Synthesis of (6*R*,11*S*)- and (6*R*,11*R*)-5,10-Methylene[11-^{1}H,^{2}H]tetrahydrofolate. Stereochemical Paths of Serine Hydroxymethyltransferase, 5,10-Methylenetetrahydrofolate Dehydrogenase, and Thymidylate Synthetase Catalysis

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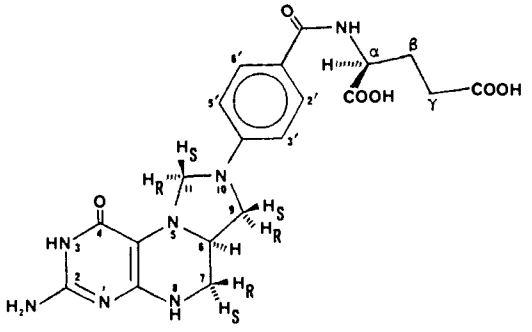
Abstract: The stereochemical courses of the reactions catalyzed by serine hydroxymethyltransferase (EC 2.1.2.1) and 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) have been determined. The method employed was to prepare the labeled intermediates (6*R*,11*R*)- and (6*R*,11*S*)-5,10-methylene[11-^{1}H,^{2}H]tetrahydrofolate by stereospecific chemical reduction and to determine their absolute stereochemistry at C11 by the measurement of proton nuclear Overhauser enhancements (NOE's). Correlation of these results with those obtained by generating (6*R*)-5,10-methylene[11-^{1}H,^{2}H]tetrahydrofolate enzymatically from (3*S*)- and (3*R*)-[3-^{1}H,^{2}H]serine with the transferase and from (6*R*)-5,10-methenyl[11-^{2}H]tetrahydrofolate via the dehydrogenase delineated the stereochemical paths of both enzymes. Serine hydroxymethyltransferase converts (3*S*)- and (3*R*)-[3-^{1}H,^{2}H]serine to (6*R*,11*S*)- and (6*R*,11*R*)-5,10-methylene[11-^{1}H,^{2}H]tetrahydrofolate, respectively, and the dehydrogenase stereospecifically removes H_R. These results also were correlated with those obtained by Tatum et al. [Tatum, C. M.; Vederas, J.; Schleicher, E.; Benkovic, S. J.; Floss, H. G. *J. Chem. Soc. Chem. Commun.* **1977**, 218–220] on thymidylate synthetase (EC 2.1.1.b) to further delineate the stereochemical course of the one carbon unit transfer and accompanying reduction catalyzed by that enzyme.

(6*R*)-5,10-Methylenetetrahydrofolate (**1**) is an important intermediate in the biosynthesis of both purines and pyrimidines.[1]



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Early stereochemical experiments by Biellman and Schuber[2,3] and by Tatum et al.[4] had shown that serine hydroxymethyltransferase catalyzes the transfer of a chiral, isotopically labeled, C3 unit from L-serine to tetrahydrofolate to form (6*R*)-5,10-methylenetetrahydrofolate with only partial stereochemical control at the C11 center. In Tatum's experiments this was measured indirectly by coupling serine hydroxymethyltransferase to 5,10-methylenetetrahydrofolate dehydrogenase. By starting with both (3*S*)- and (3*R*)-[3-^{1}H,^{3}H]serine, and analyzing for the amount of label retained as 5,10-methenyltetrahydrofolate and the amount transferred to $NADP^+$, we determined the relative stereochemical distribution of tritium at C11 (3:1) of the intermediate 5,10-methylene[11-^{1}H,^{3}H]tetrahydrofolate. Although these experiments demonstrated enzyme-catalyzed epimerization of the product aminal, they could not elucidate the absolute stereochemical course of either the transferase or the dehydrogenase-catalyzed reactions.

Similar coupling experiments employing serine hydroxymethyltransferase and thymidylate synthetase were reported by Floss and Benkovic.[5] In this case it was shown that (3*S*)- and (3*R*)-[3-^{2}H,^{3}H]serine generated (*S*)- and (*R*)-methyl-[7-^{1}H,^{2}H,^{3}H]deoxythymidine monophosphate, respectively. However, as in the previous experiment, the absolute configuration of the intermediate 5,10-methylene[11-^{2}H,^{3}H]tetrahydrofolate was unknown, and therefore it was not possible to more fully elucidate the mechanism of thymidylate synthetase or to predict the three-dimensional orientation of the substrates on the enzyme surface.

In order to solve these problems, we have investigated several ways to synthesize chiral 5,10-methylene[11-^{1}H,^{2}H]tetrahydrofolate and to determine the absolute stereochemistry at C11. These include enzymatic conversion of stereospecifically labeled (3*R*)- and (3*S*)-[3-^{2}H]-L-serine to 5,10-methylene[11-^{1}H,^{2}H]tetrahydrofolate, enzymatic reduction of 5,10-methenyl[11-^{2}H]tetrahydrofolate with NADPH, and chemical reduction of 5,10-methenyltetrahydrofolate with $NaBD_4$ or $NaBH_4$ in the presence of *N*,*N*-diethylaniline.[4]

The ^{1}H NMR spectrum of 5,10-methylenetetrahydrofolate has been obtained at 300 MHz by Poe et al.,[6] and all seven protons on C6, C7, C9, and C11 were resolved and spin–spin coupling constants determined. This information allowed Poe to predict the solution conformation of the tetrahydropyrazine and imidazolidine rings and also confirmed Tatum's[4] observation that the diastereotopic protons at C11 have a chemical shift difference of approximately 1.1 ppm. Measurement of steady-state proton nuclear Overhauser enhancements (NOEs) of C11–H of the chemically reduced species have allowed us to assign the chemical shifts of both diastereotopic protons. Correlation of these results with those of the enzymatic syntheses has permitted determination of the stereochemistry of the 5,10-methylenetetrahydrofolate dehydrogenase reaction, as well as provided valuable information

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0002-7863/84/1506-1833$01.50/0

on the mechanisms of the reactions catalyzed by serine hydroxymethyltransferase and thymidylate synthetase.

Experimental Section

Materials. Folic acid, $NADP^+$, ATP, isocitrate dehydrogenase type IV (specific activity = 5.5 units/mg at pH 7.4, 37 °C), DL-isocitrate, D-glucose-6-phosphate dehydrogenase (430 units/mg at pH 7.4, 25 °C), and D-glucose 6-phosphate were purchased from Sigma Chemical Co. Sodium borodeuteride (98 atom % D), sodium borohydride, and D_2O (≥99.996 atom % D) were obtained from Aldrich Chemical Co. (6*S*)-Tetrahydrofolate was prepared enzymatically by the methods of Mathews and Huennekens[7] and Blakley[8] and purified by column elution from (diethylaminoethyl)cellulose (DEAE-cellulose) employing a gradient from 0.2 to 0.7 M triethylammonium bicarbonate and 0.1 M β-mercaptoethanol, pH 8.0, as described by Rabinowitz et al.[9] Sodium [1-^{2}H]formate was prepared by hydrolysis of KCN in D_2O as reported by Smith.[10] (2*S*,3*S*)-[3-^{2}H]Serine and (2*S*,3*R*)-[2,3-$^2H_2$]serine were synthesized by the method of Slieker and Benkovic.[11] Serine hydroxymethyltransferase was a generous gift of Dr. LaVerne Schirch, Medical College of Virginia, Richmond, VA, and chicken liver trifunctional enzyme was obtained from Dr. Gail Wasserman, The Pennsylvania State University.

Methods. High-resolution proton NMR spectra were obtained on a Brüker WM-360 spectrometer operating at 360.13 MHz, with sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the internal standard. Deuterium NMR spectra were run on the same instrument at 55.28 MHz, operating without field frequency lock. Proton relaxation measurements were made on a degassed solution using the inversion recovery (180°–τ–90°–T) sequence, and T_1's were calculated by a nonlinear least-squares analysis. There were no systematic deviations consistent with a contribution from cross relaxation in the magnetization recovery. Proton NOE measurements were obtained by difference of the free induction decays (FIDs) collected on and off resonance, employing a standard microcomputer sequence provided by Brücker Instruments. For these measurements a 90° pulse (7.5 μs) was used with a 4000-Hz sweep width, and the FIDs were averaged into a 32K data block. To effect the steady-state NOE, a saturating rf field was applied for 20 s at the resonance frequency of interest using a decoupler power of 40 L. NOE samples were dissolved in 700 μL of 0.1 N NaOD (99.8 atom % D) and lyophilized to dryness. The deuterium-exchanged product was then redissolved in the same volume of 99.996 atom % D D_2O and deoxygenated by four freeze–thaw cycles under vacuum before sealing in an NMR tube.

(6*R*)-5,10-Methenyl[9,9,11-$^2H_3$]tetrahydrofolate (2). [9,9-$^2H_2$]Folic acid was prepared by the method of Khalifa et al.[12] and was converted to (6*R*)-[9,9-$^2H_2$]tetrahydrofolate as described under Materials for the nonlabeled species. (6*R*)-5,10-Methenyl[9,9,11-$^2H_3$]tetrahydrofolate was prepared from (6*R*)-[9,9-$^2H_2$]tetrahydrofolate and [1-^{2}H]formate by direct enzymatic formylation. This reaction is catalyzed by the 10-formyltetrahydrofolate synthetase activity of the trifunctional protein, and upon acidification of the reaction mixture, the product 10-formyltetrahydrofolate rapidly cyclizes to 5,10-methenyltetrahydrofolate. Poe et al.[13] reported that the C11 amidinium proton is reasonably acidic and can slowly exchange with solvent, even at neutral or slightly alkaline pH. Therefore, the synthesis was performed in D_2O. Typically, the following compounds were dissolved in 5 mL of D_2O and lyophilized to dryness to remove exchangeable protons: 60 μmol of $MgCl_2$ (anhydrous), 40 μmol of sodium [1-^{2}H]formate, 200 μmol of NH_4Cl, 40 μmol of disodium adenosine triphosphate (Na_2ATP), and 100 μmol of Tris base. To the residue was added 10 mg of (6*R*)-[9,9-$^2H_2$]tetrahydrofolate (free acid) and 2 mL of deoxygenated D_2O, under N_2. The pH was adjusted to an electrode reading of 7.4 at 25 °C with 1.0 N NaOD. The reaction was initiated by adding 15 μL of trifunctional protein (100 units mL^{-1} dehydrogenase activity) and the solution was incubated at 37 °C under Ar. Five-microliter aliquots were removed at 1-h intervals and quenched into 1.0 mL of 1 N HCl to measure at 350 nm (ϵ = 25 100 M^{-1} cm^{-1}) the 5,10-methenyltetrahydrofolate formed. Approximately 70% conversion was obtained after 3 h. To the reaction mixture was added 200 μL of 6 N HCl, and after 30 min at room temperature the solution was loaded onto a phosphocellulose column (Bio-Rad Cellex-P, 1.5 cm × 25 cm) in the H^+ form. The product was eluted with 0.1 M HCl and fractions were monitored by measuring the UV absorbance at 350 nm. Fractions (8 mL) containing 5,10-methenyl[9,9,11-$^2H_3$]tetrahydrofolate (11–26 inclusive) were pooled and lyophilized to dryness. The residue was dissolved in H_2O and then lyophilized to give 8 mg of the zwitterionic form.[14] The ^{1}H NMR spectrum of this material confirmed deuterium incorporation into the C11 position at a level of 98% of one deuterium.

(6*R*)-5,10-Methenyl[9,9-$^2H_2$]tetrahydrofolate (3). The method of Rowe[15] was employed to directly formylate (6*R*)-[9,9-$^2H_2$]tetrahydrofolate nonenzymatically in 98% formic acid containing β-mercaptoethanol. Column purification was performed as described for the enzymatic synthesis.

(6*R*)-5,10-Methenyl[11-^{2}H]tetrahydrofolate (4). The enzymatic method described above for **2** was employed with the substitution of tetrahydrofolate for [9,9-$^2H_2$]tetrahydrofolate.

Conversion of (2*S*,3*S*)-[3-^{2}H]- and (2*S*,3*R*)-[2,3-$^2H_2$]Serine to 5,10-Methylene[11-^{1}H,^{2}H]tetrahydrofolate. To 1 mL of deoxygenated D_2O (99.8 atom % D) was added 40 mg of (6*S*)-tetrahydrofolate, and the material was brought into solution by the addition of 1.3 N NaOD to a final pD of 7.5. Equal volumes of this solution (0.5 mL) were added to 4.7 mg each of (2*S*,3*S*)-[3-^{2}H]- and (2*S*,3*R*)-[2,3-$^2H_2$]serine, and the solutions were filtered into two NMR tubes. The initial concentration of tetrahydrofolate was 55 mM and that of L-serine, 90 mM. Control ^{1}H NMR spectra were obtained, and then the reaction was begun by the addition of 2 units of serine hydroxymethyltransferase (10 μL volume) that had been dialyzed against 50 mM potassium phosphate, 0.1 mM pyridoxal-5-phosphate, and 5 mM dithiothreitol in D_2O. The sample tube was returned to the spectrometer and scans were resumed. Acquisition was terminated after approximately 20, 40, 60, 100, and 120 min. Initially, the C2′,6′–2H and C3′,5′–2H resonances of tetrahydrofolate were visible at 7.65 and 6.73 ppm, respectively, but in time the corresponding signals of 5,10-methylenetetrahydrofolate grew in at 7.67 and 6.56 ppm. Also visible were the C11–H singlets of the bridging methylene unit at 4.74 and 3.47 ppm. In 0.1 N NaOD, Poe et al.[6] determined the chemical shifts of the C11 protons to be 5.01 and 3.89 ppm, but did not report their values at neutral pH. Presumably ionization of the N_3 amide hydrogen, which has a pK_a of approximately 10,[16] results in a downfield shift in the C11–2H resonance positions. Deuterium NMR of 5,10-methylene[11-$^2H_2$]tetrahydrofolate (made from tetrahydrofolate and [$^2H_2$]formaldehyde) at neutral pH also confirmed this assignment with broad resonances appearing at approximately 4.7 and 3.6 ppm (relative to acetone-d_6 = 2.07 ppm).

Enzymatic Reduction of (6*R*)-5,10-Methenyl[11-^{2}H]tetrahydrofolate. The preparative enzymatic reduction of **4** was performed in deoxygenated D_2O at a pH electrode reading of 8.1. The dehydrogenase was shown to decrease in activity significantly above pH 8.5, but it was thought necessary to perform the reduction at higher than normal pH to slow down possible acid-catalyzed epimerization of the aminal. The following compounds were dissolved in 2.0 mL of deoxygenated D_2O and added to 13.3 μmol of 5,10-methenyl[11-^{2}H]tetrahydrofolate: 2.4 μmol of $NADP^+$, 42 μmol of D,L-isocitrate, 54 μmol of $MgCl_2$, 200 μmol of Tris, and 20 μmol of ascorbic acid. The pH was adjusted to a meter reading of 8.1 with 0.1 M NaOD. The reaction was inititated by adding 30 μL of isocitrate dehydrogenase (2.3 units at pH 7.5 and 25 °C) and 100 μL of concentrated trifunctional protein (10 units of dehydrogenase activity in the forward direction at pH 7.5 and 25 °C). The progress of the reaction was monitored by quenching 10-μL aliquots into 100 mM Tris buffer, pH 7.5, and measuring the change in OD_{312}.[17] which increased from 0.50 to 0.86 over a 3.5-h period. The reaction mixture was then loaded onto a (triethylaminoethyl)cellulose column (1.5 × 22 cm) equilibrated with 500 mL of 0.005 M triethylammonium bicarbonate, pH 9.6, and eluted with a linear gradient from 0.005 M–0.4 M triethylammonium bicarbonate, pH 9.6, 500 mL.[18] The elution profile, monitored at 295 nm, showed two major peaks. The second, more intense, peak, centered at fraction 21, was pooled and shown to have the same UV spectrum as 5,10-methylenetetrahydrofolate (λ_{max} = 295 nm). The pooled fractions were lyophilized to dryness, redissolved in deoxygenated 0.1 N NaOD, and filtered into an NMR tube under nitrogen.

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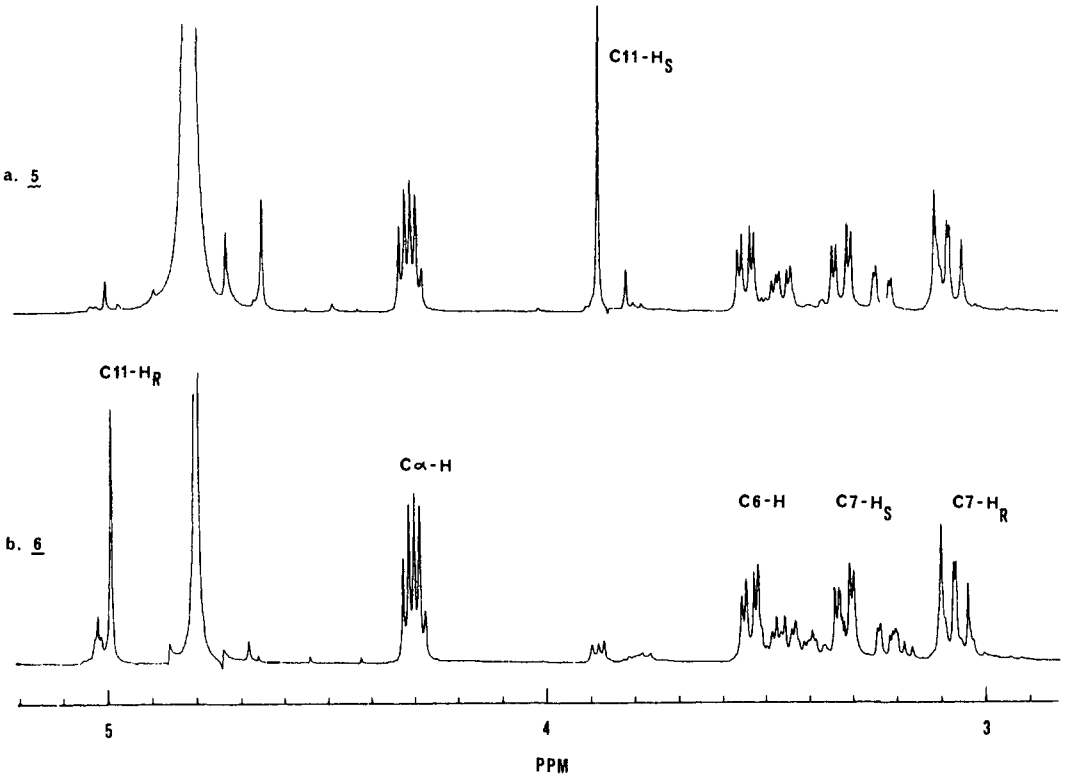

Figure 1. 360.13-MHz Proton NMR spectra in 0.1 N NaOD of (a) (6*R*,11*R*)-5,10-methylene[9,9,11-$^2H_3$]tetrahydrofolate (**5**) and (b) (6*R*,11*S*)-5,10-methylene[9,9,11-$^2H_3$]tetrahydrofolate (**6**). The resonances at 3.22 and 3.48 ppm are C7-H$_S$ and C7-H$_R$, respectively, of 5-methyltetrahydrofolate, which was present at a level of 40% when the reduction was performed in the presence of *N*,*N*-diethylaniline. In the presence of pyridine, however, no 5-methyltetrahydrofolate was observed.

Chemical Reduction of 5,10-Methenyltetrahydrofolate. Essentially the procedure of Tatum[4] was employed with the substitution of $NaBD_4$ for $NaBT_4$: both pyridine (2.5 M) and *N*,*N*-diethylaniline (1.5 M) were tried, and the acid precipitation step was omitted. Both diastereomers of (6*R*)-5,10-methylene[11-^{1}H,^{2}H]tetrahydrofolate were prepared in the presence of *N*,*N*-diethylaniline by reduction of **2** with $NaBH_4$ and **3** with $NaBD_4$ to give methylene species **5** and **6**, respectively. The pyridine reduction product (C11-2H) was assayed with 5,10-methylenetetrahydrofolate dehydrogenase by the method of Ramasastri and Blakley[19] and was shown to be oxidized at the same rate and to the same extent as (6*R*)-5,10-methylenetetrahydrofolate prepared from (6*S*)-tetrahydrofolate and formaldehyde.

Results

Assignment of C11 Protons. As is shown in Figure 1, the chemical reduction of 5,10-methenyltetrahydrofolate with $NaBH_4$ is highly stereospecific. Reduction of **2** with $NaBH_4$ gave (6*R*)-5,10-methylene[9,9,11-$^2H_3$]tetrahydrofolate (**5**) with protium predominating in the upfield position (3.87 ppm:4.99 ppm, 92:8). Conversely, reduction of the 11-^{1}H species (**3**) with $NaBD_4$ gave product **6** containing protium in the downfield position in equivalent diastereomeric excess (Figure 1 a,b). The reduction of **3** with $NaBD_4$ was also performed using pyridine as the organic base. In this case the same diastereomer was predominant, but the diastereomeric ratio was only 75:25. These product distributions are considerably greater than those reported by Tatum[4] (78:24 for *N*,*N*-DEA, 50:50 for pyridine), using a similar procedure and are probably due to our omission of Tatum's acid precipitation of the reduction product, which may have caused a significant degree of epimerization at C11.

The absolute assignment of the two C11 protons was determined by measurement of proton nuclear Overhauser enhancements. In order to simplify the spectrum of the product and to decrease the number of $I = {}^1/_2$ nuclei that could contribute to the relaxation of C11-H, both C9 protons were substituted by deuterium. Since deuterium has a small magnetogyric ratio and relaxes efficiently through a quadrapole mechanism, its effect on the relaxation of the remaining protons is negligible. From measurements of the approximate internuclear distances with a space-filling model, it was clear that C7-H$_R$ was reasonably close to C11-H$_S$ ($r \cong 2.5$ Å). By measuring the T_1's of **5** (see Table I), it was shown that both C7 protons have significantly shorter relaxation times (0.39, 0.38 s) than either C6-H (1.8 s) or C11-H (1.1 s). Therefore, although irradiation of C7-H*R* would be expected to result in a significant enhancement of C7-H$_S$ due to proximity (r = 1.74

Table I. T_1 values for **5**

proton	T_1, s	proton	T_1, s
C2′,6′-2H	1.83	C7-H$_S$	0.39
C3′,5′-2H	1.14	C7-H$_R$	0.38
Cα-H	1.69	Cγ-2H	0.52
C11-H	1.11	Cβ-H	0.38
C6-H	1.35	Cβ-H	0.35

Table II. Proton Nuclear Overhauser Enhancements of **5** and **6**

	irradiated				
obsd	C3′,5-2H	C11-H	C6-H	C7-H$_S$	C7-H$_R$
		5			
C2′,6′-2H	7.1				
C3′,5′-2H		3.2			
C11-H	5.5				4.9
C6-H				4.7	4.5
C7-H$_S$			1.5		8.1
C7-H$_R$		2.2		11	
		6			
C2′,6′-2H	7.2				
C3′,5-2H		4.5			
C11-H	7.8				
C6-H				2.4	2.9
C7-H$_S$					6.4
C7-H$_R$				12	

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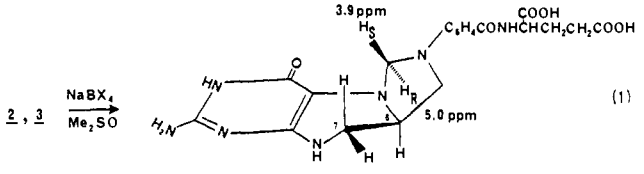
Å), a NOE at C11–H_S should also be observed as a result of its longer T_1. NOE experiments were performed on both diastereomers at C11 as described in the Experimental Section. The results are presented in Table II. Irradiation of C7–H_R of **5** gave a 4.9% enhancement of C11–H (at 3.87 ppm), while saturation of C11–H generated a 2.2% NOE of C7–H_R. No NOEs of C11–H (4.99 ppm) were observed when either C6–H, C7–H_S, or C7–H_R of **6** were irradiated. Since C11–H_R is quite removed from any protons on the imidazolidine or tetrahydropyrazine rings (C6–H to C11–H_R ≅ 4.1 Å, C7–H_S to C11–H_R ≅ 5.6 Å), no enhancements would be expected. Therefore, these data indicate that the upfield resonance of the C11 bridge of 5,10-methylenetetrahydrofolate corresponds to H_S, while the downfield position is H_R, so that the absolute configuration at C11 for **5** and **6** is as shown in eq 1. This is in agreement with the earlier



(1)

5: X = H_S = ^{1}H, H_R = ^{2}H

6: X = H_S = ^{2}H, H_R = ^{1}H

prediction by Poe, Jackman, and Benkovic.[6] Both C11 protons are approximately equidistant from the amide oxygen at C4, but H_S, shifted 1.1 ppm upfield relative to H_R, is apparently shielded due to the anisotropy of the carbonyl bond.[20]

These data may also be used to estimate the internuclear distance between C11–H_S and C7–H_R, provided that (a) at least one internuclear distance is known independently (in this case, the distance between C7–H_S and C7–H_R calculated from a presumed C–H bond length of 1.07 Å and an H–C–H bond angle of 109°), (b) the correlation times of the internuclear vectors are equal, and (c) the molecule is completely rigid. Assumption c is reasonable since the imidazolidine–tetrahydropyrazine ring fusion precludes conformational change, and assumption b probably introduces only minor errors since the C7–H_S–C7–H_R and C7–H_R–C11–H_S relaxation vectors cannot reorient independently of each other. For three loosely coupled nonequivalent spins, eq 2 describes the ratio of internuclear distances in terms

$$\left(\frac{r_{ax}}{r_{am}}\right)^6 = \left(\frac{\gamma_x^3}{\gamma_m^3}\right)\left(\frac{f_a(m) + f_a(x)f_x(m)}{f_a(x) + f_a(m)f_m(x)}\right) \qquad (2)$$

a = C7–H_R; m = C7–H_S; x = C11–H_S

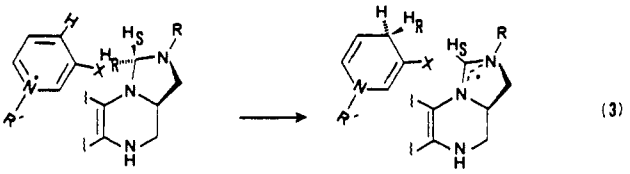
r_{ij} = distance in Å between nuclei i and j

$f_i(j)$ = the nuclear Overhauser enhancement of spin i when j is saturated

of γ and the measured NOEs.[21] Substituting the enhancements for **5** (Table II) and the calculated distance between C7–H_R and C7–H_S of 1.74 Å into eq 2, one obtains the value of 2.3 Å for r_{ax}, the distance between C7–H_R and C11–H_S. This compares favorably to the value of approximately 2.5 Å predicted from molecular models.

Enzymatic Synthesis of Chiral (6*R*)-5,10-Methylene[11-^{1}H,^{2}H]tetrahydrofolate. When serine hydroxymethyltransferase and both (3*S*)- and (3*R*)-[3-^{2}H]serine were employed, (6*R*)-5,10-methylene[11-^{1}H,^{2}H]tetrahydrofolate was synthesized enzymatically as described in the Experimental Section. With (3S)-serine as the starting point, the product distribution after 40 min (the earliest point at which it could be measured accurately) was approximately 60% 11*S* and 40% 11*R*. After 120 min, the distribution was 50:50, or complete epimerization at C11. Analogously, (3*R*)-serine gave the opposite product ratio, with an initial excess of 11*R* over 11*S*. These results are consistent with those of Tatum,[4] who showed that chemical reduction of (6*R*)-5,10-methenyltetrahydrofolate with $NaBT_4$ gave the same diastereomer of (6*R*)-5,10-methylene[11-^{3}H]tetrahydrofolate as was produced from (3*S*)-[3-^{3}H]serine by serine hydroxymethyltransferase. Since the data presented above prove that the $NaBT_4$ reduction product must have been 11*S*, the enzymatic conversion of (3*S*)-serine to (6*R*,11*S*)-5,10-methylenetetrahydrofolate is confirmed. The rate at which epimerization of C11 was shown to occur also supports Tatum's[4] contention that the enzyme does not exhibit tight stereochemical control over its reaction.

Enzymatic reduction of (6*R*)-5,10-methenyl[11-^{2}H]tetrahydrofolate by the NADPH-requiring 5,10-methylenetetrahydrofolate dehydrogenase from chicken liver gave a 20% excess of the 11*S* species. This confirms that the dehydrogenase abstracts the more accessible proton, H_R, from the methylene bridge as shown in eq 3. The partial racemization presumably occurred



(3)

R = C_6H_4CO–Glu

R' = Ribose-5-pyrophosphoryl-(5'-adenosine-2'-phosphate)

X = $CONH_2$

during the 3.5-h incubation period and the column purification step.

Discussion

Tatum et al.[4] had originally shown that reduction of (6*R*)-5,10-methenyltetrahydrofolate with $NaBT_4$ in the presence of either *N*,*N*-diethylaniline or pyridine gave (6*R*)-5,10-methylene[11-^{3}H]tetrahydrofolate possessing a 76:24 and 50:50 ratio, respectively, of diastereomers at C11. Our experiments show that the stereospecificity of the reduction is actually much higher (92:8 and 75:25, respectively), and that an acidic workup resulted in the partial racemization observed.[4] One possible explanation for this high stereospecificity is that the aromatic base can coordinate with the electron-deficient imidazolinium ring, preferably on the less sterically hindered side. This might involve stacking of the aromatic rings and preclude the attack of borohydride on what would be in the absence of *N*,*N*-diethylaniline, the less hindered side. Pyridine, which is less basic and not as bulky, might simply be less effective at coordinating with the amidine or in showing a preference for one face of the imidazolinium ring. Support for the theory that the base is affecting the accessibility of hydride to one of the faces of the imidazolinium ring is also provided by the NMR experiments described above, where it was demonstrated that chemical reduction of (6*R*)-5,10-methenyl-[11-^{2}H]tetrahydrofolate by $NaBH_4$ generated (6*R*,11*R*)-5,10-methylene[11-^{1}H,^{2}H]tetrahydrofolate but that enzymatic reduction of the same starting material gave the opposite diastereomer. If one assumes that the dehydrogenase transfers hydride from the less hindered side, it is puzzling that $NaBH_4$, in the presence of *N*,*N*-diethylaniline, chooses the opposite face.

Since the NOE experiments described under Results defined the assignments of the C11 diastereotopic hydrogens, it is now possible to correlate these results with those of the enzymatic studies. As is shown in Figure 2, (3*S*)-[3-^{2}H]serine (used as an example) exists on serine hydroxymethyltransferase in a Schiff base linkage with pyridoxal 5-phosphate (**7**), which stabilizes the glycine anion resulting from a retro-aldol cleavage.[22] Although shown in a conformation where all substituents are eclipsed, the actual conformation is unknown. For this reason it is impossible to predict the relative orientation of the resulting one-carbon

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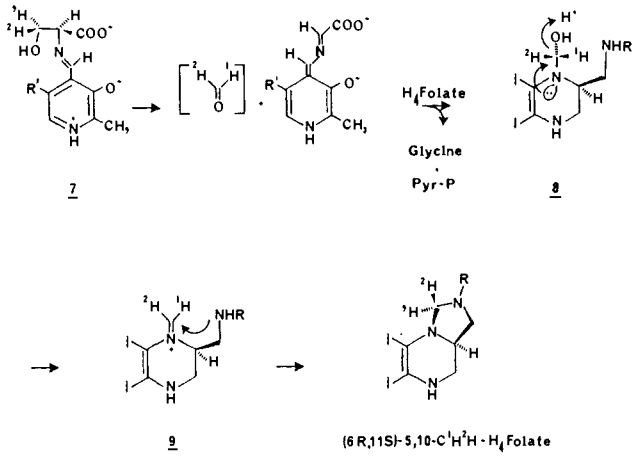

Figure 2. Proposed stereochemical path of serine hydroxymethyltransferase. R = *p*-C_6H_4CO-Glu; R′ = $CH_2OPO_3H^-$.

formaldehyde equivalent whether it exists as free formaldehyde or as an enzyme-bound thiohemiacetal.[23] If free formaldehyde, the 25% crossover of label from each serine stereoisomer into the 5,10-methylenetetrahydrofolate argues against complete torsional symmetry owing to some degree of enzymatic restraint. Either species must then produce the N^5-hydroxymethyl derivative (**8**) predicted from the work of Kallen and Jencks on the kinetics of formation of 5,10-methylenetetrahydrofolate from formaldehyde and tetrahydrofolate.[24] The stereochemistry at C11 is depicted as *S*, although this is arbitrary. However, elimination of H_2O must give the *Z* isomer of the N^5-iminium species (**9**) since the *S* stereochemistry at C6 requires attack of N10 from above the C11–N5 plane to give predominantly (11*S*)-5,10-methylene[11-^{1}H,^{2}H]tetrahydrofolate.[25] Although rotation of an intermediate may be responsible for the observed epimerization, the observed preference for the erythro isomer in the cleavage of L-allothreonine/L-threonine and erythro-/threo-β-phenylserine by the same enzyme in the absence of tetrahydrofolate[22,26] suggests that the transferase accommodates two different conformations of serine at the β carbon. Thus the formaldehyde equivalent might be released initially in two orientations relative to tetrahydrofolate, likewise resulting in the observed crossover.

Although serine hydroxymethyltransferase clearly exhibits partial stereochemical control over the conversion of serine to 5,10-methylenetetrahydrofolate in the first turnover, the approach to chemical equilibrium is concomitant with isotopic equilibration (i.e., complete scrambling at C11) as observed in the NMR experiments. Since the overall equilibrium constant is only 10–12,[27] the substrates and products can undergo rapid interconversion on the enzyme surface with partial epimerization occurring with each turnover, and this obviates this method for synthesis of the desired chiral 5,10-methylene derivative.

One can also correlate these results with the stereochemical information already known about the mechanism of thymidylate synthetase.[5] Figures 3 and 4 depict the proposed stereochemical pathway for the conversion of (6*R*,11*S*)-5,10-methylene[11-^{2}H,^{3}H]tetrahydrofolate to (*S*)-methyl[*methyl*-^{1}H,^{2}H,^{3}H]TMP. The first step in Figure 3 presumes the antiperiplanar opening[25] of the imidazolidine ring, since the lone pair of electrons on N5

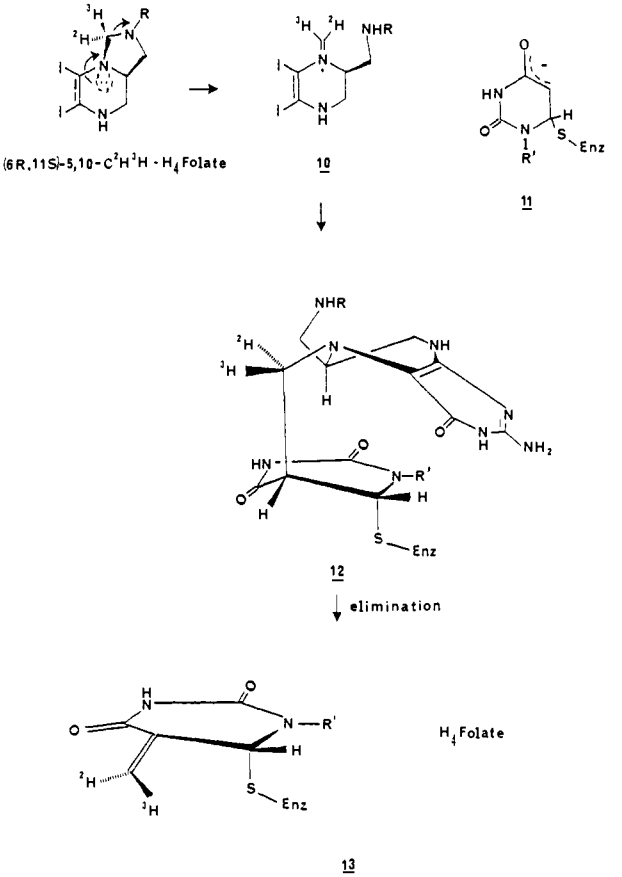

Figure 3. Proposed stereochemical path for formation of **13** by thymidylate synthetase. R = *p*-C_6H_4CO-Glu; R′ = 2′-deoxyribose 5′-phosphate.

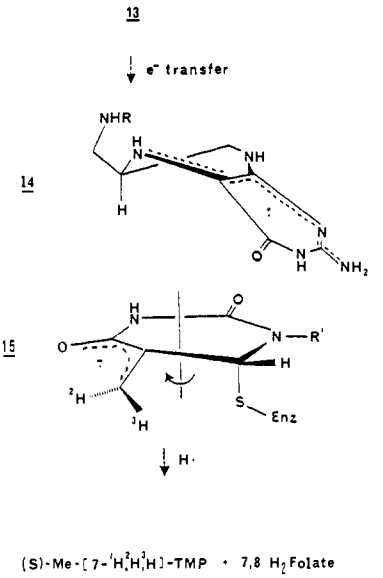

Figure 4. Proposed mechanism for conversion of **13** to TMP and 7,8-dihydrofolate. R = *p*-C_6H_4CO-Glu; R′ = 2′-deoxyribose 5′-phosphate.

is almost antiperiplanar to the breaking C11–N10 bond. Since the conformational restraints of the fused imidazolidine–tetrahydropyrazine ring system preclude inversion at N5,[8] this would seem to eliminate the possibility of a syn elimination. The resulting N^5-iminium cation **10** must then have the *Z* configuration, as shown. Nucleophilic attack by enzyme-bound dUMP (**11**)[16] on C11 can then occur from the *si* face to give the ternary complex (**12**) followed by elimination to give enzyme-bound 5-CH_2dUMP and tetrahydrofolate (**13**).

(23) A mechanism involving dehydration of serine to α-aminoacrylate followed by a Michael addition of N5 from tetrahydrofolate is precluded since α-methylserine is a substrate for the enzyme (Schirch, L.; Diller, A. *J. Biol. Chem.* **1971**, *246*, 3961–3966).

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(25) Ring closure represents a disfavored 5-endo-trig cyclization (Baldwin, J. E. *J. Chem. Soc., Chem. Commun.* **1976**, 734–736); however there are compelling kinetic[24] and stereochemical (Kirby, A. J. "The Anomeric Effect and Stereoelectronic Effects at Oxygen"; Springer-Verlag: New York, 1983; pp 91–94) arguments in favor of the dehydration–addition ring closure sequence.

(26) Schirch, L.; Gross, T. *J. Biol. Chem.* **1968**, *243*, 5651–5655.

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The depicted conformation of complex **12** is based on ^{19}F NMR studies performed on the ternary complex of FdUMP-5,10-methylenetetrahydrofolate-thymidylate synthetase in both the native[28] and denatured[28,29] states. These measurements of the C5-F analogue of **12** confirmed the fluorine atom to be in a pseudoequatorial position in the native enzyme complex, with C6-H also equatorial, and the Enz-S and C11-CH_2 bridge moieties in a trans-diaxial conformation.[28] Indirect measurement of J_{HF} values between C5-F and C11-2H suggested that the C5-F bond (and therefore presumably the C5-H bond of the native species) was neither syn nor anti to the C11-N5 bond. Thus, whatever the conformation of the ternary complex, the enzyme must induce a conformation change prior to elimination.

The formation of the methyl group has been postulated to occur through the transfer of C6-H as a hydride equivalent to the exocyclic C5-methylene of the uracil ring mainly on the basis of model reactions.[30] This results in oxidation of the tetrahydropterin ring to the 7,8-dihydro form in a single two-electron step. However, tetrahydrofolate or tetrahydropterin oxidation by O_2, Br_2,[31] or ferricyanide[32] proceeds through a two-electron oxidation to quininoid dihydrofolate (or quininoid dihydropterin). The quininoid species then undergoes spontaneous rearrangement to the more stable 7,8-dihydro tautomer.[31] On chemical grounds the direct oxidation to 7,8-dihydrofolate necessitated by hydride transfer in the thymidylate synthetase reaction is less likely. Furthermore, Ehrenberg[33] has shown by electron spin resonance spectroscopy that a radical cation is formed in a chemical oxidation of tetrahydropterins, thus indicating that the two-electron oxidation to quininoid dihydropterin probably occurs in two one-electron steps.

These steps can then be extrapolated to the proposed mechanism for thymidylate synthetase shown in Figure 4. The placement of the two rings within **13** in a stacked conformation positions them for electron transfer. The first step in Figure 4 involves transfer of one electron from tetrahydrofolate to 5-methylene-dUMP to generate the radical cation **14** and the radical anion **15**. The enzyme could then rotate the methylene carbon directly under C6-H and facilitate abstraction of H· along a linear transition pathway. From this it is clear that attack of **11** must occur on the *si* face of **10** (i.e., the same side as C6-H). Transfer of H· from C6 of **14** to **15** then completes the reduction. Elimination of enzyme-SH finally gives (*S*)-methyl[7-^{1}H,^{2}H,^{3}H]-TMP (**16**) and 7,8-dihydrofolate.

Both the hydride and H· mechanisms are of course compatible with the stereochemical evidence.[34] It is our intent to suggest the possibility that quininoid species, which have been directly observed in hydroxylation reactions catalyzed by enzymes that require tetrahydrobiopterin,[16] may also be involved in one-carbon transfers involving tetrahydrofolate.

Acknowledgment. We thank Drs. Lloyd M. Jackman and Charles W. DeBrosse for their helpful suggestions and discussions and the National Institutes of Health for financial support (GM 24129).

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(34) Transfer of C6-H as a proton would require a prior two-electron reduction of 5-methylene-dUMP to generate a nonresonance stabilized carbanion at C7 of the incipient thymidine ring and is disfavored on those grounds.
(35) **Note Added in Proof**: The stereoselectivity of the chemical reduction may also stem from a stereoelectronic component with hydride approaching C11 antiperiplanar to the lone pair on N5, whose preferred orientation results from the favored cis ring junction in the product (Cieplak, A. S., personal communication).

The Walling, El-Taliawi, and Zhao[1] "Carbonyl Effect" in Radical Brominations Is an Example of HBr Reversal. It Is Not Relevant to π and σ Radical Chemistry

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Received July 27, 1983

Abstract: Walling, El-Taliawi, and Zhao[1] claim that our "S_π chemistry" is reproduced in photobrominations employing Br_2 in the presence of carbonyl compounds (no NBS). Photobromination of alkanes by *N*-bromosuccinimide (NBS) shows selectivities that vary, but which show limiting values for significant ranges of reaction conditions. We have attributed these different limiting values to three reaction paths which differ in involving either Br·, S_σ, or S_π as the intermediate hydrogen-abstracting radical. Walling, El-Taliawi, and Zhao (WEZ) have reported selectivities they believe are attributable to a carbonyl-bromine intermediate rather than our S_π. Evidence is now presented to show that the WEZ results are due to HBr in varying amounts with *or without* carbonyl compounds present. This represents a fourth type of selectivity and one that is sensitive to HBr concentrations and probably arises in their systems from the following reaction: R + HBr $\rightleftarrows$ RH + Br·. There is no carbonyl effect. This fourth type had been deliberately excluded from our work, so that reactions involving HBr reversal are not relevant to our published work on succinimidyl chemistry. Thus, the selectivities (per H basis) for the neopentane/methylene chloride competitions stand without modification, as published earlier: 0.067, 1.0, and 17 for Br·, S_π, and S_σ, respectively.

Walling, El-Taliawi, and Zhao (WEZ) have stirred up a controversy[1] by challenging our hypothesis that both π and σ states are required to explain the thermal chain reactions of succinimidyl radicals generated in halogen abstraction reactions from *N*-bromo- or *N*-chlorosuccinimide.[2] The method of private approach having failed, it has been our reluctant duty to examine the evidence on which they based their claims. There are some areas of agreement.

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