Stereochemistry of reduction of methylenetetrahydrofolate to methyltetrahydrofolate catalyzed by pig liver methylenetetrahydrofolate reductase

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make further predictions about biosynthetic processes leading to manumycin group antibiotics. There appear to be two separate enzymes, or enzyme systems, that are essential for the incorporation of the natural mC_7N unit as a central starter unit. One of them, an amide synthase, is responsible for the connection of the upper side chain to the C2 amino group. The other activates the mC_7N unit carboxyl group (CoA transferase) for the subsequent chain-extension process via the polyketide pathway. The amide synthase and the CoA transferase act in a noncoupled fashion. It seems very likely that the amide synthase and/or the polyketide synthase have tight substrate specificities, since only a few artificial precursors were acceptable to them. The fact that feeding experiments with 3-hydroxybenzoic acid produced only a lower chain elaborated analogue demonstrates that an amino group is necessary for attachment of the upper chain carboxylic acid. The enzyme involved appears to be able to synthesize only amides but not esters. The polyketide synthase involved in lower chain formation is highly specific in its action, and termination of elongation appears to be under strict control of the synthase or, less likely, of a second amide synthase that is responsible for connecting the activated trienecarboxylic acid to the C_5N unit. While the precursor-directed biosynthesis studies were conducted only with the manumycin producer, due to the correspondence of all the isotope feeding data for **1** and **2**, it is likely that these predictions can be extended to other members of the manumycin group of antibiotics.

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Supplementary Material Available: Pulse-decoupled ^{13}C NMR spectra and 2D INADEQUATE spectra of **1** and **2** labeled from [U-$^{13}C_3$]glycerol (5 pages). Ordering information is given on any current masthead page.

Stereochemistry of Reduction of Methylenetetrahydrofolate to Methyltetrahydrofolate Catalyzed by Pig Liver Methylenetetrahydrofolate Reductase

Maria A. Vanoni,[†] Sungsook Lee,[‡] Heinz G. Floss,[‡] and Rowena G. Matthews*

Contribution from the Department of General Physiology and Biochemistry, University of Milan, 20133 Milan, Italy, the Department of Chemistry, University of Washington, Seattle, Washington 98195, and the Biophysics Research Division and Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109.
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Abstract: Methyltetrahydrofolate; chirally labeled with deuterium and tritium in the methyl group, was synthesized from L-(3*R*)- or L-(3*S*)-[3-^{3}H]serine, tetrahydrofolate, and NADPH by coupling the reactions of pig liver methylenetetrahydrofolate reductase and serine hydroxymethyltransferase in deuterated aqueous buffer. The stereochemistry at the N^5 methyl group of the methyltetrahydrofolate products was determined by chemical degradation of methyltetrahydrofolate to methylamine and conversion to acetate. L-(3*R*)-[3-^{3}H]Serine is converted to (*S*)-acetate, which is derived from (*methyl-R*)-methyltetrahydrofolate, and L-(3*S*)-[3-^{3}H]serine is converted to (*R*)-acetate. The reaction proceeds with ~75% stereospecificity. The results indicate that reduction of the methylene group of methylenetetrahydrofolate takes place with addition of hydrogen to the more sterically accessible face of the pteridine; this is the same face from which methylenetetrahydrofolate dehydrogenase abstracts a hydride equivalent and it is the face attacked by enzyme-bound dUMP in the thymidylate synthase reaction (Slieker, L. J.; Benkovic, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 1833-1838). To determine the degree to which racemization of methylenetetrahydrofolate was occurring in solution during the coupled synthesis of methyltetrahydrofolate, the stereochemical course of the serine hydroxymethyltransferase reaction was investigated under the same conditions in a coupled reaction with methylenetetrahydrofolate dehydrogenase, as initially described by Tatum and co-workers (Tatum, C. M.; Benkovic, P. A.; Benkovic, S. J.; Potts, R.; Schleicher, E.; Floss, H. J. *Biochemistry* **1977**, *16*, 218-220). In contrast to the results reported earlier, we observed greater than 95% stereospecificity in this coupled reaction, suggesting that little or no racemization associated with the production of methylenetetrahydrofolate from serine occurred under the conditions employed for synthesis of methyltetrahydrofolate.

Methylenetetrahydrofolate reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of CH_2-H_4folate to CH_3-H_4folate according to eq 1.

$$\text{NADPH} + \text{CH}_2\text{-H}_4\text{folate} \rightarrow \text{NADP}^+ + \text{CH}_3\text{-H}_4\text{folate} \quad (1)$$

Previous studies have shown that the reaction proceeds via a ping-pong kinetic mechanism in which $NADP^+$ release from the enzyme precedes the binding of CH_2-H_4folate.[1,2] The enzyme catalyzes the transfer of the 4*S* hydrogen of NADPH to the flavin coenzyme, FAD.[3] Reduction of CH_2-H_4folate in deuterated aqueous buffer occurs with the incorporation of one deuteron from solvent into the methyl group of CH_3-H_4folate.[4,5] It is not known

* Address correspondence to this author at: Biophysics Research Division, The University of Michigan, 2200 Bonisteel Blvd., Ann Arbor, MI 48109.
[†] University of Milan.
[‡] University of Washington.

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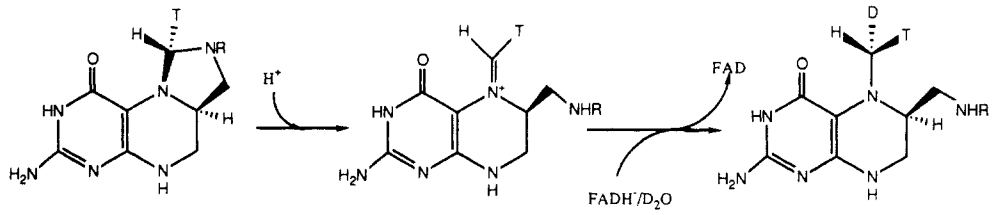
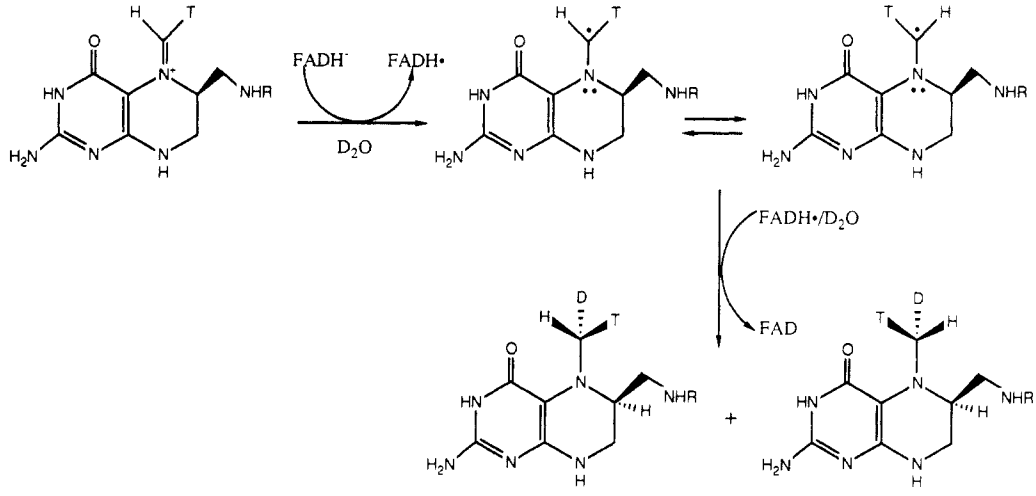
Scheme I. Postulated Mechanism for Two-Electron Reduction of Methylenetetrahydrofolate to Methyltetrahydrofolate by Methylenetetrahydrofolate Reductase



(11*R*)-5,10-CH_2-H_4folate 5-iminium cation (*R*-methyl)CH_3-H_4folate

Scheme II. Postulated Mechanism for One-Electron Reduction of Methylenetetrahydrofolate to Methyltetrahydrofolate by Methylenetetrahydrofolate Reductase

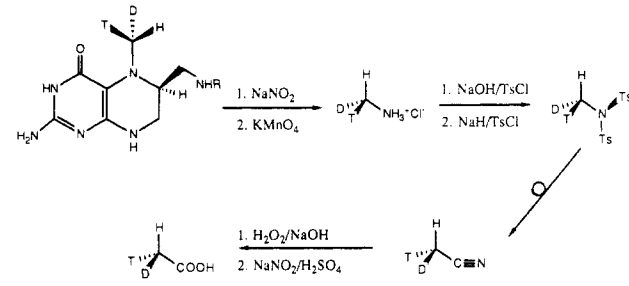


whether the solvent-derived hydron is introduced from an exchangeable position on the flavin cofactor or from a general-base catalyst. However, the enzyme also catalyzes the reduction of quinoid dihydropteridines that lack a substituent at N^5 and this observation suggests transfer of electrons/protons to the pteridine ring rather than hydride transfer to the exocyclic methylene group of CH_2-H_4folate.[6] Enzymatic reduction of CH_2-H_4folate presumably requires the intermediate formation of the 5-iminium cation[7] (Scheme I), although this has not been demonstrated. Neither is it known whether the reduction proceeds in one-electron or two-electron steps.

We now present the results of experiments that were designed to determine the stereochemistry of reduction of CH_2-H_4folate to CH_3-H_4folate catalyzed by pig liver methylenetetrahydrofolate reductase. Structural studies on (6*R*)-CH_2-H_4folate[8,9] have shown that the imidazolidine ring protrudes at an angle to the plane of the pteridine ring, so that the face of the pteridine opposite the imidazolidine ring is more sterically accessible to bulky reactants. Both the hydride transfer from CH_2-H_4folate to $NADP^+$ catalyzed by methylenetetrahydrofolate dehydrogenase and the attack of enzyme-bound dUMP catalyzed by thymidylate synthase occur at the more sterically accessible face of the pteridine ring.[9] The observation of reduction by hydrogen transfer to the less sterically accessible face of the pteridine ring (i.e., the face opposite the hydrogen at C^6) might suggest proton rather than hydride transfer to the methylene group. The observation of complete racemization of the methylene group during enzymatic reduction might suggest radical intermediates in the reaction of methylenetetrahydrofolate reductase (Scheme II).

Our approach involved the enzymatic synthesis of CH_3-H_4folate from (3*R*)- or (3*S*)-[3-^{3}H]serine in deuterated aqueous buffer

Scheme III. Sequence of Reactions Used To Convert Methyltetrahydrofolate to Acetate



using pig liver serine hydroxymethyltransferase and (6*S*)-H_4folate to generate (6*R*,11*R*)- or (6*R*,11*R*)-CH_2-H_4folate, respectively[9,10] and concomitant reduction of the resultant CH_2-H_4folate to CH_3-H_4folate with NADPH and an excess of pig liver methylenetetrahydrofolate reductase. The configuration of the N^5 methyl group of CH_3-H_4folate was then determined by degradation to methylamine[11,12] and conversion of methylamine to acetate[13] for chirality analysis.[14-16] Because our reaction conditions differed somewhat from those employed by Tatum et al.[10] in coupling the serine hydroxymethyltransferase reaction to methylenetetrahydrofolate dehydrogenase, it was necessary to repeat their experiments under our reaction conditions to correct for any

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Table I. Stereochemical Analysis of the Serine Hydroxymethyltransferase Reaction

substrate	incub time, min	% dpm ^3H CH^+-H_4folate	% dpm ^3H NADPH	f_{NADPH}	$f_{CH^+\text{-}H_4folate}$
(3*R*)-[3-^{3}H]serine/H_2O	1	0.4	7.2	0.95	0.05
	6	1.8	27.7	0.94	0.06
	20	4.4	48.3	0.92	0.08
		av values		0.94 ± 0.02	0.06 ± 0.02
(3*S*)-[3-^{3}H]serine/H_2O	1	6.8	0.4	0.06	0.94
	6	23.4	0.3	0.01	0.99
	20	55.9	5.0	0.08	0.92
		av values		0.05 ± 0.04	0.95 ± 0.04

time, min	$f_{NADPH(R)}$	$f_{NADPH(S)}$	$f_{CH^+\text{-}H_4folate(R)}$	$f_{CH^+\text{-}H_4folate(S)}$
1	0.95	0.05	0.06	0.94
6	0.99	0.01	0.07	0.93
20	0.91	0.09	0.08	0.92
av values	0.95 ± 0.04	0.05 ± 0.04	0.07 ± 0.01	0.93 ± 0.01

substrate	time, incub min	% dpm ^3H CH^+-H_4folate	% dpm ^3H NADPH	f_{NADPH}	$f_{CH^+\text{-}H_4folate}$
(3*R*)-[3-^{3}H]serine/D_2O	20	3.9	91.5	0.96	0.04
(3*S*)-[3-^{3}H]serine/D_2O	20	91.1	3.5	0.04	0.96

time, min	$f_{NADPH(R)}$	$f_{NADPH(S)}$	$f_{CH^+\text{-}H_4folate}$	$f_{CH^+\text{-}H_4folate(S)}$
20	0.96	0.04	0.04	0.96

racemization of CH_2-H_4folate that might be occurring in solution during our experiments. We have observed a much greater stereochemical control of the serine hydroxymethyltransferase reaction than has been previously reported.[10,17,18]

Results

Determination of the Stereochemistry of the Reaction Catalyzed by Pig Liver Serine Hydroxymethyltransferase. We wished to determine the extent of racemization of CH_2-H_4folate under conditions that would permit optimal coupling of serine hydroxymethyltransferase with methylenetetrahydrofolate reductase. Although Tatum and co-workers[10] had shown that phosphate buffer and low pH accelerate the racemization of CH_2-H_4folate, the K_m value of methylenetetrahydrofolate reductase for CH_2-H_4folate increases as the pH is raised above 7,[2] or if cationic buffers are used instead of phosphate buffer.[6] Accordingly, we decided to investigate racemization of CH_2-H_4folate in dilute phosphate buffer, 10 mM, at pH 7 and included 2-mercaptoethanol to trap dissociated formaldehyde by thiohemiacetal formation.

Reaction mixtures containing varying amounts of methylenetetrahydrofolate dehydrogenase and serine hydroxymethyltransferase were incubated in the presence of radiolabeled serines and then injected onto the HPLC for analysis. The radioactivity of each fraction was measured by scintillation counting. When L-(3*R*,*S*)-[3-^{3}H]serine was the substrate, radioactivity was associated with both the CH^+-H_4folate and NADPH peaks and with a third peak eluting at ~27 min. This peak has the same retention time as $NADP^+$ and 10-formyltetrahydrofolate, which coelute in the analytic protocol employed for product separation. 10-Formyltetrahydrofolate is the expected product of the cyclohydrolase activity of the trifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase/10-formyltetrahydrofolate synthetase protein used in these incubations. However, nonenzymatic hydrolysis of CH^+-H_4folate is extremely slow in the buffer employed for these experiments, and the cyclohydrolase activity of the trifunctional enzyme from pig liver is inhibited at the $NADP^+$ concentration (180 μM) used for the coupled reaction. The radiolabel found in the third peak was shown to be associated with $NADP^+$ by conversion to NADPH on incubation with glucose 6-phosphate dehydrogenase and glucose 6-phosphate. The appearance of radiolabel in $NADP^+$ was greatest when incubations were performed in the presence of high levels of methylenetetrahydrofolate dehydrogenase, and when L-(3*R*)-[3-^{3}H]serine was the substrate, i.e., conditions that result in the majority of the tritium from serine being incorporated into (4*R*)-[4-^{3}H]-NADPH. Control experiments suggested that the tritiated $NADP^+$ was formed by a contaminant of the trifunctional protein that exhibits *S*-specific NADPH oxidase activity. For our calculations of tritium transferred or retained in the products of the methylenetetrahydrofolate dehydrogenase reaction, the radioactivity associated with $NADP^+$ was ascribed to the NADPH peak. This assumption was checked by comparing the results of determinations of the fraction of tritium transferred from CH_2-H_4folate to NADPH in samples that were incubated prior to HPLC analysis with glucose 6-phosphate dehydrogenase and glucose 6-phosphate and in samples that had not been so treated. The results obtained by the two methods agreed within experimental error (data not shown).

When the coupled serine hydroxymethyltransferse/methylenetetrahydrofolate dehydrogenase reaction was carried out in the presence of racemic L-[3-^{3}H]serine, the label was equally distributed between CH^+-H_4folate and NADPH. A set of experiments was performed in which both the incubation time and the ratio of serine hydroxymethyltransferase activity to methylenetetrahydrofolate dehydrogenase activity were changed using L-(3*R*)-[3-^{3}H]serine. When the reaction was properly coupled, a sufficient excess of methylenetetrahydrofolate dehydrogenase activity over serine hydroxymethyltransferase activity was present to ensure very low steady-state concentrations of the intermediate CH_2-H_4folate. This resulted in low levels of tritium in the fraction eluting from the HPLC column at 32 min (the retention time of CH_2-H_4folate) and a lack of dimedone-extractable counts in the void volume, where tritiated formaldehyde formed by dissociation from CH_2-H_4folate elutes. It was found that a ratio of methylenetetrahydrofolate dehydrogenase to serine hydroxymethyltransferase activity[19] of 5 or more was sufficient to lower the concentration of CH_2-H_4folate to undetectable levels, and activity ratios of 8 were used for all further experiments.

Table I summarizes a set of experiments in which the tritiated products of coupled serine hydroxymethyltransferase/methylenetetrahydrofolate dehydrogenase reactions were analyzed in both protonated and deuterated solvent. Identical results were obtained

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(19) Units of activity for both enzymes were expressed as micromoles of CH_2-H_4folate formed or consumed per minute at 25 °C in 10 mM potassium phosphate buffer, pH 7.0. Where reactions were to be run in D_2O, activities of the enzymes were also measured in D_2O.

Table II. Stereochemical Analysis of the Methylenetetrahydrofolate Reductase Reaction

substrate	incub time, min	% rxn	F value (acetate)	% ee[16]
(3R)-[3-^{3}H]serine	10	47	37	45 S
	30	73	38	41 S
(3S)-[3-^{3}H]serine	30	77	66, 63[a]	50 R
	60	93	66, 64[b]	52 R

[a] Results of duplicate degradations of the same CH_3-H_4folate sample. [b] Results of duplicate enzymatic preparations of CH_3-H_4folate.

whether the reaction was carried out in H_2O or D_2O, and whether the results were analyzed by the method of Battersby[20] or by simple calculation of the fractions of tritium retained in CH^+-H_4folate or transferred to NADPH. In agreement with earlier results of Tatum et al.,[10] the bulk of tritium from (3R)-[3-^{3}H]serine was transferred to NADPH, while the bulk of tritium from (3S)-[3-^{3}H]serine was retained in CH^+-H_4folate. However, in contrast to these earlier experiments we observed much greater stereochemical control, with only ~10% racemization associated with the coupled reactions.

Measurement of the Secondary Tritium Kinetic Isotope Effect of the Reaction of Serine Hydroxymethyltransferase. The two methods applied to monitor the degree of retention of stereochemistry of the coupled reaction gave identical results, suggesting that no secondary tritium isotope effect on V/K was associated with the serine hydroxymethyltransferase reaction. The secondary tritium kinetic isotope effect on the transfer of the β-carbon of serine to H_4folate during the reaction catalyzed by pig liver serine hydroxymethyltransferase was directly determined by using a mixture of [U-^{14}C]serine and (3R,S)-[3-^{3}H]serine and measuring fractional conversions of both bulk and tritiated substrate with the ^{14}C label as a tracer for reaction of the bulk substrate. An average secondary tritium kinetic isotope effect on V/K of 1.005 ± 0.018 was calculated, indicating that within experimental error no secondary tritium kinetic isotope effect was associated with the transfer of the β-carbon of serine to H_4folate.

Analysis of the Stereochemistry of the Methylenetetrahydrofolate Reductase Reaction. [*Methyl*-$^2H_1$,^{3}H]CH_3-H_4folate was produced enzymatically by coupling the reactions of pig liver serine hydroxymethyltransferase and pig liver methylenetetrahydrofolate reductase in D_2O, using mixtures of [3-^{14}C]serine and (3R)- or (3S)-[3-^{3}H]serine as described in their experimental procedures, and the chirality of the methyl group was analyzed after conversion to acetate. The results of these analyses are shown in Table II. In coupling these reactions, we again employed a 8:1 ratio of methylenetetrahydrofolate reductase activity to serine hydroxymethyltransferase activity.[19] Under these conditions, no CH_2-H_4folate could be detected in the reaction mixture. The average F value[16] obtained with L-(3R)-[3-^{3}H]serine was 65 ± 2 regardless of the extent of reaction in the sample from which CH_3-H_4folate was isolated. When the 3S enantiomer of serine was used, an average F value of 37 ± 1 was obtained, and this value was again independent of the extent of the reaction.

Discussion

As shown in Scheme III, the chemical conversion of the methyl group of CH_3-H_4folate to acetate occurs with an inversion of configuration in the conversion of ditosylated methylamine to acetonitrile. Thus (3R)-[3-^{3}H]serine is converted enzymatically to (*methyl-R*)-[*methyl*-$^2H_1$,^{3}H]CH_3-H_4folate, which yields (S)-acetate. Serine hydroxymethyltransferase from rabbit liver has previously been shown to convert (3R)-[3-^{3}H]serine to (11R)-5,10-methylene[11-^{3}H]H_4folate,[9,10] and methylenetetrahydrofolate dehydrogenase from *Saccharomyces cervisiae* stereospecifically removes H_R from the methylene group.[22] Methylenetetrahydrofolate dehydrogenase from pig liver also abstracts H_R from the methylene group of 5,10-methylene[11-^{3}H]H_4folate,[23] and the experimental results summarized in Table I indicate that serine hydroxymethyltransferase from pig liver transfers the β-carbon of serine to H_4folate with the same stereochemistry as the rabbit liver enzyme. Scheme I depicts the proposed stereochemical pathway for the reduction of 5,10-methylene[11-^{3}H]H_4folate catalyzed by methylenetetrahydrofolate reductase. If we assume an antiperiplanar ring opening of CH_2-H_4folate to generate a 5-iminium cation,[9] reduction of the iminimum cation must be occurring with incorporation of a deuterium from solvent from the *re* face of C^{11} to generate (*methyl-R*)-[*methyl*-$^2H_1$,^{3}H]-CH_3-H_4folate.

The observed conversion of the β-carbon of serine to the methyl group of CH_3-H_4folate proceeds with approximately 50% racemization at the transferred carbon. Apparent racemization could result during the transfer of the β-carbon of serine to H_4folate, since the serine hydroxymethyltransferase reaction is reported to result in the loss of ~50% of the stereochemical purity of the transferred carbon center.[10] However, our analysis of the stereochemistry of the reaction catalyzed by serine hydroxymethyltransferase from pig liver indicates only about a 10% loss of stereochemistry at the transferred carbon (Table I). Since these experiments were conducted under the same conditions as the coupled serine hydroxymethyltransferase/methylenetetrahydrofolate reductase reactions used to generate chirally labeled CH_3-H_4folate for stereochemical analysis, we do not believe that the racemization associated with formation of CH_3-H_4folate derives primarily from the serine hydroxymethyltransferase reaction. Racemization could be occurring at the level of the free intermediate, CH_2-H_4folate, which can undergo reversible hydration to form the carbinolamine. However, since both the coupled serine hydroxymethyltransferase/methylene tetrahydrofolate dehydrogenase and serine hydroxymethyltransferase/methylene tetrahydrofolate reductase reactions were conducted with an 8-fold excess of dehydrogenase or reductase activity over serine hydroxymethyltransferase activity, and the levels of CH_2-H_4folate were undetectable under these circumstances, this does not seem to be a likely explanation of the racemization associated with formation of CH_3-H_4folate. Several other possibilities for racemization remain. The reduction of CH_2-H_4folate to CH_3-H_4folate catalyzed by methylenetetrahydrofolate reductase is associated with a large isotope effect governing the incorporation of solvent hydrogen into the methyl group (k_D/k_T = 2.75, corresponding to k_H/k_D = 10).[5] Although we attempted to ensure that the reduction was carried out in fully deuterated solvent, by repeatedly exchanging all the assay reagents with deuterated buffer, any hydrogen in the solvent would be preferentially incorporated into the methyl group of CH_3-H_4folate, and the resultant [*methyl*-$^1H_2$,^{3}H]CH_3-H_4folate would contribute to the apparent racemization associated with the reaction. It is also possible that the methylenetetrahydrofolate reductase reaction itself results in racemization or in exchange of the protons of the methylene group with those of solvent. Less than 8.5% exchange of the methylene protons of CH_2-H_4folate during the enzyme-catalyzed reduction in D_2O was previously estimated to occur.[5] Furthermore, overall reversal of the methylenetetrahydrofolate reductase reaction, which could contribute to racemization, is minimized in the presence of NADPH because this substrate irreversibly reduces the enzyme-bound flavin.[3] However, as indicated in Scheme II, one-electron reduction of the 5-iminium cation by the flavin hydroquinone anion could result in substantial racemization without exchange. Finally, the possibility cannot be dismissed that the chemical degradation procedure used to excise the methyl group from CH_3-H_4folate and convert it into acetate is accompanied by some racemization. This procedure has been used before with this[21] and other substrates,[11,12] but there are no cases in which the product had very high enantiomeric excess, which would validate its complete stereospecificity. Given

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the technical difficulty of these experiments and the possibility of multiple small effects contributing to the loss of stereochemical integrity, we cannot be sure that any racemization derives from the methylenetetrahydrofolate reductase reaction itself.

As shown in Scheme I, addition of a hydrogen from solvent to the *re* face of the 5-iminium cation is occurring from the more sterically accessible face. Stereochemical analysis of the methylenetetrahydrofolate dehydrogenase reaction[9,10] indicates that removal of a hydride equivalent from CH_2-H_4folate also occurs from this side, as does attack of dUMP on the 5-iminium cation in the thymidylate synthase reaction, and transfer of a hydride equivalent from C^6 of the pteridine ring to the exocyclic methylene group of 5-CH_2-dUMP.[10,24]

We have found ~90% retention of stereochemical integrity associated with the transfer of the β-carbon of serine to H_4folate catalyzed by serine hydroxymethyltransferase from pig liver, in contrast to the ~50% loss of chirality observed by Tatum et al.[10] These differences may arise because enzymes from different species were used in these two experiments, or possibly because coupling between serine hydroxymethyltransferase and methylenetetrahydrofolate dehydrogenase is better when both enzymes are from the same species. Similar stereochemical control was observed in either D_2O or H_2O. However, it should be noted that the coupled serine hydroxymethyltransferase/thymidylate synthase reaction used by Tatum et al.[24] to synthesize dTMP for stereochemical analysis resulted in dTMP of unexpectedly high stereochemical purity, with (3*R*)-[3-^{2}H,^{3}H]serine being converted to acetate derived from dTMP with an *F* value of 69, and (3*S*)-[3-^{2}H,^{3}H]serine forming acetate with an *F* value of 31. Taken together with our results, these results suggest that serine hydroxymethyltransferase enzymes from both rabbit and pig liver exhibit a high degree of stereochemical control and that loss of stereochemical integrity associated with coupled reactions involving these enzymes most probably derives from the experimental protocol rather than from the rotation of a "free formaldehyde" intermediate in the reaction sequence involved in the transfer of the β-carbon of serine to H_4folate. The lack of stereochemical control in reactions involving serine hydroxymethyltransferase measured in tissue slices[17,18] or in vivo[25,26] most probably is observed because the enzymes responsible for consumption of CH_2-H_4folate are not present in sufficiently high concentration to prevent racemization of this molecule in solution.

Experimental Section

Preparation and Storage of Enzymes. Methylenetetrahydrofolate reductase was purified from pig liver as previously described.[28] Purified pig liver cytoplasmic serine hydroxymethyltransferase[29] was the kind gift of Dr. Leodis Davis. Purified pig liver trifunctional enzyme,[30] a multifunctional protein with methylenetetrahydrofolate dehydrogenase, methylidene tetrahydrofolate cyclohydrolase, and 10-formyltetrahydrofolate synthetase activities, was obtained from Dr. Robert MacKenzie. Methylenetetrahydrofolate reductase was stored in 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA, and 10% glycerol; serine hydroxymethyltransferase was dialyzed prior to use against 10 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA; the trifunctional enzyme was dialyzed against 10 mM potassium phosphate buffer, pH 7.2, containing 0.4 mM DTT and 20% glycerol.

Enzyme Activity Assays. Methylenetetrahydrofolate reductase activity was measured by the NADPH-menadione reductase assay described previously.[28] Serine hydroxymethyltransferase activity was assayed spectrophotometrically by using β-phenylserine[29] or radiometrically by using H_4folate and serine labeled in the β-position as the substrates, and monitoring the production of labeled CH_2-H_4folate after extraction of the methylene group as a formaldehyde–dimedone complex.[28]

Materials. All radiolabeled compounds were purchased from Amersham except for L-(3*R*)- and L-(3*S*)-[3-^{3}H]serine, which were synthesized as described by Floss et al.[27] (6*S*)-*H*$_4$folate was prepared as previously described.[28] D_2O (99.9% D-atom excess) was purchased from Sigma or from Cambridge Nuclear Laboratories. All other chemicals were used without further purification and were the finest available grade.

Serine Hydroxymethyltransferase–Methylenetetrahydrofolate Dehydrogenase and Serine Hydroxymethyltransferase–Methylenetetrahydrofolate Reductase Coupled Assays. For the stereochemical analyses described in this paper, it was desirable to decrease the concentration of CH_2-H_4folate in solution by employing a large excess of the activity of the enzyme consuming this substrate, as compared to the activity of serine hydroxymethyltransferase, which generates this substrate. This strategy minimizes the opportunity for racemization of CH_2-H_4folate that is chirally labeled in the methylene group by reducing the time available for dissociation into free formaldehyde and tetrahydrofolate or formation of the carbinolamine intermediate[7] and then re-formation of CH_2-H_4folate. The degree of coupling of the serine hydroxymethyltransferase–methylenetetrahydrofolate dehydrogenase reaction was determined in several ways: the rate of production of CH^+-H_4folate was monitored spectrophotometrically at varied concentrations of the two enzymes; the level of free formaldehyde or CH_2-H_4folate was determined at several time points by the dimedone assay[28] using serine labeled in the β-position or alternatively the reaction products were separated by reversed-phase HPLC as described below and analyzed for the presence of either labeled formaldehyde or labeled CH_2-H_4folate. Reaction mixtures contained 10 mM potassium phosphate buffer, pH 7.0 45 mM 2-mercaptoethanol, 180 μM $NADP^+$, 0.09 mM EDTA, 100–400 μM (6*S*)-H_4folate or 200–800 μM (6-*R*,*S*)-H_4folate, and 50–100 μM L-serine (specific radioactivity, ~80 000 dpm/nmol when labeled serine was used). For an adequately coupled reaction system the rate of formation of CH^+-H_4folate was proportional to serine hydroxymethyltransferase activity and independent of methylenetetrahydrofolate dehydrogenase activity and no free formaldehyde or CH_2-H_4folate was detectable by either dimedone extraction of the assay mixture or analyses of the HPLC elution profiles. To determine the conditions for adequate coupling of serine hydroxymethyltransferase and methylenetetrahydrofolate reductase the reactions were carried out in 10 mM potassium phosphate buffer, pH 7.0, 0.09 mM EDTA, 200 μM NADPH, 380 μM (6*S*)-H_4folate, and 50–100 μM L-serine labeled in the β-position. The amounts of free formaldehyde and CH_2-H_4folate were determined either by dimedone extraction of aliquots of the reaction mixture or by analyzing the HPLC elution profiles of the labeled reaction products.

Analytical Separation of the Reaction Components. The components of the coupled serine hydroxymethyltransferase–methylenetetrahydrofolate dehydrogenase or serine hydroxymethyltransferase–methylenetetrahydrofolate reductase reaction systems were separated by injecting 50–100-μL aliquots of the reaction mixture on a 5 μ Altex Ultrasphere ODS HPLC column (0.46 × 25 cm). The column was equilibrated with 5 mM tetrabutylammonium phosphate (Pic A, Waters) and eluted with a 0–30% propanol gradient.[31] The absorbance at 254 nm of the eluate was monitored continuously and 0.5- or 1-mL fractions were collected and analyzed spectrophotometrically or radiometrically. Retention times for the analytical separation were as follows: serine and formaldehyde, 3 min; CH^+-H_4folate, 24 min; $NADP^+$ and 10-CHO-H_4folate, 27 min; CH_2-H_4folate, 32 min; NADPH, 42 min.

Purification of CH_3-H_4folate. Tetrabutylammonium phosphate was shown to interfere with the analysis of the stereochemistry of the N^5-methyl group of methyltetrahydrofolate produced by the coupled serine hydroxymethyltransferase–methylenetetrahydrofolate reductase reaction system and it was difficult to remove residual tetrabutylammonium phosphate quantitatively. Therefore we developed a different HPLC chromatographic separation of the reaction components. Aliquots of the reaction mixture (100–1000 μL) were injected on a 5 μ Altex Ultrasphere ODS HPLC column equilibrated with 10 mM acetic acid (buffer A) and run at a flow rate of 1 mL/min. The column was eluted for 6 min with 100% buffer A, then for 30 min with a linear gradient from 0 to 15% buffer B (10 mM acetic acid in 100% methanol), and for 50 min with 15% buffer B. The absorbance of the eluate was monitored continuously at 254 nm and 1-mL fractions were collected. CH_3-H_4folate-containing fractions elute with a retention time of 50 min, while serine elutes at 30 min, CH_2-H_4folate at 8 min, $NADP^+$ at 31 min, and NADPH at 41 min. The CH_3-H_4folate-containing fractions were pooled and lyophilized to remove the solvent.

Analysis of the Stereochemical Course of Pig Liver Serine Hydroxymethyltransferase. The stereochemical course of the serine hydroxy-

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methyltransferase reaction was determined essentially as described by Tatum et al. for the rabbit liver enzyme,[10] by measuring the amount of tritium in the products of the methylenetetrahydrofolate dehydrogenase reaction, NADPH and CH^+-H_4folate, after incubation of the assay mixture for a given time and HPLC separation of the reaction products. The data were analyzed according to the method described by Battersby,[20] whose analysis requires that two reaction mixtures containing the different stereoisomers of the substrate, in our case L-(3*R*)-[3-^{3}H]serine and L-(3*S*)-[3-^{3}H]serine, be brought to the same percent conversion. The distribution of tritium label in the products is calculated by using eq 2 and 3, where $f_{NADPH(R)}$ is the fraction of tritium transferred from

$$f_{NADPH(R)} = {}^3H_{NADPH(R)}/({}^3H_{NADPH(R)} + {}^3H_{NADPH(S)}) \quad (2)$$

$$f_{CH^+\text{-}H_4folate(R)} = {}^3H_{CH^+\text{-}H_4folate(R)}/({}^3H_{CH^+\text{-}H_4folate(R)} + {}^3H_{CH^+\text{-}H_4folate(S)}) \quad (3)$$

CH_2-H_4folate to NADPH when (3*R*)-[3-^{3}H]serine is used; ${}^3H_{NADPH(R)}$ is the fraction of total tritium associated with NADPH in the reaction carried out in the presence of (3*R*)-serine; $f_{CH^+\text{-}H_4folate(R)}$ is the fraction of tritium retained in CH^+-H_4folate with the 3*R* isomer of serine; and ${}^3H_{CH^+\text{-}H_4folate(R)}$ is the fraction of total tritium associated with CH^+-H_4-folate in the reaction carried out in the presence of L-(3*R*)-[3-^{3}H]serine. The same expressions containing *S* as the subscript indicate that (3*S*)-[3-^{3}H]serine was the substrate in the coupled reactions.

The distribution of tritium label was routinely calculated from the results of each HPLC run by using eq 4 and 5, where the subscript *R*

$$f_{NADPH(R)} = {}^3H_{NADPH(R)}/({}^3H_{NADPH(R)} + {}^3H_{CH^+\text{-}H_4folate(R)}) \quad (4)$$

$$f_{CH^+\text{-}H_4folate(R)} = 1 - f_{NADPH(R)} \quad (5)$$

indicates that (3*R*)-[3-^{3}H]serine was the substrate. The same equations with *S* as the subscript were used to calculate the results when (3*S*)-[3-^{3}H]serine was the substrate. Where there is no secondary isotope effect on the serine hydroxymethyltransferase reaction, use of eq 2 and 3 or 4 and 5 should give identical *f* values.

Determination of the Secondary Tritium Kinetic Isotope Effect on the Reaction of Pig Liver Serine Hydroxymethyltransferase. The secondary tritium kinetic isotope effect on *V*/*K* during transfer of the β-carbon group of serine to H_4folate during the reaction catalyzed by pig liver serine hydroxymethyltransferase was measured by using L-(3*R*,*S*)-[3-^{3}H,U-^{14}C]serine. An assay mixture containing 10 μmol of potassium phosphate buffer, pH 7.0, 400 nmol of $NADP^+$, 380 nmol of H_4folate, 45 μmol of 2-mercaptoethanol, and 50 nmol of L-(3*R*,*S*)-[3-^{3}H,U-^{14}C]-serine (80 000 dpm/nmol) in 900 μL was prepared. A 360-μL aliquot was equilibrated at 25 °C and the reaction was started by addition of 0.032 unit of serine hydroxymethyltransferase. At intervals 50-μL aliquots were withdrawn. Aliquots were quenched by transfer to test tubes containing 300 μL of dimedone (3 mg/mL in 1 M acetate, pH 4.5), and 450 μL of water. The amount of radiolabeled CH_2-H_4folate product was measured after extraction of the dimedone–formaldehyde complex into toluene. Two 45-μL aliquots were withdrawn from the assay mixture before addition of serine hydroxymethyltransferase and added to test tubes containing 450 μL of water, 300 μL of dimedone solution, and 0.004 unit of serine hydroxymethyltransferase. The amount of radiolabel extracted with dimedone provided the blank value for our calculations. Finally, two aliquots of the reaction mixture were added to tubes containing 0.012 and 0.016 unit of serine hydroxymethyltransferase and were incubated for 30 min at 25 °C. The amount of label extracted with dimedone from these aliquots was used to determine the radiolabel associated with 100% conversion of serine to CH_2-H_4folate. The experimental data were analyzed by using eq 6,[32] where ${}^T(V/K)$ is the observed

$${}^T(V/K) = \log (1 - F_1)/\log (1 - F_2) \quad (6)$$

secondary tritium kinetic isotope effect on *V*/*K*; F_1 is the fractional conversion of unlabeled substrate and is determined from the fraction of ^{14}C-labeled serine converted to formaldehyde after correction for nonenzymatic release of label; and F_2 is the fractional conversion of tritiated substrate, which is determined from the fraction of [^{3}H]serine converted to formaldehyde after correction for the nonenzymatic release of label.

Preparation of Labeled CH_3-H_4Folate Samples for Stereochemical Analysis. Since one solvent proton is incorporated into the methyl group of the product during the reduction of CH_2-H_4folate to CH_3-H_4folate catalyzed by methylenetetrahydrofolate reductase, deuterated solvent was used for the coupled serine hydroxymethyltransferase–methylenetetrahydrofolate reductase reactions in which chirally labeled CH_3-H_4folate was generated from (3*R*)- or (3*S*)-[3-^{3}H]serine. A mixture of L-[3-^{14}C]serine and (3*R*)- or (3*S*)-[3-^{3}H]serine was lyophilized and resuspended in 100 μL of D_2O. The lyophilization and resuspension in D_2O were repeated twice. Buffers were prepared by dissolving the reagents in D_2O and adjusting the pD to 7.0 with solutions of 1 M KOH or 1 M HCl in D_2O. The pD was measured by correcting the pH meter reading, assuming pD = pH + 0.4.[33] Purified (6*R*)-H_4folate was exchanged into deuterated solvent by chromatography on a 2-mL DEAE-cellulose column equilibrated in 5 mM Tris chloride buffer in D_2O, pD = 7.2, containing 100 mM 2-mercaptoethanol. The column was rinsed with 10 volumes of equilibrating buffer and (6*R*)-H_4folate was eluted with the same buffer containing 1 M KCl. Methylenetetrahydrofolate reductase was equilibrated with deuterated buffer by five cycles of 10-fold dilution and reconcentration in a Centricon 30 microconcentrator. Methylenetetrahydrofolate dehydrogenase (0.5 mL) and serine hydroxymethyltransferase (0.5 mL) were dialyzed overnight against 100 mL of deuterated storage buffer. Alternatively, serine hydroxymethyltransferase was equilibrated with deuterated buffer by repeated dilution and concentration cycles as described for the reductase. NADPH (10 mM) was prepared in 20 mM unneutralized Tris in D_2O, and $NADP^+$ was directly dissolved in D_2O. The reaction mixture for the coupled synthesis of CH_3-H_4folate from serine consisted of 10 mM potassium phosphate buffer, pD = 7.0, 0.01 mM EDTA, 50 μM serine [either L-[3-^{14}C]serine (12 000 dpm/nmol) and (3*R*)[3-^{3}H]serine (59 000 dpm/nmol) or L-[3-^{14}C]serine (14 300 dpm/nmol) and (3*S*)-[3-^{3}H]serine (76 800 dpm/nmol)], 300 μM (6*S*)-H_4folate, 300 μM NADPH, and 42 mM 2-mercaptoethanol in 1000 μL total volume. After equilibration of the reaction mixture at 25 °C, the reaction was started by addition of 9 units of methylenetetrahydrofolate reductase and then 0.003 unit of serine hydroxymethyltransferase, in this order. Aliquots were removed after 10, 30, or 60 min at 25 °C in the dark, and the solutions were injected on the HPLC to determine the extent of reaction and isolate the CH_3-H_4folate product. Peak CH_3-H_4folate fractions were pooled, equilibrated with nitrogen, frozen, and lyophilized. Recovery of CH_3-H_4folate from HPLC purification was essentially quantitative.

Chirality Analysis of CH_3-H_4folate. The N^5 methyl group of methyltetrahydrofolate was excised from the tetrahydrofolate molecule as methylamine by permanganate oxidation following diazotization under acidic conditions.[11,12] The methylamine was recovered as the hydrochloride salt and converted to acetic acid.[13] The average yield of CH_3-NH_2 was 47% (based on the yield of purified CH_3-H_4folate starting material) and the average yield of acetate was 19%. The acetate was then enzymatically converted to malate and incubated with fumarase, and the tritium released to solvent was quantitated.[14-16] The *F* value represents the percentage of the tritium of malate released to water in the fumarase reaction; it is related to the enantiomeric excess by eq 7.

$$\%\ ee = [|50 - F|/29] \times 100 \quad (7)$$

F values of >50 represent excess *R* and <50 represent excess *S* configuration.[16] Under the conditions of analysis used here,[16] *F* values are reproducible to within ±2.

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