Hydroxylated Derivatives of 5-Methyl-5,6,7,8-tetrahydrofolate*

G. R. Gapski,† J. M. Whiteley, and F. M. Huennekens‡

ABSTRACT: Oxidation of 5-methyl-5,6,7,8-tetrahydrofolate (I) with H_2O_2 at pH 6 produces, *via* 5-methyldihydrofolate (II) as an intermediate, a yellow product (III) which can be isolated either as the crystalline acid or as the barium salt. At pH 7, III exhibits λ_{max} at 278 mμ with a shoulder at 350 mμ. That no major portion of I has been lost during its conversion into III is indicated by elemental analyses and the proton magnetic resonance spectra. When I labeled with ^3H at C-6 is converted into III, there is essentially full retention of the label. The atom per cent excess of ^{18}O in III when $[^{18}O]H_2O_2$ is used demonstrates that III contains one more oxygen atom than I. The proton magnetic resonance spectrum of (III) indicates the presence of a methylene group at C-7 and a single proton at C-6. Reduction of III by borohydride or by catalytic hydrogenation (1 mole of H_2/mole of III) yields another new compound (IV) which, at pH 7, has a λ_{max} at 297 mμ and is characterized by a blue fluorescence. Structures are proposed for III and IV.

Oxidation of 5-methyltetrahydrofolate (I) by O_2 (Donaldson and Keresztesy, 1962), or by H_2O_2 in the presence of peroxidase (Gupta and Huennekens, 1967), yields a methyldihydrofolate (II) that has been postulated to be 5-methyl-5,6-dihydrofolate (Donaldson and Keresztesy, 1962; Larrabee *et al.*, 1963; Scrimgeour and Vitols, 1966). In the H_2O_2-dependent reaction, another product, separable from II by chromatography on DEAE-cellulose, was also observed (Gupta and Huennekens, 1967). This second oxidation product, designated hereafter as III, had a distinct yellow color due to a weak absorption band in the 350- to 400-mμ region. The present investigation was undertaken to determine the structure of III.

In the previous study (Gupta and Huennekens, 1967), treatment of I with H_2O_2 and peroxidase at pH 8 produced III and II in a ratio of about 1:5. By modifying the conditions (increasing the concentration of H_2O_2, prolonging the reaction time, and lowering the pH to 6), it has now been found that III can be increased at the expense of II. In addition, peroxidase has been omitted, since it does not appear to affect the course of the reaction. Under these modified conditions, both I and II are converted in good yield into III, which suggests that the course of the overall reaction is I → II → III.

Guided by these preliminary experiments, III has been synthesized on a larger scale, purified by chromatography on DEAE-cellulose, and isolated as either the crystalline free acid or the barium salt. The absorption spectra of III at various pH values are shown in Figure 1 and its nonidentity with I and II is verified by paper and thin-layer chromatography (Table I). Elemental analyses provided molecular formulas of $C_{20}H_{25}N_7O_7$ (mol wt 475.4) and $C_{20}H_{23}N_7O_7Ba \cdot 2H_2O$ (mol wt 646.7) for the free acid and the barium salt, respectively. These data indicated that no major portion of I, whose molecular formula is $C_{20}H_{25}N_7O_6 \cdot 2H_2O$ (Gupta and Huennekens, 1967), had been lost during its transformation to III. This conclusion was strengthened by the identification of *p*-aminobenzoylglutamate as a product resulting from alkaline permanganate degradation of III, by the retention of the ^{14}C label when I-5-$^{14}CH_3$ was converted into III, and by the proton magnetic resonance spectrum of III.

Previous studies (Scrimgeour and Vitols, 1966) with appropriately labeled compounds had shown that, when I is oxidized to II, hydrogen is lost from C-7, but not from C-6. It was of interest, therefore, to determine whether the latter hydrogen is still retained after the transformation of I (or II) to III. Accordingly, I was synthesized with ^3H at C-6 and, to provide an internal marker, additionally labeled with ^{14}C in the 5-methyl group. When this doubly labeled I was converted into III, both labels were present in the latter, although the apparent specific activity was about 10% lower than in the precursor, I. However, the fact that *both* specific activities decreased by the same amount suggested that this was probably due to uncertainties in the extinction coefficients and/or purity of I and III and that, in fact, no loss in label had occurred.

From the molecular formulas given above, it appeared as if III contained an additional atom of oxygen (relative to I). It was desirable, however, to obtain more direct evidence on this point. Therefore, I was oxidized to III with $[^{18}O]H_2O_2$ and the atom per cent excess of ^{18}O in the product indicated that one oxygen had been incorporated. Although treatment of pteridines with 30% H_2O_2 in formic acid has been reported to give the 5-*N*-oxides (Pfleiderer and Hutzenlaub, 1965), the additional oxygen in III was most probably in the form of a hydroxyl group, rather than as an *N*-oxide. The *N*-oxide structure was excluded by the inability of III to be converted into I by catalytic hydrogenation (see below), and by the absence of HCHO when III was subjected to a procedure that oxidatively demethylates *N*-methyl tertiary amine *N*-oxides (Ferris *et al.*, 1968).

Although II is reduced to I by mercaptoethanol (Donaldson and Keresztesy, 1962), III is resistant to this reductant. It can be reduced, however, by borohydride or by catalytic hydrogenation; in the latter process, 1 mole of H_2 was consumed per mole of III reduced. The reduced form of III is a colorless material IV, whose spectra are shown in Figure 2. Unlike I and II, IV is unaffected by treatment with H_2O_2. Compound

* From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received March 10, 1971.* This work was supported by grants from the National Cancer Institute, National Institutes of Health (CA 6522 and CA 11778), and a contract with the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health (PH-43-65-14).

† Present address: Terra-Marine Bioresearch, P.O. Box 2208, La Jolla, Calif. 92037.

‡ To whom to address correspondence.

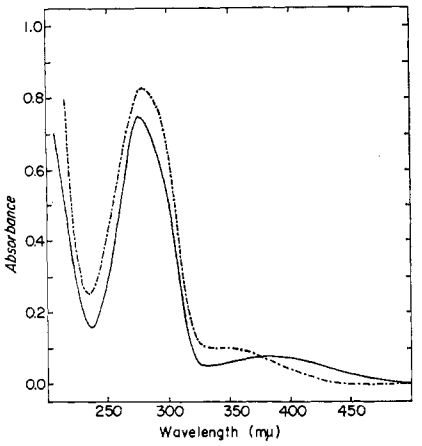


FIGURE 1: Absorption spectra of III. (———) pH 1, 0.1 M HCl; (—·—) pH 7, 0.1 M phosphate and pH 13, 0.1 M NaOH. Concentration of III was 16.6 mg/l. at pH 1 and 22.2 mg/l. at pH 7 and 13.

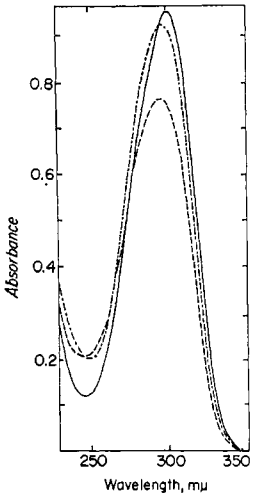


FIGURE 2: Absorption spectra of IV. (———) pH 1, 0.1 M HCl; (—·—) pH 7, 0.1 M phosphate; (— — —) pH 13, 0.1 M NaOH. Concentration of IV was 24.6 mg/l. at pH 1 and 7 and 18.4 mg/l. at pH 13.

IV is further distinguished from I, II, and III by its chromatographic behavior and blue fluorescence (*cf.* Table I), and by its analytical values, which are consistent with a molecular formula of $C_{20}H_{27}N_7O_7 \cdot 1.5H_2O$.

The structures of III and IV were further delineated by comparing their proton magnetic resonance spectra to those of the reference compounds, (I) and *p*-aminobenzoylglutamate (Figure 3). Retention of the *p*-aminobenzoylglutamate moiety in III is apparent from the aromatic protons at 7.79 and 6.96 ppm and the glutamate α, γ, and β protons at 4.94, 2.74, and 2.50 ppm, respectively. Compound III also shows a three-proton singlet at 3.37 ppm due to the *N*-methyl group. Since this signal occurs at 3.05 ppm in I, it would seem that the 5-methyl group is subjected to a different perturbing effect in the two compounds. The two-proton signal at 3.94 ppm in III is attributed to the C-9 methylene group, since free rotation would permit both protons to experience a similar environment. The broad, weak signal at 4.10 ppm is due to the C-6 proton. The two related doublets centered at 4.30 and 4.80 ppm each show a coupling constant of 14 Hz. In a spin-decoupling experiment, irradiation at a resonance frequency

TABLE I: Chromatography of 5-Methyltetrahydrofolate and Related Compounds.

Compound	R_F Value: Thin Layer[a]	R_F Value: Paper[b]	Appearance under Ultraviolet Light
5-Methyltetrahydrofolate (I)	0.23	0.70	Quenching
5-Methyldihydrofolate (II)	0.50	0.89	Quenching
III	0.61	0.88	Quenching
IV	0.64	0.77	Blue fluorescent
p-Aminobenzoylglutamate	0.47	0.90	Quenching

[a] 0.1 M phosphate buffer, pH 7. [b] 1 M NaCl.

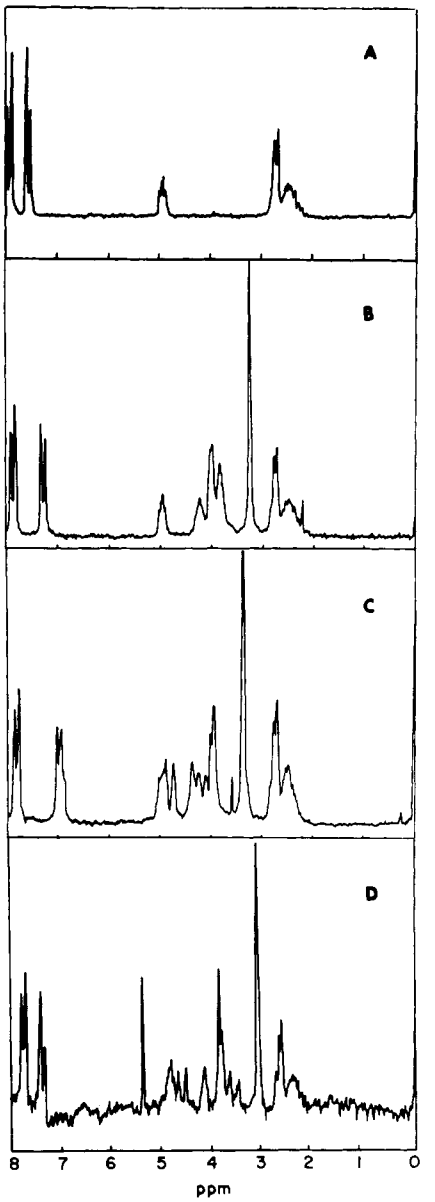


FIGURE 3: Proton magnetic resonance spectra of *p*-aminobenzoylglutamate (panel A), (I) (panel B), (III) (panel C), and (IV) (panel D) in CF_3COOD. Tetramethylsilane was the internal standard.

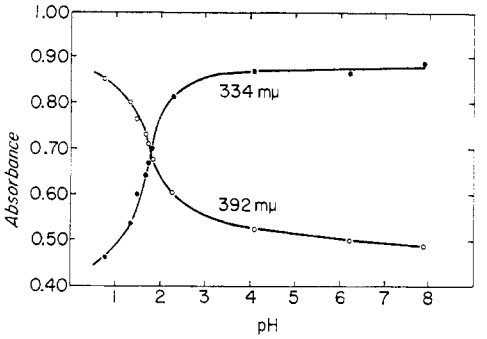
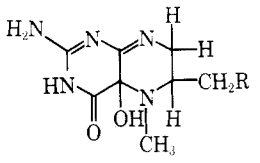


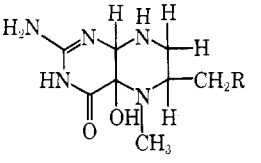
FIGURE 4: Effect of pH upon absorbance of III at 392 and 334 mμ. Absorbances of solutions of III (0.2 mg/ml) at 392 and 334 mμ were determined at the indicated pH values.

corresponding to 4.80 ppm caused the 4.30-ppm doublet to collapse to a singlet. Likewise, irradiation at a frequency corresponding to 4.30 ppm produced the same effect upon the 4.80-ppm doublet. This verified the existence of an AB system and, from the magnitude of the coupling constant, geminal rather than vicinal protons were indicated. These observations are consistent with the presence of a methylene group at C-7 in III.

The proton magnetic resonance spectrum of IV shows a pattern similar to that of III with respect to the aromatic and glutamate protons, the *N*-methyl group, the C-9 protons and the C-6 proton. The doublets at 4.63 and 3.60 ppm were also shown by spin decoupling to be part of an AB system, *viz.*, the C-7 protons. The only additional feature in the spectrum of IV is the one-proton singlet at 5.43 ppm, which is attributed to a proton at C-8a. Pteridine structures characterized by a proton at C-8a have been suggested previously by Viscontini and Okada (1967). Thus, based upon the proton magnetic resonance spectra, the analytical data, and the ^{18}O-, ^{14}C-, and ^{3}H-labeling experiments, the following structures (in which R = *p*-aminobenzoylglutamate) are postulated for III and IV.



III



IV

The above structure explains the following anomalies in the absorption spectrum of III. (a) The 278-mμ absorption band of III has an unusually low extinction coefficient at pH 7 (ϵ 17.7 $\times$ 10^3 M^{-1} cm^{-1}) compared to the ϵ_{290} values of I and II which are 31.7 and 31.2 $\times$ 10^3 M^{-1} cm^{-1}, respectively (Gupta and Huennekens, 1967). The pteridine portion apparently contributes little to the spectrum of III since *N*-methyl-*p*-aminobenzoylglutamate itself has an ϵ_{291} value of 15.0 $\times$ 10^3 M^{-1} cm^{-1} (Fu *et al.*, 1965) and (b) As shown in Figure 4, spectrophotometric titration of III reveals a pK_a of 1.8, due probably to protonation at N-1 or at the 2-amino group. In contrast, both I and tetrahydrofolate show a spectrally determined pK_a near 5 which has been attributed to protonation at N-5. These observations are consistent with the proposed structure of III, since protonation of N-5 (which is not part of the chromophoric system) would not be expected to change the absorption spectrum.

Some preliminary studies have been carried out to determine whether III or IV have any biological activity. Compound I, a naturally occurring folate coenzyme, supports the growth of *Lactobacillus casei*, *Streptococcus faecalis*, or *Pediococcus pentasaceus*, and is the substrate for the methionine synthetase from *Escherichia coli* K-12, but III and IV are devoid of activity in each of these systems.[1,2] However, these compounds may serve as useful models for hydroxylated pteridines that have been considered as possible intermediates in the enzymatic conversion of phenylalanine into tyrosine (Kaufman *et al.*, 1970; Viscontini, 1970).

Experimental Section

Materials. Chemicals were obtained from the following sources: folic acid (Calbiochem), platinum oxide (Engelhard Industries), 2-ethylanthraquinone (Aldrich Chemical Co.), 30% H_2O_2 (Matheson Coleman & Bell), [^{3}H]$NaBH_4$ (sp act. 102 mCi/mmole) (Nuclear-Chicago), [^{14}C]HCHO (sp act. 12 mCi/mmole) (New England Nuclear Corp.), oxygen gas (containing 53.72% $^{18}O_2$ and 0.267% $^{17}O_2$) (Miles Laboratories, Inc.), Bio-Solv BBS-3 (Beckman Instruments), butyl-PBD (Packard Instrument Co.), and DEAE-cellulose (Whatman DE22) and cellulose (Whatman CF11) (W & R Balston, Ltd.).

7,8-Dihydrofolic acid was prepared according to the procedure of Blakley (1960). 5-Methyldihydrofolic acid was synthesized by the method of Gupta and Huennekens (1967), or by oxidation of 5-methyltetrahydrofolic acid with ferricyanide by the procedure described below. Tetrahydrofolic acid was prepared by the procedure of Hatefi *et al.* (1960), except that hydrogenation was performed in a Parr apparatus at 35 psi for 6 hr at room temperature.

5-Methyltetrahydrofolate was synthesized according to Gupta and Huennekens (1967), except that reduction with borohydride was allowed to proceed for 90 min at room temperature. The product was purified by chromatography on DEAE-cellulose, lyophilized, partially dissolved in a small amount of 0.02 M mercaptoethanol, and lyophilized again to remove residual ammonium acetate which causes the material to be hygroscopic.

[6-^{3}H]Tetrahydrofolate was prepared by reducing 7,8-dihydrofolate with a mixture of KBH_4 and [^{3}H]$NaBH_4$ and isolating the product by chromatography on DEAE-cellulose (Scrimgeour and Vitols, 1966). [6-^{3}H]Tetrahydrofolate was treated with [^{14}C]HCHO and KBH_4 and the resulting product, [5-$^{14}CH_3$,6-^{3}H]5-methyltetrahydrofolate, was isolated by the procedure of Gupta and Huennekens (1967).

Methods. Absorption spectra were obtained with a Cary Model 14 recording spectrophotometer. Proton magnetic resonance spectra were determined on a Jeol instrument, Model JNM-PS-100 (courtesy of Dr. J. Rivier, Salk Institute for Biological Studies).

Column chromatography was performed in a cold room (*ca.* 5°). Ascending paper chromatography was carried out on Whatman No. 1 paper using (unless otherwise indicated) 0.1 M phosphate buffer (pH 7) as the solvent system. Fluorescent or quenching components were visualized under ultraviolet light in a Chromato-vue. PABG[3] was detected by spraying the paper with *p*-dimethylaminobenzaldehyde using the procedure of Wollish *et al.* (1961). Thin-layer chromatography was carried out on Baker-flex cellulose PEI-F (J. T. Baker Chemi-

[1] Unpublished results of Dr. N. Harding.
[2] Unpublished results of Dr. J. Galivan.
[3] Abbreviation used is: PABG, *p*-aminobenzoylglutamate.

cal Co.) using (unless otherwise indicated) 1 M NaCl as the solvent system.

Microanalyses were performed by the Spang Microanalytical Laboratories. ^{18}O analyses, according to the method of Rittenberg and Ponticorvo (1956), were performed by West Coast Technical Services, Inc. Radioactivity was determined with a Beckman liquid scintillation counter; the scintillation fluid consisted of 5 g of butyl-PBD and 100 ml of Bio-Solv BBS-3 diluted to 1 l. with toluene.

Synthesis of III from 5-Methyltetrahydrofolate. A pH 6.0 solution of 5-methyltetrahydrofolate (1.5 g) in 50 ml of 0.1 M ammonium acetate was added to a mixture of 50 ml of 0.1 M ammonium acetate and 5.0 ml of 30% H_2O_2; the pH of the latter mixture had also been adjusted to 6.0 using 1 N acetic acid. After the reaction had been allowed to proceed for 1 hr at room temperature, the solution was lyophilized. The residue was dissolved in 25 ml of cold water and chromatographed on a 2.4 × 44 cm column of DEAE-cellulose using 500 ml of water followed by 0.1 M ammonium acetate for elution. The first liter of the ammonium acetate effluent was discarded; thereafter, 19-ml fractions were collected automatically. Fractions exhibiting λ_{max} at 282 mμ (tubes 6–65) were combined and lyophilized. The yellow solid was dissolved in 50 ml of methanol and the solution was refluxed for 16 hr. (This procedure converts an unidentified minor contaminant into a product which elutes before III in the subsequent chromatographic step.) After removal of methanol, the yellow residue was dissolved in 20 ml of water and the pH was adjusted to 7.5 with 1 M NH_4OH. The solution was chromatographed on a 3.8 × 50 cm column of DEAE-cellulose. The column was eluted with 1 l. of water and the effluent was discarded. Elution was continued with 0.1 M ammonium acetate and 20-ml fractions were collected automatically. The contents of tubes 70–90 (λ_{max} at 270 mμ) were discarded. Fractions 162–227 (λ_{max} at 278 mμ) were pooled and lyophilized to yield 424 mg of III. The product was crystallized from methanol and dried over P_2O_5 at 100° for 6 hr *in vacuo*, mp 287° with decomposition; R_F 0.88 (quenching). At pH 1, λ_{max} 275 and 392 mμ (ϵ 21.3 × 10^3 and 2.13 × 10^3 M^{-1} cm^{-1}), λ_{min} 236 and 334 mμ; at pH 7, λ_{max} 278 mμ (ϵ 17.8 × 10^3 M^{-1} cm^{-1}), λ_{min} 235 mμ; at pH 13, λ_{max} 278 mμ (ϵ 18.0 × 10^3 M^{-1} cm^{-1}), λ_{min} 235 mμ. *Anal.* Calcd for $C_{20}H_{25}N_7O_7$: C, 50.52; H, 5.30; N, 20.62. Found: C, 50.18; H, 4.88; N, 20.41.

The barium salt of III was prepared as follows. Compound III (52 mg) was dissolved in 10 ml of water and treated with 0.5 ml of 0.5 M $BaCl_2$. The dropwise addition of 25 ml of methanol with stirring produced a fine precipitate which was collected on a Millipore filter. The barium salt was dissolved in 1 ml of water and reprecipitated with 25 ml of methanol. The product was dried at 100° for 6 hr over P_2O_5 *in vacuo* (40 mg). *Anal.* Calcd. for $C_{20}H_{23}N_7O_7Ba \cdot 2H_2O$: C, 37.10; H, 4.21; N, 15.14. Found: C, 37.66; H, 3.71; N, 14.85.

Synthesis of 5-Methyldihydrofolate from 5-Methyltetrahydrofolate. 5-Methyltetrahydrofolate (50 mg) was dissolved in 10 ml of deaerated 0.5 M phosphate buffer (pH 5.6). Potassium ferricyanide (95 mg) was added and the reaction allowed to proceed at room temperature for 30 min under argon. The solution was diluted to 100 ml with cold water and chromatographed on a 1.8 × 33 cm column of DEAE-cellulose. Elution was initiated with 250 ml of 0.1 M ammonium acetate and continued with a gradient (0.1–0.5 M, 750 ml each) of the same salt. 5-Methyldihydrofolate (λ_{max} at 290 and 248 mμ) emerged between 650 and 800 ml in the elution profile. These fractions were pooled and lyophilized: yield 26 mg; 56 μmoles (78%).

Synthesis of III from 5-Methyldihydrofolate. 5-Methyldihydrofolate (69 mg) was dissolved in 25 ml of 0.1 M ammonium acetate containing 0.5 M H_2O_2. The pH was adjusted, if necessary, to 4.7 and the solution was allowed to stand at room temperature for 1 hr. After lyophilization, the yellow residue was dissolved in 25 ml of cold water and chromatographed on a 2.8 × 28 cm column of DEAE-cellulose, as described in a previous section: yield 41 mg.

Quantitative Hydrogenation of III. PtO_2 (205 mg) suspended in 25 ml of water was reduced in a quantitative hydrogenation apparatus. Compound III (204 mg, 0.36 mmole) was dissolved in 1.0 ml of water and introduced into the flask by means of a syringe through a self-seal rubber stopper. Hydrogenation was carried out at 1 atm. The initial, rapid uptake of H_2 was essentially complete after 60 min (8.4 ml corresponding to the consumption of 1 mole of hydrogen/mole of III. The catalyst was removed by filtration, and the colorless filtrate was lyophilized. The product had an R_F of 0.77 (blue fluorescence) and λ_{max} at 297 mμ (pH 7).

Synthesis of IV

A. CATALYTIC HYDROGENATION OF III. Compound III (1.82 g) was dissolved in 100 ml of water and, after addition of PtO_2 (250 mg), the mixture was hydrogenated at 35 psi for 6 hr at room temperature in a Parr apparatus. After removal of the catalyst by filtration, the solution was lyophilized. The white residue (at pH 7, λ_{max} 297 mμ) was dissolved in 25 ml of water and chromatographed on a 3.8 × 45 cm column of DEAE-cellulose. The column was eluted with 0.1 M ammonium acetate and 15-ml fractions were collected automatically. Fractions exhibiting λ_{max} at 297 mμ and λ_{min} at 252 mμ (tubes 110–215) were pooled and lyophilized.

The white residue (984 mg) was dissolved in 10 ml of water, diluted with 10 ml of 95% ethanol, and the resulting solution chromatographed on a 3.8 × 47 cm column of cellulose that had been washed previously with 2 l. of 95% ethanol. The column was eluted with 1.8 l. of 95% ethanol and the eluate discarded. Gradient elution (95% ethanol–water, 1 l. each) was then applied and 15-ml fractions were collected automatically. Fractions exhibiting λ_{max} at 297 mμ (tubes 40–60) were pooled and taken to dryness *in vacuo* at 30°: yield 540 mg; R_F 0.77 (blue fluorescence). The product was crystallized from methanol. *Anal.* Calcd for $C_{20}H_{27}N_7O_7 \cdot 1.5H_2O$: C, 47.61; H, 5.99; N, 19.44. Found: C, 47.39; H, 5.53; N, 19.42. At pH 1, λ_{max} 302 mμ (ϵ 18.7 × 10^3 M^{-1} cm^{-1}), λ_{min} 246 mμ; at pH 7, λ_{max} 297 mμ (ϵ 19.0 × 10^3 M^{-1} cm^{-1}), λ_{min} 250 mμ; and at pH 13, λ_{max} 296 mμ (ϵ 21.0 × 10^3 M^{-1} cm^{-1}), λ_{min} 247 mμ.

B. REDUCTION OF III WITH BOROHYDRIDE. Compound III (474 mg) was dissolved in 100 ml of water and the pH was adjusted to 7.2 with 0.1 N NaOH. Three 100-mg portions of KBH_4 were added with stirring at 10-min intervals. The reaction was allowed to proceed for 30 min after the last addition of KBH_4, and the pH of the solution was adjusted to 7.0 with 5 N acetic acid. The solution was diluted to 500 ml with cold water and chromatographed on a 2.8 × 30 cm column of DEAE-cellulose, as described above; yield 284 mg.

Degradation of III and IV to PABG. Compound III (50 mg) was added to 10 ml of 0.1 N NaOH containing 50 mg of $KMnO_4$, and the mixture was heated at 60° for 2 hr. Ethanol (1 ml) was added and the dense brown precipitate was removed by filtration through Celite. The pH of the filtrate was adjusted to 5.0 with 1 N HCl and the solution was lyophilized. The residue was extracted with 20 ml of absolute ethanol, and

the ethanol was evaporated to recover PABG (R_F 0.80 with butanol–acetic acid water (5:2:3); at pH 7, λ_{max} 268 mμ; yield, 26%, based upon ϵ 15.8 × 10^3 M^{-1} cm^{-1} (Kallen and Jencks, 1966)). When this procedure was repeated with IV, the yield of PABG was 14%. In a control experiment in which 50 mg of PABG was subjected to this procedure, only 4% was recovered.

Attempted Demethylation of III. Compound III (200 mg) and $FeSO_4 \cdot 7H_2O$ (462 mg) were dissolved in 25 ml of deaerated 0.5 N H_2SO_4. The solution was refluxed for 7 hr under argon, cooled, and added to 25 ml of a solution prepared by dissolving 0.66 g of 2,4-dinitrophenylhydrazine in 3.5 ml of concentrated H_2SO_4, adding 5.5 ml of water, and diluting the solution to 175 ml with 2 N HCl. A small amount of residue, obtained by filtration, was extracted with $CHCl_3$. Thin-layer chromatography on silica gel S-coated sheets (Macherey-Nagel & Co.) with the use of $CHCl_3$–CCl_4 (1:1) failed to reveal any 2,4-dinitrophenylhydrazone of HCHO (R_F 0.36).

Oxidation of [5-^{14}C,6-^{3}H]5-Methyltetrahydrofolate to III. Doubly labeled 5-methyltetrahydrofolate (54 mg, sp act. 1.52 × 10^5 dpm/μmole for ^{3}H and 2.54 × 10^4 dpm/μmole for ^{14}C, based upon ϵ_{290} 31.7 × 10^3 M^{-1} cm^{-1}) was oxidized with 0.5 M H_2O_2 and the resulting product III was purified by chromatography on DEAE-cellulose, as described above. Compound III had a specific activity of 1.33 × 10^5 dpm/μmole for ^{3}H and 2.24 × 10^4 dpm/μmole for ^{14}C, based upon ϵ_{278} 17.8 × 10^3 M^{-1} cm^{-1}.

Synthesis of [^{18}O]H_2O_2. The following procedure was adapted from a commercial synthesis of H_2O_2 (Cech, 1952). 2-Ethylanthraquinone (4.0 g) was reduced by $NaBH_4$ according to the procedure of Panson and Weill (1957), and the resulting hydroquinone was dried over P_2O_5 *in vacuo*. A two-neck, round-bottom flask was fitted with two connecting tubes–one with a stopcock for evacuating the flask and another leading to a 200-ml bulb of gaseous oxygen that contained 53.72% $^{18}O_2$. A magnetic stirring bar, 50 ml of benzene and 1.0 ml of 1.0 M phosphate buffer (pH 6.0) were added to the flask and the contents were frozen. After introduction of 2-ethylanthrahydroquinone (4.0 g), the flask was evacuated, flushed with argon several times, and evacuated again. The contents were allowed to thaw, and the oxygen in the bulb was released into the system *via* the break seal. The reaction mixture was stirred for 1.5 hr. After the flask was opened, the aqueous phase was immediately removed and filtered, and its volume was adjusted to 1.0 ml with the pH 6.0 buffer. Titration of an 0.05-ml aliquot with potassium permanganate showed that the H_2O_2 concentration was 1.7 M.

Synthesis of III Using [^{18}O]H_2O_2. To 0.9 ml of the [^{18}O]H_2O_2 (1.7 M, 53.7 atom % ^{18}O) was added 0.17 mmole of I. After remaining at room temperature for 2 hr, the solution was lyophilized. The product, III, was isolated by chromatography on DEAE-cellulose according to the procedure described above. Compound III was further purified by paper chromatography on Whatman No. 3MM paper (previously washed with water) with methanol–water (4:1) as the solvent system. The yellow material at R_F 0.80 was eluted with water and the solution was lyophilized. Analysis of the residue gave values of 5.687, 0.070, and 94.243 atom % for ^{18}O, ^{17}O, and ^{16}O, respectively.

Acknowledgments

The authors are indebted to Dr. V. S. Gupta for his contributions to the early phases of this work and to Mrs. Wendy Fong for expert assistance.

References

Blakley, R. L. (1960), *Nature (London) 188*, 231.
Cech, J. (1952), *Chem. Abstr. 46*, 3226*f*.
Donaldson, K. O., and Keresztesy, J. C. (1962), *J. Biol. Chem. 237*, 3815.
Ferris, J. P., Gerwe, R. D., and Gapski, G. R. (1968), *J. Org. Chem. 33*, 3493.
Fu, S. C. J., Renier, M., and Loo, T. L. (1965), *J. Org. Chem. 30*, 1277.
Gupta, V. S. and Huennekens, F. M. (1967), *Arch. Biochem. Biophys. 120*, 712.
Hatefi, Y., Talbert, P. T., Osborn, M. J. and Huennekens, F. M. (1960), *Biochem. Prepn. 7*, 89.
Kallen, R. G., and Jencks, W. P. (1966), *J. Biol. Chem. 241*, 5845.
Kaufman, S., Storm, C. B., and Fisher, D. B. (1970), *in* Chemistry and Biology of Pteridines, Iwai, K., Akino, M., Gato, M., and Iwanami, Y., Ed., Tokyo, International Academic Printing Co., Ltd., p 209.
Larrabee, A. R., Rosenthal, S., Cathou, R. E., and Buchanan, J. M. (1963), *J. Biol. Chem. 238*, 1925.
Panson, G. S., and Weill, C. E. (1957), *J. Org. Chem. 22*, 120.
Pfleiderer, W., and Hutzenlaub, W. (1965), *Angew. Chem., Int. Ed. Engl. 4*, 1075.
Rittenberg, D., and Ponticorvo, L. (1956), *Intern. J. Appl. Radiat. Isotopes 1*, 208.
Scrimgeour, K. G., and Vitols, K. S. (1966), *Biochemistry 5*, 1438.
Viscontini, M. (1970), *in* Chemistry and Biology of Pteridines, Iwai, K., Akino, M., Goto, M., and Iwanami, Y., Ed., Tokyo, International Academic Printing Co., Ltd., p 217.
Viscontini, M., and Okada, T. (1967), *Helv. Chim. Acta 50*, 1492.
Wollish, E. G., Schmall, M., and Hawrylyshyn, M. (1961), *Anal. Chem. 33*, 1138.