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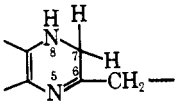
The Reduction of Folate by Borohydride*

K. G. Scrimgeour and Karin Smith Vitols†

ABSTRACT: After reduction of folate by borohydride, the products have been examined by chromatography on columns of DEAE-cellulose. In addition to unreacted folate, the only compounds observed under a variety of conditions were tetrahydrofolate and dihydrofolate; the latter product could be obtained in yields as high as 30%. Contrary to our previous results, dihydrofolate produced by this method appeared to be identical with the material resulting from the reduction of folate with hydrosulfite. Experiments with tritium-labeled borohydride provided evidence that the hydride ion adds first to carbon atom 7 of the pyrazine ring during reduction of folate to dihydrofolate, and then to carbon atom 6 during reduction of dihydrofolate to tetrahydrofolate. In this manner, dihydrofolate-7-[^{3}H] and tetrahydrofolate-6-[^{3}H], -7-[^{3}H], and -6,7-[^{3}H] have been prepared. Labeled tetrahydrofolates have been used for the preparation of 5-methyltetrahydrofolate-6-[^{3}H] and -7-[^{3}H]. When 5-methyltetrahydrofolate-7-[^{3}H] was oxidized with O_2 or with H_2O_2 in the presence of peroxidase, the 5-methyldihydro product had a much lower specific activity than its precursor. Conversely, oxidation of 5-methyltetrahydrofolate-6-[^{3}H] was accompanied by no loss of label. Thus, when 5-methyltetrahydrofolate is oxidized under these conditions, the product appears to be 5-methyl-5,6-dihydrofolate.

The pyrazine ring of folic acid can be reduced by treatment with hydrosulfite or borohydride and by catalytic hydrogenation. In each instance, the specific reaction conditions employed determine whether the end product is dihydrofolate or tetrahydrofolate. Thus, when folate is treated with hydrosulfite at room temperature, dihydrofolate is obtained (Futterman, 1957; Blakley, 1960), but if the reaction is carried out at 75°, tetrahydrofolate is the main product (Silverman and Noronha, 1961). Similarly, dihydrofolate and tetrahydrofolate, respectively, are obtained when folate is hydrogenated over platinum oxide in alkaline or acidic solutions (O'Dell *et al.*, 1947; Hatefi *et al.*, 1960). Mathews and Huennekens (1963) demonstrated that borohydride readily reduced dihydrofolate, but not folate. By modifying the original reaction conditions of Mathews and Huennekens (1963), we were able to achieve a reaction of folate with borohydride (Smith *et al.*, 1963). A product, isolated by precipitation at pH 2.5, was postulated to be an isomer of dihydrofolate not identical with the hydrosulfite-produced material. Hillcoat and Blakley (1964), however, found that reduction of folate by borohydride yielded primarily tetrahydrofolate along with a small amount (*ca.* 3%) of dihydrofolate. The latter material was indistinguishable by high voltage paper electrophoresis from the product of hydrosulfite reduction. Evidence has been presented by Pastore *et al.* (1963) and by Hillcoat and Blakley (1964) that hydrosulfite-reduced dihydrofolate is the 7,8-isomer (I).



I

We have now reinvestigated the reduction of folate by borohydride under various conditions. The reaction

* From the Division of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California. *Received June 8, 1965; revised February 24, 1966.* This work was supported by Grants AM 8124-01 from the National Institute of Arthritis and Metabolic Diseases and CY 6522-03 from the National Cancer Institute, National Institutes of Health.

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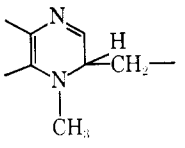
products were separated by chromatography on DEAE-cellulose using the method of Mathews and Huennekens (1960). By carefully selecting the reaction conditions, dihydrofolate could be accumulated in yields as high as 30%. Tritium-labeled borohydride was used to prepare labeled dihydrofolate and tetrahydrofolate. One atom of tritium is incorporated during the folate → dihydrofolate reaction, and a second atom is incorporated during the dihydrofolate → tetrahydrofolate reaction. Assuming that the tritium is bound to carbon rather than to nitrogen, the present data also support the 7,8-structure for dihydrofolate.

Donaldson and Keresztesy (1962) and Larrabee *et al.* (1963) have presented evidence that 5-methyldihydrofolate, resulting from oxidation of 5-methyltetrahydrofolate, is the 5,6-dihydro isomer. Reduction of folate and dihydrofolate by tritium-labeled borohydride have enabled us to prepare tetrahydrofolate-6-[^{3}H] and -7-[^{3}H]. The latter were converted to the correspondingly labeled 5-methyltetrahydrofolates. Oxidation of 5-methyltetrahydrofolate-6-[^{3}H] to the dihydro stage occurs without loss of label. In contrast, there is considerable loss of radioactivity when 5-methyltetrahydrofolate-7-[^{3}H] is oxidized. These results provide independent evidence for the 5,6-dihydro structure (II) in 5-methyldihydrofolate.



II

Experimental Section

Materials. DEAE-cellulose, a product of the Carl Schleicher and Schuell Co., was washed with 1.0 M Na_2HPO_4 until the absorbancy of the supernatant fluid was <0.050 at 260 mμ, washed with water until the pH of the suspension was 7, and stored in water. 2-Mercaptoethanol was purchased from Eastman Organic Chemicals; folic acid, aminopterin, and horse radish peroxidase were obtained from California Corp. for Biochemical Research. The folic acid was homogeneous when subjected to column chromatography as described below, and it showed a dry-weight purity of 90% from absorbancy measurements (ϵ 28 × 10^3 M^{-1} cm^{-1} at 280 mμ in neutral solution). Potassium borohydride (93%) and sodium borohydride[^{3}H] were obtained from Metal Hydrides Incorp. and from New England Nuclear Corp., respectively.

Tracer Techniques. Tritium was counted in a Packard Tri-Carb Model 314 EX scintillation instrument using Bray's solvent (Bray, 1960). The counting was done on samples sufficiently small to give no appreciable quenching (<0.1 ml in 10 ml of solvent). Solid sodium borohydride[^{3}H] (200 mc/mmole) was mixed uniformly with four parts of the nonlabeled compound so that the final specific activity was 40 mc/mmole which was equivalent to 12 × 10^6 cpm/μmole at 13% counting efficiency.

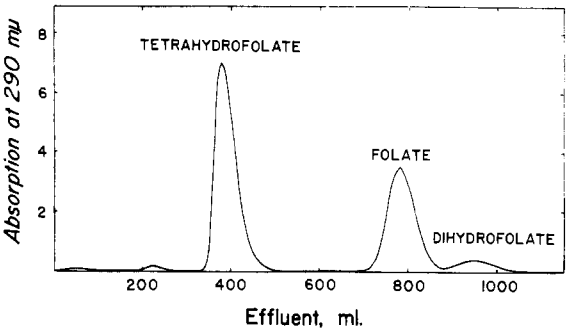


FIGURE 1: Elution profile of the reduction products of folate under standard reaction conditions.

Standard Reaction Conditions. Unless otherwise specified, the standard reaction mixture contained 34 μmoles of folate dissolved in 3 ml of 0.066 M Tris buffer,[1] pH 7.8. KBH_4 (15 mg) was added to start the reaction. After 10 min, 0.3 ml of 1 N HCl was added to destroy unreacted borohydride. The solution was neutralized rapidly with 1 N NaOH, mixed with 15 ml of 0.13 M ammonium acetate–0.2 mercaptoethanol, and chromatographed immediately on DEAE-cellulose.

Chromatographic Procedures. The above solution (18–20 ml) was chromatographed on a 2 cm × 15 cm column of DEAE-cellulose. Elution was accomplished in two steps: (a) with a linear gradient using 500 ml of 0.13 M ammonium acetate (pH 6.9) in the mixing flask and 500 ml of 0.40 M ammonium acetate (pH 6.9) in the reservoir; and (b) with 0.40 M ammonium acetate. 2-Mercaptoethanol was added to these solutions to a final concentration of 0.2 M. Ten-milliliter fractions were collected with a volumetric fraction collector.

Figure 1 illustrates a typical elution profile obtained by chromatography of the reaction products after borohydride treatment of folic acid. The three major peaks correspond to tetrahydrofolate, folate, and dihydrofolate. These compounds were identified by their absorption spectra at pH 7, and by their correspondence in the elution pattern to the positions of authentic standards.

Folate, dihydrofolate, and tetrahydrofolate have essentially the same extinction coefficient at 290 mμ (ϵ 26 × 10^3 M^{-1} cm^{-1}), and the mole fraction of each compound could be determined from planimetric measurements of the areas of the peaks. The recovery of the material in the three fractions, in all experiments, was greater than 95%.

5-Methyldihydrofolate and 5-methyltetrahydrofolate were separated by chromatography on 2 × 15 cm columns of DEAE-cellulose. Elution was performed with a linear gradient in which 250 ml each of 0.13 and 0.40 M ammonium acetate was present in the flasks; mercaptoethanol was omitted from the solutions.

[1] All values refer to the initial pH of the solution. The pH increased by about 1 unit during the reaction owing to hydrolysis of borohydride.

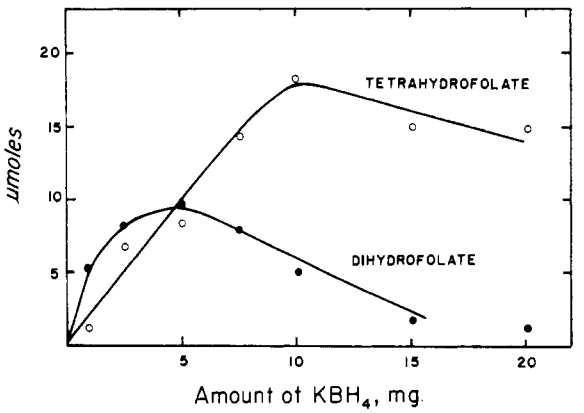


FIGURE 2: Effect of KBH_4 concentration on the reduction of folate to dihydro- and tetrahydrofolate.

Methyldihydrofolate is eluted prior to methyltetrahydrofolate.

Preparation of [^3H]-Labeled Compounds. Dihydrofolate-7-[^{3}H] was prepared by reduction of folate under standard conditions except that 3 mg of NaB^3H_4 was used. The solution was chromatographed on DEAE-cellulose, and tubes containing only dihydrofolate were pooled (*ca.* 230 ml). To this solution was added 375 mg of KBH_4 and reduction was allowed to proceed for 10 min at 37°. Carrier tetrahydrofolate (60 mg) dissolved in 10 ml of 0.03 M 2-mercaptoethanol was added, and the solution was lyophilized.

Tetrahydrofolate-6-[^{3}H] was prepared by treating 30 mg of dihydrofolate with 3 mg of NaB^3H_4, and the product was separated from extraneous radioactivity by column chromatography and lyophilization. Before conversion to the methyl derivative, tetrahydrofolate-6-[^{3}H] (*ca.* 30 mg) was admixed with 40 mg of carrier tetrahydrofolate.

5-Methyltetrahydrofolate-6-[^{3}H] and -7-[^{3}H] were synthesized from tetrahydrofolate-6-[^{3}H] and -7-[^{3}H], respectively, by the method of Mangum (1963).

Oxidation of 5-Methyltetrahydrofolate. Methyltetrahydrofolate (15–20 μmoles in 2 ml of 0.02 M phosphate buffer, pH 7.5) was oxidized by addition of 0.5 ml of 0.3% H_2O_2 and 1 mg of peroxidase (Ho, 1963). After standing for 10 min at room temperature, the product was separated by chromatography. Some samples of methyltetrahydrofolate were oxidized by adjustment to pH 9 and gassing with oxygen for 18 hr. The peroxidation method is preferable because of its speed and the lack of degradation products.

Results

Factors Affecting Relative Amounts of Dihydrofolate and Tetrahydrofolate. The effects of borohydride concentration, time, temperature, and pH upon the yields of dihydrofolate and tetrahydrofolate were examined. At a fixed reaction time of 10 min, the dihydrofolate–tetrahydrofolate ratio was most favorable when the amount of borohydride was 5 mg or less (Figure 2).

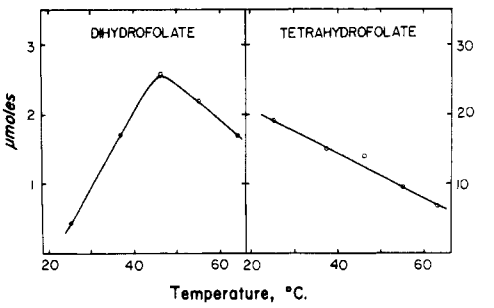


FIGURE 3: Effect of temperature on the reduction of folate by KBH_4.

The optimal over-all yield (10 μmoles) of dihydrofolate was obtained with 5 mg of reductant.

The influence of temperature on the production of dihydrofolate and tetrahydrofolate is shown in Figure 3. The amount of dihydrofolate increased as the temperature was raised to about 45°, and thereafter decreased. The production of tetrahydrofolate, however, decreased linearly with increasing temperature. Hydrolysis of borohydride according to eq 1 (Schlesinger *et al.*, 1953) is temperature dependent. The data in Figure 4 illustrates the rate of disappearance of borohydride under standard conditions and at the indicated temperatures.

$$BH_4^- + H^+ + 3H_2O \longrightarrow H_3BO_3 + 4H_2 \quad (1)$$

The yield of dihydrofolate could also be increased by lowering the initial pH (Table I). Experiments were not performed at pH values below 5.7 because of the insolubility of folic acid. The rate of reaction is markedly decreased in phosphate buffer as compared to Tris buffer.

In the above experiments, and especially in the

TABLE I: Effect of pH and Buffer on the Production of Dihydrofolate and Tetrahydrofolate.

Buffer (0.066 M)	Initial pH	Unreacted Folate (μmoles)	Dihydrofolate (μmoles)	Tetrahydrofolate (μmoles)
Phosphate	5.7	28.2	1.6	4.2
	6.4	28.6	1.1	4.3
	7.0	29.5	0	4.5
Tris	7.0	13.1	3.9	17.0
	7.8	15.3	1.9	16.8
	8.5	19.0	1.4	13.6

preparation of tritium-labeled compounds, it was essential to know whether all dihydrofolate in reaction mixtures was formed by reduction of folate rather than by reoxidation of tetrahydrofolate. As a control experiment, tetrahydrofolate was incubated with borohydride at 37° for 10 min, and the solution was examined by chromatography. The only material present was tetrahydrofolate, which was recovered quantitatively.

Reduction of Folate and Dihydrofolate with [^3H]-Borohydride. Experiments were performed with tritium-labeled borohydride to determine whether the hydride ion was transferred to a carbon or to a nitrogen of the pyrazine ring during reduction of folate and dihydrofolate. When folate was treated under standard conditions with NaB^3H_4, radioactivity that was not exchangeable with water appeared in both dihydrofolate and tetrahydrofolate. For each of these compounds, the profiles of radioactivity and of absorbancy at 290 mμ coincided in the elution diagram. In Table II, the starting material was folate in expt 1 and 2, and dihydrofolate in expt 3. The specific activity of the borohydride was 3×10^6 cpm/μequiv of hydride. Experiments 1 and 2, therefore, demonstrate a transfer of one atom of radioactive hydride during the folate → dihydrofolate conversion and two atoms of tritium during the folate → tetrahydrofolate reaction. In expt 3, when dihydrofolate was reduced with labeled borohydride, the resulting tetrahydrofolate, as expected, contained one atom of tritium. In all of these experiments, there appeared to be no isotopic discrimination against tritium in the borohydride reduction of the folate compounds. This is in contrast to the marked dilution of tritium from ^3H_2O encountered in hydrosulfite reduction of folate to dihydrofolate (Pastore *et al.*, 1963).

TABLE II: Incorporation of Tritium from Sodium [^{3}H]-Borohydride into Dihydrofolate and Tetrahydrofolate.

Expt	Starting Material[a]	Specific Activity of Products: Dihydrofolate (cpm/μmole)	Specific Activity of Products: Tetrahydrofolate (cpm/μmole)
1	Folate	2.7×10^6	5.6×10^6
2	Folate	2.5×10^6	4.9×10^6
3	Dihydrofolate	...	2.8×10^6

[a] The starting materials were reduced with 2–5 mg of sodium [^{3}H]borohydride which had a specific activity of 12×10^6 cpm/μmole (3×10^6 cpm/μequiv of hydride). The products, dihydrofolate and tetrahydrofolate, were separated by column chromatography and then rechromatographed prior to counting.

Characterization of Borohydride-Produced Dihydrofolate. Dihydrofolate synthesized by reduction of folate by borohydride was judged to be identical with the hydrosulfite product by the following criteria: (a) both compounds had identical ultraviolet absorption spectra when examined at pH 1, 7, and 12; (b) the compounds could not be distinguished by their behavior on DEAE columns or on paper chromatograms using the three solvents described earlier by Mathews and Huennekens (1963) for the hydrosulfite product; (c) dihydrofolate-[^{3}H], prepared by reduction of folate with labeled borohydride, was admixed with unlabeled dihydrofolate prepared by the hydrosulfite method, and after three recrystallizations of the mixture at pH 2.8 there was no change in the specific activity; and (d) both dihydrofolates reacted with similar rates and consumed the same amount of TPNH when assayed with the chicken liver dihydrofolic reductase (Table III).

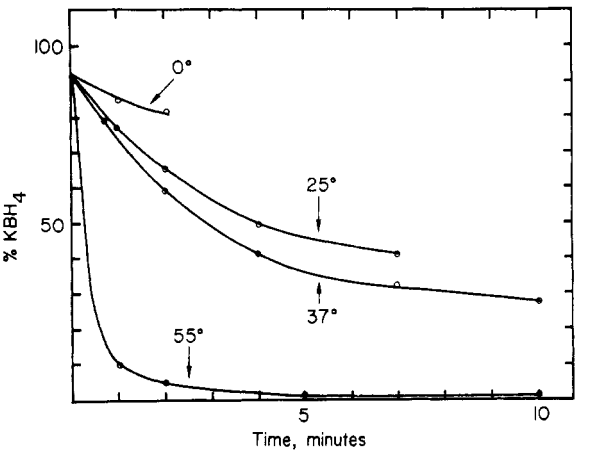


FIGURE 4: Effect of temperature on the hydrolysis of KBH_4 under standard reaction conditions. For each point, 100 mg of KBH_4 was dissolved in 20 ml of 0.066 M Tris buffer, pH 7.8, and incubated in a water bath at the indicated temperature. At the appropriate time, the residual borohydride in the sample was estimated by the method of Lyttle *et al.* (1952).

TABLE III: Enzymatic Assay of Dihydrofolate Preparations.[a]

Source of Dihydrofolate	Dihydrofolate Reduced (μmole)	Initial Rate (mμmoles/min)
Borohydride reduction of folate	0.039	6.0
Dithionite reduction of folate	0.040	6.8

[a] In each experiment, 0.038 μmole of dihydrofolate (standardized spectrophotometrically at 282 mμ) was used.

TABLE IV: Oxidation of 5-Methyltetrahydrofolate-6-[^{3}H] and -7-[^{3}H].[a]

	Specific Activity of 5-Methyldihydrofolate (cpm/μmole)
A. Oxidation of 5-methyltetrahydrofolate-6-[^{3}H] (1.3×10^6 cpm/μmole)	
Expt 1	1.3×10^6
Expt 2	1.4×10^6
Expt 3	1.3×10^6
B. Oxidation of 5-methyltetrahydrofolate-7-[^{3}H] (7.5×10^4 cpm/μmole)	
Expt 1	5.1×10^4
Expt 2	4.8×10^4
Expt 3	4.7×10^4

[a] In expt 1 and 2 (in both parts A and B), oxidation was performed with H_2O_2 and peroxidase. In expt 3, 5-methyltetrahydrofolate at pH 9 was treated with oxygen.

Oxidation of Tritium-Labeled 5-Methyltetrahydrofolate. Table IV summarizes a series of experiments in which 5-methyltetrahydrofolate, labeled with tritium either at C-6 or at C-7, was oxidized to 5-methyldihydrofolate. The same results were obtained whether oxidation was carried out with H_2O_2–peroxidase at pH 7.5 or with oxygen at pH 9. As shown in part A, no tritium was lost when the 6-labeled methyltetrahydrofolate was oxidized. Conversely, oxidation of the 7-labeled compound resulted in an appreciable loss of radioactivity (0.7 atom/mole of starting material).

Discussion

In a previous investigation of the reduction of folate by borohydride, we described a material that appeared to be a new isomer of dihydrofolate (Smith *et al.*, 1963). This assumption rested principally upon the finding that various samples of product, when subjected to catalytic hydrogenation, consumed *ca.* 1 mole of hydrogen/mole of the folate compound. However, the nuclear magnetic resonance spectrum[2] of the material was not significantly different from that of folate itself. Despite this unexpected result, there was little doubt that folate was being reduced by borohydride. For example, there was a change in the folate spectrum in the region of 350 mμ after the addition of borohydride. Chromatographic analysis of folate–borohydride reaction mixtures on columns of DEAE-cellulose revealed the presence of both tetrahydrofolate and folate, but when the products were reprecipitated from acid solution, only folate was observed. In some instances, a small amount of dihydrofolate was also observed in the elution profiles. Conditions have been developed in the present investigation for the accumulation of dihydrofolate, which has been shown by chemical and enzymatic methods to be identical with dihydrofolate obtained by hydrosulfite reduction of folate.

The reduction of folate by borohydride

$$\text{folate} \xrightarrow{BH_4^-} \text{dihydrofolate} \xrightarrow{BH_4^-} \text{tetrahydrofolate}$$

is a complex system because the second step is much more rapid than the first and because borohydride is being constantly removed by hydrolysis. The yield of dihydrofolate can be increased by: (a) using low concentrations of borohydride; (b) raising the temperature; and (c) decreasing the pH. Each of these conditions tends to prevent excess borohydride from reducing dihydrofolate to tetrahydrofolate. However, even under conditions found empirically to be most favorable, the yield of the intermediate, dihydrofolate, did not exceed 30% of the starting material.

Tracer studies using [^{3}H]borohydride as the reductant revealed that the dihydrofolate formed contained one atom of nonexchangeable tritium. Control experiments showed that the radioactive dihydrofolate arose from the reduction of folate, rather than from oxidation of tetrahydrofolate. If it is assumed that the nonexchangeable tritium is carbon bound, the structure of the product must be either 5,6-dihydrofolate or 7,8-dihydrofolate. The 5,6-dihydro form has been ruled out previously by the observation that dihydrofolate does not contain an asymmetric center at carbon 6. Therefore, the nonexchangeable radioactivity must be located at carbon 7 because dihydrofolate cannot have a hydrogen at carbon 6. The 7,8-structure of dihydrofolate deduced from the present study agrees with the conclusions of Pastore *et al.* (1963) and of Hillcoat and Blakley (1964) who carried out nuclear magnetic resonance measurements and tracer experiments on the hydrosulfite reduction product of folic acid.

During the reduction of dihydrofolate to tetrahydrofolate, the hydride ion must be added to the only available position, *viz.*, carbon 6. Thus, in Table II the products are dihydrofolate-7-[^{3}H] and tetrahydrofolate-6,7-[^{3}H] in expt 1 and 2, and tetrahydrofolate-6-[^{3}H] in expt 3. In a separate experiment, tetrahydrofolate-7-[^{3}H] was synthesized by reduction of dihydrofolate-7-[^{3}H] with nonlabeled borohydride.

Two atoms of hydrogen are added to the pyrazine ring in each step in the reduction of folate to tetrahydrofolate. Because the hydrogen coming from borohydride adds to a carbon atom of the ring (to carbon 7 in dihydrofolate formation; to carbon 6 in the dihydrofolate to tetrahydrofolate conversion), the second hydrogen atom in each step must be attached to the corresponding nitrogen atom (position 8 and position 5, respectively). These nitrogen-bound hydrogens would

[2] Kindly performed by Dr. W. V. von Philipsborn.

be expected to be exchangeable, and presumably are derived from the protons of the solvent.

The experiments with [^{3}H]borohydride also provide independent evidence that 7,8-dihydrofolate is an obligatory intermediate in the reduction of folate to 5,6,7,8-tetrahydrofolate. This is shown by the fact that the specific activity of tetrahydrofolate (*cf.* expt 1 and 2, Table II) is almost exactly twice that of dihydrofolate; this could not have occurred if 5,8-dihydrofolate were a transient intermediate in the reduction. Thus, reduction of folate by hydrosulfite or by borohydride produces the same intermediate, 7,8-dihydrofolate, even though different reaction mechanisms are probably involved (Albert and Matsuura, 1962). The pyridine nucleotide dependent reduction of folate to tetrahydrofolate, catalyzed by dihydrofolic reductase, may also proceed *via* 7,8-dihydrofolate, although this compound has not yet been detected as an intermediate in the enzymatic system.

The availability of tetrahydrofolate labeled with tritium at carbons 6 or 7 has made it possible to investigate the mechanism of oxidation of 5-methyltetrahydrofolate to 5-methyldihydrofolate. Donaldson and Keresztesy (1962) and Larrabee *et al.* (1963) have suggested that the structure of 5-methyldihydrofolate, prepared in this manner, is 5-methyl-5,6-dihydrofolate. Their conclusions were based on the lack of racemization at carbon 6 during oxidation and subsequent reduction. The data in part A of Table III confirm the finding that the hydrogen at position 6 was not lost during oxidation. The situation is more complex when the 7-labeled methyltetrahydrofolate is used since two atoms of hydrogen are bound to this position. Therefore, no more than 50% of the label at position 7 should be lost. The data in part B of Table III show that about 35% of the label (*i.e.*, 0.7 atom/mole) was indeed lost from the 7-position. The discrepancy from the expected 50% loss is due most probably to isotopic discrimination. The present experiments are consistent, therefore, with the 5-methyl-5,6-dihydrofolate structure but this conclusion awaits verification by nuclear magnetic resonance spectroscopy of the crystalline material.

Acknowledgment

The authors are indebted to Dr. F. M. Huennekens for his advice on this problem, to Dr. J. M. Whiteley for the recrystallization of radioactive dihydrofolate, to Dr. D. J. Vonderschmitt for determining the stability of borohydride, to Dr. R. L. Blakley for communicating his results to us prior to publication, and to Dr. W. von Philipsborn of the Organisch-Chemisches Institut at the University of Zurich for performing the nuclear magnetic resonance measurements.

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