′-dibromofolic acid yielded a rapid uptake in deuterium gas over the first 1–1.5 h, followed by a slower consumption parallel to that observed in the catalytic exchange folate. Estimation based on the equation of state for an ideal gas ($PV = nRT$) indicated 2.2 mol of D_2 gas consumed/mol of dibromofolate within 90 min. This suggests that catalytic dehalogenation as well as reduction of the pteridine ring system was occurring during the initial stages of reaction. Subsequent gas consumption presumably involved continued reduction of the pteridine moiety to form dihydrofolate and potentially tetrahydrofolate.

DISCUSSION

The procedure reported here is a significant improvement in stable-isotopic methods because it permits the synthesis of [3′,5′-$^2H_2$]folic acid with complete labeling of the 3′,5′-positions and no detectable labeling elsewhere on the folate molecule. Other advantages of this method, compared to the previous procedure (Gregory and Toth, 1988a), include a shorter reaction time than previously observed (6 vs 66 h) and a greater yield (35% vs 15–20%). The factors responsible for the markedly increased rate of the reaction are unclear in view of the fact that the catalyst used in each study was from the same lot. The comparatively low yields in these methods are partially due to formation of dihydrofolate and incomplete separation of the [$^2H_2$]folate from dihydrofolate during preparative ion-exchange chromatography. In view of the inexpensive nature of folic acid and the small quantity of deuterium gas employed, this yield is acceptable.

Potential racemization of the glutamyl α-carbon is of concern when alkaline conditions are employed in synthetic reactions. The NMR signals for the α-protons do not exhibit any significant difference, which is consistent with the retention of the chirality of this carbon. The results of the microbiological assay, which responds only to pteroyl-L-glutamates, further support the stereochemical purity of the [$^2H_2$]folate product.

The NMR spectral data of this study confirm the high degree of specificity of labeling of the 3′,5′-positions, which is in contrast to the distribution of tritium obtained under analogous reaction conditions (Zakrzewski et al., 1970; Evans et al., 1979). Although Evans et al. (1979) observed that the extent of tritium labeling of C-7 and C-9 varied

markedly among batches of palladium/$CaCO_3$ catalyst employed, consistent labeling of these positions was observed. The NMR spectra reported here (Figure 1) for [$^2H_2$]folic acid and the catalytic exchange folate confirm previous observations that little or no labeling of C-7, C-9, or other positions occurs by deuterium–proton exchange under the conditions of this reaction. Whether the difference in labeling specificity between tritium and deuterium reflects a true isotopic effect or a difference in the nature of the catalysts employed is unclear.

In summary, this method of synthesis represents a significant improvement in the preparation of [$^2H_2$]folic acid for in vivo use with human subjects. This form of [$^2H_2$]-folic acid is also suitable as a substrate in chemical and/or enzymatic synthesis of other [$^2H_2$]folate compounds having a comparably high degree of deuterium labeling.

ACKNOWLEDGMENT

This study was supported by Grant 88-37200-3620 from the Competitive Research Grants Office, U.S. Department of Agriculture. I thank Drs. Sandra Bonetti and John Gander, Department of Microbiology and Cell Science, University of Florida, for assistance in the acquisition of NMR spectra.

LITERATURE CITED

Bier, D. M. The use of stable isotopes in metabolic investigation. Bailliere's Clin. *Endocrinol. Metab.* **1987**, *1*, 817.

Cosulich, D. B.; Seeger, D. R.; Fahrenbach, M. J.; Roth, B.; Mowat, J. H.; Smith, J. M.; Hultquist, M. E. Analogs of pteroylglutamic acid. VI. 3′,5′-Diholopteroyl derivatives. *J. Am. Chem. Soc.* **1951**, *73*, 2554.

Evans, E. A.; Kitcher, J. P.; Warrell, D. C.; Elvidge, J. A.; Jones, J. R.; Lenk, R. Tritium nuclear magnetic resonance spectroscopy. Part 12. Patterns of labelling in tritiated folic acid and methotrexate. *J. Labelled Compd. Radiopharm.* **1979**, *16*, 697.

Gregory, J. F.; Toth, J. P. Preparation of folic acid specifically labeled with deuterium at the 3′,5′-positions. *J. Labelled Compd. Radiopharm.* **1988a**, *25*, 1349.

Gregory, J. F.; Toth, J. P. Chemical synthesis of deuterated folate monoglutamate and in vivo assessment of urinary excretion of deuterated folates in man. *Anal. Biochem.* **1988b**, *170*, 94.

Gregory, J. F.; Toth, J. P. Stable-isotopic methods for in vivo investigation of folate absorption and metabolism. In *Evaluation of Folic Acid Metabolism in Nutrition and Disease*; Picciano, M. F., Stokstad, E. L. R., Gregory, J. F., Eds.; Alan R. Liss: New York, 1989; in press.

Gregory, J. F.; Sartain, D. B.; Day, B. P. F. Fluorometric determination of folacin in biological materials using high performance liquid chromatography. *J. Nutr.* **1984**, *114*, 341.

Gregory, J. F.; Bailey, L. B.; Toth, J. P.; Cerda, J. J. Stable isotopic methods for assessment of folate bioavailability. *Am. J. Clin. Nutr.* **1990**, *51*, 212.

Hachey, D. L.; Palladino, L.; Blair, J. A.; Rosenberg, I. H.; Klein, P. D. Preparation of pteroylglutamic acid-3′,5′-$^2H_2$ by acid catalyzed exchange with deuterium oxide. *J. Labelled Compd. Radiopharm.* **1978**, *14*, 479.

Hachey, D. L.; Wong, W. W.; Boutton, T. W.; Klein, P. D. Isotope ratio measurements in nutrition and biomedical research. *Mass Spectrom. Rev.* **1987**, *6*, 289.

Phillips, D. R.; Wright, A. J. A. Studies on the response of *Lactobacillus casei* to different folate monoglutamates. *Br. J. Nutr.* **1982**, *47*, 183.

Poe, M. PMR characteristics of folic acid and analogs. *Methods Enzymol.* **1980**, *66*, 483.

Zakrzewski, S. F.; Evans, E. A.; Phillips, R. F. On the specificity of labeling in tritiated folic acid. *Anal. Biochem.* **1970**, *36*, 197.

Received for review August 17, 1989. Accepted November 20, 1989.