Stereochemistry of Methylene Transfer Involving 5,10-Methylenetetrahydrofolate†

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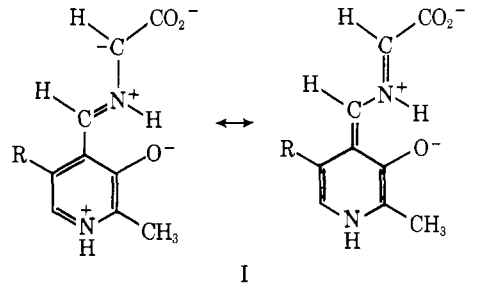
ABSTRACT: The stereochemistry of the transfer catalyzed by rabbit liver serine transhydroxymethylase (EC 2.1.2.1) of the prochiral hydroxymethyl group from serine to tetrahydrofolate to form 5,10-methylenetetrahydrofolate was studied. Initial kinetic studies on labeled 5,10-methylenetetrahydrofolate showed that nonenzymatic racemization of the prochiral methylene center was buffer dependent and was slow under the conditions employed. Specifically tritiated (3*R*)- and (3*S*)-serines were employed to study the transfer reaction. Reactions were carried out under various conditions and the stereochemistry of the methylene carbon of the 5,10-methylenetetrahydrofolate produced was determined. The enzyme was shown to be partially stereospecific for this transfer reaction, proceeding with a loss of about 50% of the stereochemical purity of the transferred carbon center. Possible mechanistic interpretations of this finding are discussed.

Serine transhydroxymethylase (EC 2.1.2.1) catalyzes the reversible reaction of eq 1:

L-serine + *l*-H_4folate[1] ⇄ *d*-5,10-CH_2-H_4folate + glycine (1)

It forms an important physiological entry from serine into the C-1 pool at the formaldehyde level and utilizes the *l*,L diastereomer of tetrahydrofolate (Blakley, 1960, 1969). The enzyme has also been found to cleave several other β-hydroxyamino acids (see Chen and Schirch, 1973a, and references therein) to glycine and the respective aldehydes, as well as aminomalonate to glycine + CO_2 (Palekar et al., 1973). Enzyme-bound pyridoxal phosphate is a required cofactor, and there is considerable evidence that the formation of the imine is a required step in the reaction and leads to the formation of a glycine anion species (I) (Schirch and Jenkins, 1964; Schirch and Slotter, 1966; Jordan and Akhtar, 1970). Serine transhydroxymethylase has been shown to exclusively remove the *pro*-2*S* hydrogen of glycine, while the *pro*-2*R* hydrogen atom is retained in serine (Besmer and Arigoni, 1968; cf. Besmer, 1970; Jordan and Akhtar, 1970; Akhtar et al., 1975).

Although a great deal of work has concentrated on elucidating the nature of the bound glycine species, relatively little is known about the nature of the aldehyde species involved in the reaction, and in fact, the exact role of H_4folate in the conversion is still in doubt. In an attempt to deal with this problem, Biellmann (Biellmann and Schuber, 1967, 1970) studied the stereochemistry of the prochiral C-3 of serine generated in tissue slices from [^{3}H]formate, presumably via 5,10-CH^+-H_4folate and 5,10-CH_2-H_4folate. They found that 72% of the tritium on the C-3 of the serine produced was in the *pro*-3*S* position. The reasons for the partial stereospecificity of the transfer were obscure. Due to our interest in the mechanisms of reactions involving tetrahydrofolate cofactors (Benkovic and Bullard, 1973) and, in particular, reactions at the formaldehyde oxidation level (Barrows et al., 1976; Farina et al., 1973) we have now reinvestigated this reaction. We have monitored the stereochemical course of the reaction employing the prochiral methylene moiety of 5,10-CH_2-H_4folate as a probe of mechanism utilizing both 5,10-CH_2-H_4folate labeled in the prochiral methylene position and serines labeled in the prochiral C-3 position with heavy isotopes of hydrogen.



I

Experimental Section

Materials

All solvents were redistilled before use. Specific activity is in micromoles milligram^{-1} minute^{-1}. *d,l*-Tetrahydrofolate, α-NADP, NADase (sp act. 1.8, 37 °C), glutamate dehydrogenase (sp act. 58, 25 °C), *Saccharomyces cerevisiae*, and α-ketoglutarate were obtained from the Sigma Chemical Co., St. Louis, Mo. NaB^3H_4 (120 mCi/mmol) and [^{14}C]glycine (106 mCi/mmol) were obtained from New England Nuclear Co., Boston, Mass. $NaBD_4$ (98% D) was obtained from British

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[1] Abbreviations used are: H_4folate, *d,l*-L-tetrahydrofolate; 5,10-CH^+-H_4folate, 5,10-methylenetetrahydrofolate; 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolate; EDTA, ethylenediaminetetraacetic acid. For brevity, designation of the L configuration for the glutamyl moiety has been omitted; however, the customary *d,l* nomenclature referring to optical rotation arising from the asymmetric C-6 in the tetrahydropyrazine ring is invoked when appropriate. Note that the sign of rotation changes from *l* to *d* upon conversion of enzymatically active H_4folate into 5,10-CH_2-H_4folate, although the absolute but unknown stereochemistry at C-6 is unchanged.

Oxygen Company, Ltd., Croyden, England, and [U-^{14}C]serine (10 mCi/mmol) and sodium [^{3}H]formate (25 mCi/mmol) were obtained from Amersham-Searle Corp., Arlington Hts., Ill. Ultraviolet (UV) scans and kinetics were performed on a Cary Model 118 or a Gilford Model 240 UV spectrophotometer. Liquid scintillation counting was done on a Packard Tri-Carb Model 3320. Nuclear magnetic resonance (NMR) spectra (100 MHz) were recorded on a JEOL PS-100-FT spectrometer. Rabbit liver serine transhydroxymethylase (sp act. 2.1, 25 °C) was obtained in crystalline form from Dr. LaVerne Schirch of Bluffton College, Bluffton, Ohio.

Methods

Synthesis of 5,10-Methenyltetrahydrofolates. The *d,l*-5,10-CH^+-H_4folate was synthesized by a modification of the procedure of Farina et al. (1973). In 40 mL of acetic acid containing 2% β-mercaptoethanol and 1.77 mL of 90% formic acid, 250 mg of *d,l*-H_4folate was dissolved. After stirring under N_2 at 70 °C in the dark for 24 h, the reaction mixture was poured into twice its volume of diethyl ether. The precipitate was washed two times with 50 mL of tetrahydrofuran and once with 50 mL of ether, and dried at 0.1 mm over KOH. Recrystallization from 0.1 N HCl/0.1 N mercaptoethanol afforded material 80–90% pure based on $\epsilon_{348} = 26.5 \times 10^3$ at pH 0 (Huennekens et al., 1963) in yields of 60–70%: UV (pH 1) λ 348 nm ($\epsilon = 23.8 \times 10^3$); NMR ($Me_2SO$-$d_6$-pyridine-$d_5$, 9:1) δ 9.78 (s, 1 H, methine H), 8.16 (d, 2 H, 2′,6′ protons, J = 8 Hz), 7.65 (d, 2 H, 3′,5′ protons, J = 8 Hz). Other resonances not assigned were at δ 4.13 (t), 3.86 (m), 3.58 (t), 2.11 (m). The *d,l*-5,10-CH^+-H_4folate was stored in vacuo over KOH, although it has been found to be stable for long periods of time at room temperature in air. *d,l*-[^{3}H]-5,10-CH^+-H_4folate was synthesized according to the above procedure using sodium [^{3}H]formate (25 mCi/mmol, 5 mCi total). Volumes were adjusted to account for the smaller amount of material. The crude product was purified by recrystallization as before.

Synthesis of d,l-5,10-CH_2-H_4Folate. Synthesis of *d,l*-5,10-CH_2-H_4folate was carried out according to Farina et al. (1973). Me_2SO was stored over 3-Å Linde sieves and under N_2. In 2.82 mL of Me_2SO and 0.72 mL of pyridine was dissolved 100 mg of *d,l*-5,10-CH^+-H_4folate. Reduction was carried out by the addition of 0.85 mL of a solution of 13.5 mg/mL $NaBH_4$ in Me_2SO. The product was precipitated by the addition of 2 vol of tetrahydrofuran. It was purified for NMR by dissolution in a minimum of 0.1 N KOD and by reprecipitation by addition of an equal volume of 0.1 N DCl. The product, assayed according to the enzymatic procedure which is specific for the *d* diastereomer (Ramasastri and Blakley, 1964b), was 50–60% active by weight (corrected for *d,l* mixture) and 70–75% based on UV ($\epsilon_{298} = 30.0 \times 10^3$ at pH 7.3): NMR (Me_2SO-d_6-pyridine-d_5, 9:1) δ 7.86 (d, 2 H, 2′,6′ protons, J = 8 Hz), 6.50 (d, 2 H, 3′,5′ protons, J = 8 Hz), 4.99 (d, 1 H, methylene proton, J = 4 Hz), 3.78 (d, 1 H, methylene proton, J = 4 Hz). Other resonances at δ 3.27, 2.90, 2.36, and 2.08 were not assigned but comprised glutamate and C-6, C-7, and C-9 proton signals.

Synthesis of d,l-5,10-CHD-H_4Folate. The above procedure was followed using $NaBD_4$ (98% D) in place of $NaBH_4$. Purification was effected by solution in 0.1 N KOD and reprecipitation by addition of 0.1 N DCl: NMR (Me_2SO-d_6-pyridine-d_5, 9:1) δ 7.86 (d, 2 H, 2′,6′ protons, J = 8 Hz), 6.50 (d, 2 H, 3′,5′ protons, J = 8 Hz), 4.96 (s, 0.5 H, methylene proton), 3.75 (s, 0.5 H, methylene proton). Other resonances not assigned were at δ 3.24, 2.87, 2.36, and 2.08 as above.

Synthesis of d,l-[^{3}H]-5,10-CH_2-H_4Folate. *d,l*-5,10-CH^+-H_4folate (15–30 mg) was dissolved in dry Me_2SO under N_2 so that the concentration was 0.07 M. Amine was then added for each reaction, to the following final concentrations: triethylamine (0.25 M), pyridine (2.5 M), indole (1.0 M), 2,6-lutidine (2.2 M), 2,3-dimethylindole (1.0 M), or *N,N*-diethylaniline (1.5 M). NaB^3H_4 (120 mCi/mmol, 0.36 M in Me_2SO) was then added dropwise with stirring until all the yellow color disappeared. The reaction was stirred for several minutes further. Except where noted, the product was precipitated by addition of 2 vol of tetrahydrofuran, washed two times with tetrahydrofuran and once with diethyl ether, and dried under vacuum. Reprecipitation with 0.1 N HCl from 0.1 N KOH solution removed a small amount of exchangeable tritium as noted in the results.

Assays for Stereochemistry of the Chiral Methylene Carbon of d,l-[^{3}H]-5,10-CH_2-H_4Folates.[2] Assay of Retained Label. Assays were carried out at 37 °C in a total volume of 1.5 mL in 0.05 M potassium phosphate (pH 7.50), containing 1 mM EDTA, 10 mM β-mercaptoethanol, 1.7 mM NADP, and sufficient 5,10-methylenetetrahydrofolate dehydrogenase (sp act. 1.5, ca. 8–16 units) purified from *Saccharomyces cerevisiae* (Ramasastri and Blakley, 1962), to complete the reaction in less than 5 min.

Reaction was initiated by the addition of 0.5 μmol of *d,l*-[^{3}H]-5,10-CH_2-H_4folate dissolved in 200 μL of 0.05 M potassium phosphate (pH 9.5). After the optical density change at 340 nm was complete, ca. 60 units of glutamate dehydrogenase (from bovine liver) was added, and, after 1 min, 40 μL of a solution 0.25 M in α-ketoglutarate and 2.5 M in NH_4Cl was added. After this reaction was complete, the reaction mixture was acidified to pH ~2 with 6 N HCl and allowed to stand 30–60 min. It was then placed on a 0.5 × 7 cm column of Bio Rex 70 (acid form) previously washed with 0.6 N HCl and H_2O to remove chloride ion. The *d*-[^{3}H]-5,10-CH^+-H_4folate was eluted with 0.1 N HCl. The elution profile showed two radioactive peaks, the second of which coincided with the peak due to the absorbance at 348 nm of *d*-5,10-CH^+-H_4folate. The fractions containing *d*-[^{3}H]-5,10-CH^+-H_4folate were pooled and lyophilized to dryness. The solid *d*-[^{3}H]-5,10-CH^+-H_4folate was redissolved in 150 μL of 0.1 N HCl, and placed on a 0.5 × 5 cm Bio Rex 70 column (acid form) and eluted as before. Aliquots of 100 μL from fractions containing *d*-[^{3}H]-5,10-CH^+-H_4folate were then counted in a liquid scintillation cocktail containing 2 mL of Soluene (Packard Instrument Co.) and 10 mL of a toluene scintillator solution, made by diluting 7 g of 2,5-diphenyloxazole and 0.6 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene to 1 L with toluene. Internal [^{3}H]toluene standards were used. Further purification of the *d*-[^{3}H]-5,10-CH^+-H_4folate by thin-layer chromatography (TLC) on cellulose MN-200 (Sigma Chemical Co.) (solvent used was the upper phase of the following mixture: 1-butanol-acetic acid-H_2O, 4:1:5, R_f 0.36) gave no change in the specific activity (expressed as disintegrations per minute micromole^{-1}, the latter measured by UV assay in 0.1 N HCl at 348 nm).

Assay of Transferred Label. Enzyme reactions catalyzed by 5,10-methylenetetrahydrofolate dehydrogenase and glutamate dehydrogenase were carried out exactly as above. The reaction mixture was then incubated in boiling water for 2.5 min and cooled to 0 °C. It was then placed on a Dowex 50 (acid form) column and eluted with H_2O. Fractions containing [^{3}H]NADP, identified by its absorbance at 260 nm, were

[2] See Figure 1 in the Results section.

pooled and the pH was adjusted to 7.0. Then 0.8 unit of NADase (from *Neurospora crassa*) was added and the hydrolysis at 35 °C of NADP was followed by KCN assay (Colowick et al., 1951). When the reaction was complete, 20 mg of nicotinamide (recrystallized) was added and the entire mixture was lyophilized. Recovered [^{3}H]nicotinamide was recrystallized from boiling benzene and dried under vacuum over KOH. Total recovery was usually 3–6 mg. It was weighed on a Cahn microbalance and counted in Bray's solvent to determine the specific activity.

Assay of Total Label; Synthesis of [^{3}H,^{14}C]Serine. Serine transhydroxymethylase (0.5 unit), ca. 23 mM *d,l*-[^{3}H]-5,10-CH_2-H_4folate, 6.8 mM [^{14}C]glycine (1.8 μCi/μmol), 1 mM EDTA, and 10 mM β-mercaptoethanol in 0.02 M potassium phosphate in a total volume of 100 μL at pH 7.50 were reacted at 25 °C for 6–20 min. The reaction was quenched with 12% Cl_3CCOOH and the serine in 10-μL aliquots was separated by ascending paper chromatography on Whatman No. 1 paper (1-butanol–acetone–diethylamine–H_2O, 10:10:2:5, R_f 0.35). The doubly labeled serine spot was excised and counted in a Soluene–toluene cocktail. Internal standardization with [^{14}C]- and [^{3}H]toluene standards was carried out, and disintegrations per minute were calculated by correcting for carbon spillover in the tritium channel.

Kinetics of Nonenzymic Racemization. Solutions 0.5 mM in *d,l*-[^{3}H]-5,10-CH_2-H_4folate in 1.0 mL were incubated at 25 °C for various times up to 30 min and were then placed in the assay system containing 5,10-methylenetetrahydrofolate dehydrogenase and NADP (in 0.5 mL) so that the final concentrations of all reagents were identical with the assay as described above. Stereochemistry of the methylene center was determined according to the procedures described above. The buffers used in the incubations were either 0.05 or 0.02 M potassium phosphate containing 10 mM β-mercaptoethanol and 1 mM EDTA at pH 7.50, and contained either KCl (μ = 1.0) or no KCl as noted in results.

Stereochemistry of Hydroxymethyl Transfer from Serine. Sequential Reactions. (2*S*,3*S*)- and (2*S*,3*R*)-[^{3}H]serines were prepared from 1-[^{3}H]glucose and 1-[^{3}H]mannose by enzymatic conversion via 3-phosphoglycerate and phosphoserine. They were diluted with [U-^{14}C]serine, assayed before use by conversion to indolmycin, and found to contain >98% of the diastereomer with the stated configuration at C-3 (Skye et al., 1974).

In a standard reaction, stereospecifically tritiated (3*S*)- or (3*R*)-3-[^{3}H,U-^{14}C]serine (0.58 mM, ^{3}H/^{14}C = 2.5–4.5, ^{14}C per reaction ~0.35 μCi), *d,l*-H_4folate (0.44 mM), EDTA (1 mM), β-mercaptoethanol (10 mM), and serine transhydroxymethylase (0.25–0.5 unit) in 0.02 M potassium phosphate (pH 7.50), in a total volume of 1.2 mL, were allowed to react at 25 °C for 5 min. An aliquot of 1.0 mL of this solution was then added to 0.5 mL of NADP and 5,10-methylenetetrahydrofolate dehydrogenase under the standard assay conditions above. Retained or transferred tritium was then determined according to the previously described assays. Internal [^{3}H]- and [^{14}C]toluene standards were used, and disintegrations per minute calculated by spillover correction. Calculation of the stereochemistry of the products is described in the Results section. Suitable controls were carried out to ensure the assay conditions were not altered from previous conditions.

Coupled Reactions. In a standard reaction *d,l*-H_4folate (0.56 mM), NADP (1.76 mM), EDTA (1 mM), and β-mercaptoethanol (10 mM) in 0.02 M potassium phosphate (pH 7.50) were placed in a cuvette with serine transhydroxymethylase and 5,10-methylenetetrahydrofolate dehydrogenase

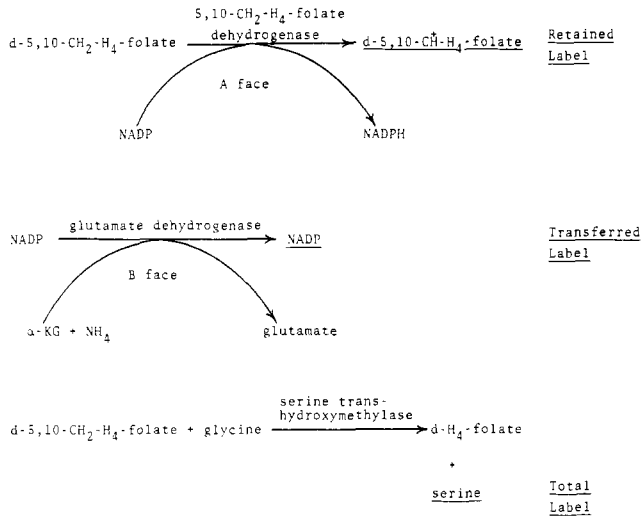


FIGURE 1: Scheme of analysis of [^{3}H]-5,10-methylenetetrahydrofolates. The amount of tritium contained in one of the heterotopic methylene positions was determined by oxidizing the methylene carbon and assaying the recovered *d*-5,10-CH^+-H_4folate. This value is referred to as *retained label*. The amount of tritium contained in the other heterotopic position was determined by reoxidizing the initially formed NADPH on the opposite face and, after hydrolysis, assaying the purified nicotinamide. This value is referred to as *transferred label*. The amount of tritium contained in both positions was determined by transferring the entire methylene unit to glycine, and assaying the recovered serine. This value is referred to as *total label*.

at 25 °C for 4 min. Enzyme concentrations employed are listed in Table III. The reaction was initiated by the addition from a 10 mM stock solution of 70 μL of specifically labeled (3*S*)- or (3*R*)-3-[^{3}H,U-^{14}C]serine to a final concentration of 0.7 mM and a final volume of 1.0 mL. Following completion of the reaction measured by the optical density change at 340 nm, α-ketoglutarate, NH_4Cl, and glutamate dehydrogenase were added as before, and the reaction mixture was acidified with 30 μL of 6 N HCl. *d*-[^{3}H,^{14}C]-5,10-CH^+-H_4folate was purified by passing the reaction mixture through Bio Rex 70, lyophilizing, and rechromatographing on Bio Rex 70 as before. The peak due to absorbance at 348 nm coincided with both ^{3}H and ^{14}C activity. Aliquots of 100 μL from the fractions containing *d*-5,10-CH^+-H_4folate were counted as above in Soluene–toluene scintillator. Further purification by TLC on cellulose MN 200 did not alter the specific activity, or the ^{3}H/^{14}C ratios.

Results[3]

Stereochemical Analysis of [^{3}H]-5,10-CH_2-H_4Folate. Figure 1 describes the analytical method utilized for the determination of the optical purity of the methylene carbon from synthetically and enzymatically tritium labeled 5,10-CH_2-H_4folate. Oxidation of *d*-5,10-CH_2-H_4folate by yeast 5,10-methylenetetrahydrofolate dehydrogenase (the enzyme is only active toward the *d*,L diastereomer) was shown (Ramasastri and Blakley, 1964b; see also the Discussion section) to result in the stereochemical transfer of one of the two heterotopic hydrogens of the methylene bridge of *d*-5,10-CH_2-H_4folate to the A face of NADP to yield *d*-5,10-CH^+-H_4folate and

[3] It is usual to discuss stereochemistry in terms of optical purity, but for the purposes of this discussion, it was found more understandable to use "isomeric distribution" to define mixtures of isomers since the absolute stereochemistry of the reactions involving the prochiral methylene carbon of 5,10-CH_2-H_4folate is unknown.

TABLE I: Analysis of *d,l*-[^{3}H]-5,10-Methylenetetrahydrofolates Synthesized with Amine Bases.

Amine	^{3}H Retained in *d*-5,10-CH$^+$-H$_4$folate[a]	^{3}H Transferred to NADPH[a]	^{3}H Total in Ser[a]	Isomeric Distribution (±5%) % *d*-5,10-CH$^+$-H$_4$folate/% NADPH
Triethylamine	1.72			47/*b*
Pyridine	1.80	1.79	3.86	50/50
Pyridine[c]	2.06	2.34	4.62	47/53
Indole	1.95	*d*	*d*	54/*b*
2,6-Lutidine	2.19			61/*b*
2,3-Dimethylindole	2.48	*d*	*d*	69/*b*
N,N-Diethylaniline	2.68	0.74	3.52	76/24

[a] dpm/μmol × 10^{-7}. [b] With one exception (see footnote *c*), all reductions were carried out with NaB^{3}H$_4$ with a specific activity of 120 mCi/mmol. Isomeric distributions are based on average total tritium incorporation of 3.60 × 10^7 dpm/μmol in product. [c] *d,l*-5,10-CH$_2$H$_4$folate synthesized from *d,l*-[^{3}H]-5,10-methenyltetrahydrofolate (4.65 × 10^7 dpm/μmol) by reduction with NaBH$_4$. [d] Low yield of 5,10-CH$_2$-H$_4$folate product.

NADPH. Therefore, the amount of tritium label originally located in each of the two heterotopic positions is directly reflected in the two products of enzymatic oxidation.

The *d*-5,10-CH$^+$-H$_4$folate from the dehydrogenase reaction was purified by column chromatography and the specific activity determined by liquid scintillation counting. Subsequent oxidation of NADPH by glutamate dehydrogenase, shown to be specific for the B face (Nakamoto and Vennesland, 1960), and cleavage with NADase yields nicotinamide containing hydrogen in the 4 position which originated in the methylene bridge of *d*-5,10-CH$_2$-H$_4$folate. This nicotinamide was then purified and counted. Finally, the total amount of label contained in both heterotopic methylene positions was determined using rabbit liver serine transhydroxymethylase to transfer the intact methylene group to glycine to form the C-3 hydroxymethyl group of serine. Serine produced in the reaction was purified by paper chromatography and counted. Under these conditions, no label is lost from the C-3 hydrogens of serine. Note in all cases one is assaying for only the enzymically active diastereomer; it is unlikely that in the synthetically tritium-labeled *d,l*-5,10-CH$_2$-H$_4$folate the *l* diastereomer has a differing ^{3}H distribution.

Thus, a complete accounting can be made for tritium from each of the two heterotopic positions, and it can be shown that the sum obtained from both is the same as that assayed as the total label transferred to serine. This serves as a check on the completeness of the dehydrogenase reaction, particularly since discrimination against tritium transfer would bias the result toward ^{3}H retained. Results varying by more than 10% were not used. Since the absolute steric course of the enzymatic oxidation is not known, the two heterotopic hydrogens in *d*-5,10-CH$_2$-H$_4$folate are referred to, respectively, as that which is *retained* (as 5,10-CH$^+$-H$_4$folate) and that which is *transferred* (to NADP) in the above assay system.

Synthetic Results. Based on the work of Farina et al. (1973) *d,l*-5,10-CH$_2$-H$_4$folate was synthesized according to eq 2:

$$d,l\text{-H}_4\text{folate} + \text{HCO}_2\text{H} \xrightarrow[70\ ^\circ\text{C}]{\text{AcOH}} d,l\text{-5,10-CH}^+\text{-H}_4\text{folate}$$

$$d,l\text{-5,10-CH}^+\text{-H}_4\text{folate} \xrightarrow[\text{Me}_2\text{SO-base}]{\text{NaBH}_4} d,l\text{-5,10-CH}_2\text{-H}_4\text{folate} \quad (2)$$

Initial work was carried out with NaBD$_4$ in the presence of pyridine to label the methylene position. The 100-MHz NMR spectra of the unlabeled *d,l*-5,10-CH$_2$-H$_4$folate and the monodeuterated *d,l*-5,10-CHD-H$_4$folate were as previously reported (Farina et al., 1975). The methylene AB doublets in *d,l*-5,10-CH$_2$-H$_4$folate are centered at δ 4.99 and 3.78 with a splitting of J = 4 Hz. Each doublet integrates to 1 H. The large shift difference is characteristic of AB or AX systems in which one proton lies above the plane and the other lies in the plane of an aromatic group (Jackman and Sternhell, 1969) and will be treated more completely in a latter publication. In *d,l*-5,10-CHD-H$_4$folate, the two doublets have been replaced by two singlets at δ 4.96 and 3.75 of approximately equal area, each equal to 0.5 H. Thus, it appears that reduction of *d,l*-5,10-CH$^+$-H$_4$folate in the presence of pyridine leads to *d,l*-5,10-CH$_2$-H$_4$folate labeled in the methylene group, which is racemic or nearly racemic at this position. An identical synthesis was carried out using *d,l*-[^{3}H]-5,10-CH$^+$-H$_4$folate and NaBH$_4$ with pyridine and the product was analyzed according to the above procedures (Table I). The finding that this material is racemic at the methylene group is consistent with the above result and further supports the validity of the assay.

A number of syntheses were then carried out using a variety of nonnucleophilic amines and NaB^{3}H$_4$. Analyses of the tritiated *d,l*-5,10-CH$_2$-H$_4$folate products are carried out according to Figure 1 and the results are shown in Table I. It was found that the relatively unhindered aromatic amine indole and the nonaromatic triethylamine also resulted in nonstereoselective reduction. However, by increasing the steric requirements around the amine nitrogen and retaining the aromatic portion of the amine, introduction of tritium predominantly into one of the two positions was accomplished. Best results were obtained with diethylaniline where an isomer distribution of 76/24 was determined. Reduction with a number of other nonnucleophilic amine bases including *m*- and *p*-*N,N*-dimethyltoluidine, 6-dimethylaminopurine, and quinoline did not give products with isomeric distributions significantly better than 60/40. Likewise, reaction conditions have been varied to optimize the reduction including the addition of 1, 3, 5, and 10% H$_2$O, variations over a factor of 10 in the amount of amine, variations in the concentration of 5,10-CH$^+$-H$_4$folate and the ratio of amine to 5,10-CH$^+$-H$_4$folate, and reduction in dimethylformamide instead of Me$_2$SO, but an isomer distribution greater than 76/24 was not achieved. Consequently, synthesis of only one of the diastereomers was completed since the material was not of sufficient optical purity to quantitate serine transhydroxymethylase stereospecificity.

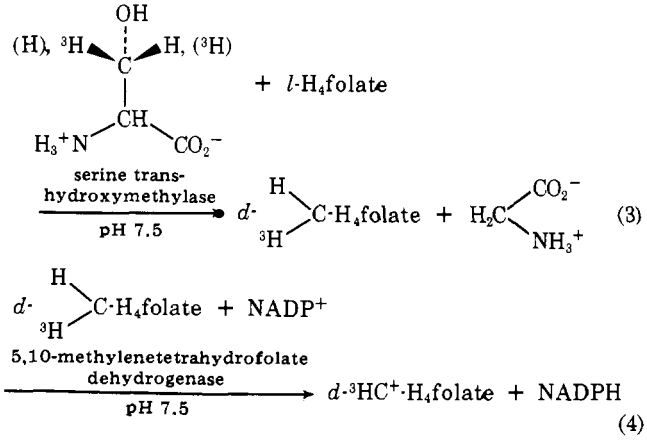
Kinetics. The five-membered ring of 5,10-CH$_2$-H$_4$folate is susceptible to protonation and ring opening, and since there has been speculation (Kisliuk, 1957; Biellmann and Schuber, 1967) that nonenzymatic racemization of the methylene center

is rapid, a study of the rates of racemization under several conditions was carried out. Kinetics were carried out on a tritiated sample of *d,l*-5,10-CH_2-H_4folate which had been analyzed by multiple determinations in each of the assay reactions and had been shown to have label distributed 76–24%, assayed as *d*-5,10-CH^+-H_4folate and nicotinamide, respectively. Samples were incubated for various times at pH 7.50 at 25 °C in either 0.02 or 0.05 M potassium phosphate containing 1 mM EDTA and 10 mM β-mercaptoethanol. After the incubation period, aliquots were withdrawn and the *d,l*-5,10-CH_2-H_4folate was assayed for isomeric distribution of the label.

Figure 2 shows the first-order plots at 0.05 and 0.02 M potassium phosphate (μ 1.0) obtained by following the decrease in tritium assayed as *retained* label. A rapid initial decrease in available tritium was observed, followed by a first-order decay. Reprecipitation of the *d,l*-5,10-CH_2-H_4folate sample with 0.1 N HCl from 0.1 N KOH eliminated the initial fast decrease, indicating the presence of a small amount of exchangeable tritium. Racemization rates can be seen to be buffer dependent from Figure 2.

Figure 3 shows the first-order rates under conditions of 0.02 M potassium phosphate, 10 mM β-mercaptoethanol, 1 mM EDTA, no KCl, for the decrease of *retained* tritium (isolated as *d*-5,10-CH^+-H_4folate) and the increase in *transferred* tritium (isolated as nicotinamide) with time. These can be seen to be parallel and are completely complimentary. The observed first-order rate constants were 0.010 and 0.009 min^{-1} for *retained* and *transferred* assays, respectively. Under these conditions of buffer, temperature, and pH, the half-life for racemization is about 70 min.

Stereochemistry of Methylene Transfer. A series of reactions was conducted in which stereospecifically tritium labeled (3*R*)- and (3*S*)-3-[^{3}H,U-^{14}C]serines were reacted with *l*-H_4folate in the presence of rabbit liver serine transhydroxymethylase to synthesize *d*-[^{3}H,^{14}C]-5,10-CH_2-H_4folate. The latter was assayed in a coupled or sequential fashion with the 5,10-methylenetetrahydrofolate dehydrogenase system. The overall sequence is illustrated in reactions 3 and 4:



Owing to interference from excess *d,l*-H_4folate in the purification procedures, we chose to optimize the serine transhydroxymethylase reaction with serine as the limiting reagent. Consequently it was necessary to first calculate the percent ^{3}H retained in the *d*-5,10-CH^+-H_4folate product isolated from *both* diastereomers of [^{3}H]serine based on ^{3}H/^{14}C ratios. The amounts of label were determined in separate experiments for each isomer. The absolute isomeric distribution was then calculated from 100 × % retention (*R* or *S*)/% retention (*R* + *S*). Since the folate product contains only one of the original serine carbons and since serine was uniformly labeled

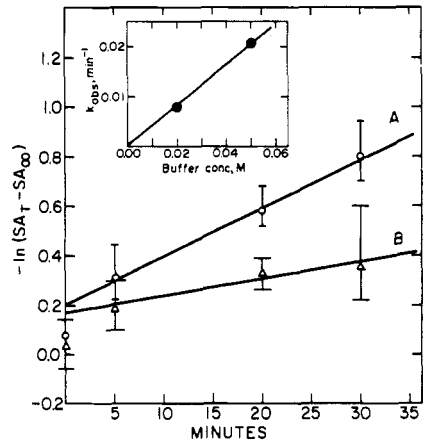


FIGURE 2: Racemization rate of *d,l*-[^{3}H]-5,10-CH_2-H_4folate at pH 7.5, assayed as tritium *retained* in *d*-5,10-CH^+-H_4folate. *d,l*-5,10-CH_2-H_4folate was assayed in the dehydrogenase assay system (see Experimental Section) following incubation at 25 °C in buffer: (curve A, ○) 0.05 M potassium phosphate–10 mM β-mercaptoethanol–1 mM EDTA, μ = 1.0 (KCl); (curve B, △) 0.02 M potassium phosphate–10 mM β-mercaptoethanol–1 mM EDTA, μ = 1.0 (KCl); insert, dependence of racemization rate on buffer concentration.

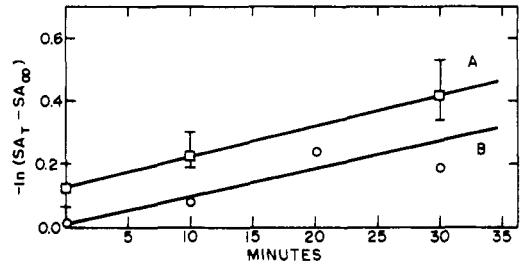


FIGURE 3: Racemization of *d,l*-[^{3}H]-5,10-CH_2-H_4folate at pH 7.5 in 0.02 M potassium phosphate–10 mM β-mercaptoethanol–1 mM EDTA with no KCl at 25 °C. (Curve A, □) *d,l*-5,10-CH_2-H_4folate, base washed, assayed (see Experimental Section) as a decrease in label in *d*-5,10-CH^+-H_4folate; (curve B, ○) *d,l*-5,10-CH_2-H_4folate, unwashed, assayed in the assay system as an increase in label in nicotinamide.

with ^{14}C, ^{3}H/^{14}C values reported for *d*-5,10-CH^+-H_4folate reflect a statistical correction which has been applied to initial experimental values. This method of calculation cancels isotope effects, in this case secondary, which may be affecting the ^{3}H/^{14}C ratios in the final products. It does not require that the reaction (i.e., utilization of [^{3}H,^{14}C]serine) be run to completion, but it does require that each reaction pair of serine diastereomers undergo the same extent of conversion, and that reaction conditions be identical (Battersby, 1972). This was the case for all data reported below. For the case where the coupled reaction is run to total completion, the isomeric distribution of the *d*-[^{3}H]-5,10-CH_2-H_4folate is directly reflected in the amount of tritium in NADPH and *d*-5,10-CH^+-H_4folate products (as was shown in the previous nonenzymatic racemization study). Since measurement of tritium in both products and total oxidation are more difficult to achieve experimentally, this procedure was carried out as a single determination in order to assess the results obtained by the ^{3}H/^{14}C method. These results are shown in Table II, illustrating that both procedures yield isomeric distributions in excellent agreement.

Table III shows the results of systems in which the dehydrogenase reaction was directly coupled to the hydroxymethyl transfer reaction, carried out under varying conditions of coupling enzyme and temperature. In all cases, a loss of initial

TABLE II: Comparison of Reactions Analyzed by Tritium Recovery and by $^3H/^{14}C$ Ratio Methods.[a]

Quantity	(3*R*)-3-[^{3}H]Ser	(3*S*)-3-[^{3}H]Ser
Serine (μCi/μmol)	2.29	2.45
% recovery ^3H		
d-5,10-CH^+-H_4folate[b]	32	64
Nicotinamide[b]	68	36
Isomeric distribution		
d-5,10-CH^+-H_4folate/NADPH (±5%)	32/68	64/36
Serine $^3H/^{14}C$	2.30	2.31
d-5,10-CH^+-H_4folate $^3H/^{14}C$[b]	0.73	1.49
% ^3H retained *d*-5,10-CH^+-H_4folate	32	65
Isomeric distribution[c]		
d-5,10-CH^+-H_4folate/NADPH (±5%)	33/67	67/33

[a] Serine transhydroxymethylase reaction run at 25 °C for 5 min followed by the addition of 5,10-methylenetetrahydrofolate dehydrogenase + NADP. Reactions run on identical serine samples. [b] Isolated and counted as described in the Experimental Section. $^3H/^{14}C$ statistically corrected due to use of [U-^{14}C]serine. [c] Based on $R + S$ % retention = 100%. This method of normalization is discussed in the text.

TABLE III: Stereochemical Results of Coupled Serine Reactions.[a]

3-[^{3}H]Ser Isomer	Enzyme Act./mL[b]	Ser $^3H/^{14}C$	*d*-5,10-CH^+-H_4folate[c] $^3H/^{14}C$	% ^3H Retained	Normalized[d] % ^3H Retained (±5%)
3*R*	0.52 unit; 25 °C	3.78	0.91	24	27
3*S*		4.02	2.58	64	73
3*R*	0.52 unit; 25 °C	4.75	0.81	17	21
3*S*		4.83	3.12	65	79
3*R*	1.57 units; 25 °C	4.75	0.83	17	21
3*S*		4.83	3.10	64	79
3*R*	5.2 units; 35 °C	3.33	0.90	27	28
3*S*		2.84	2.02	71	72
3*R*				Av % retention	24
3*S*					76

[a] 5,10-Methylenetetrahydrofolate dehydrogenase reaction directly coupled with serine transhydroxymethylase reaction (0.34 unit/mL, 25 °C; 1.3 units/mL, 35 °C). [b] Coupling enzyme activity; temperature. [c] $^3H/^{14}C$ statistically corrected due to use of [U-^{14}C]serine. [d] See text for discussion. Based on $R + S$ % retention = 100%.

stereochemistry to approximately the same extent occurred. Thus, the stereochemical result is independent of dehydrogenase concentration as well as temperature. In this set of experiments the dehydrogenase enzyme is initially present whereas for those described in Table II the dehydrogenase is added after a 5-min incubation period. These results are not identical because they do not include the small percent racemization due to dilution of the stereochemically pure serine pool owing to the back reaction, or the slight contribution of the nonenzymatic process. The results for the two experimental sets display the expected relationship to one another.

Table IV contains the results of reactions carried out with various concentrations of serine transhydroxymethylase that were calculated to cause different amounts of equilibration of the labeled serine pool (the latter figures are approximate values based on the initial reaction rates for serine to glycine conversion). Increasing amounts of equilibration can be seen to cause greater loss of stereochemistry and eventually lead to racemate (54 ± 5, 46 ± 5%) at 100% equilibration. Table V contains results of preincubation experiments with the coupling enzyme, carried out in order to help rule out its participation in racemizing the methylene center. Inspection reveals that the presence of the coupling enzyme over a 5-min period followed by addition of cofactor yields the same percent retention as an experiment where both dehydrogenase and cofactor are added concurrently after 5 min.

Finally, the reverse of reaction 3 was run employing *d,l*-[^{3}H]-5,10-CH_2-H_4folate having an isomeric distribution of 76/24 (used for kinetic studies). In separate experiments, it was determined that, under the reaction conditions employed, the system had reached both chemical and isotopic equilibrium before quenching. The serine synthesized was analyzed by conversion to indolmycin (Skye et al., 1974) and was found to be completely racemic (Table VI). Not listed in Table VI is the result of incubating the same diastereomer under identical conditions in the presence of 3 units of the serine transhydroxymethylase enzyme for 10 min, but with the dehydrogenase enzyme absent. Subsequent assay for retained label showed no increase in racemization of the [^{3}H]-5,10-CH_2-H_4folate above that caused by nonenzymatic racemization.

Discussion

The mechanism of the reaction may be thought of in terms of two classes of pathways that are stereochemically distinguishable. The first class is comprised of mechanisms which feature the complete or partial rotation of substrates or reaction intermediates containing an asymmetric center in competition with their rate of interconversion so that a partial or total loss

TABLE IV: Stereochemical Results of Serine Reactions at 25 °C as a Function of Serine Transhydroxymethylase Concentration.[a]

3-[^{3}H]Ser Isomer	Enzyme Act. (% Ser Pool Equilibrated)[b]	Ser $^3H/^{14}C$	*d*-5,10-CH^+-H_4folate[c] $^3H/^{14}C$	% ^{3}H Retained	Normalized[d] % ^{3}H Retained (±5%)
3*R*	0.17 unit	3.78	1.04	27	31
3*S*	(<5)	4.35	2.65	61	69
3*R*	3.7 units	3.33	1.12	33	43
3*S*	(80)	2.84	1.30	46	57
3*R*	7.5 units	3.33	1.47	44	54
3*S*	(100)	2.84	1.08	38	46

[a] Serine transhydroxymethylase reaction run for 5 min followed by addition of 5,10-methylenetetrahydrofolate dehydrogenase. Amounts of transhydroxymethylase enzyme were increased to increase the flux in the fixed time period to give the approximate percent of serine pool equilibration as shown. [b] The latter was approximated from the turnover number for the enzyme under these conditions. [c] $^3H/^{14}C$ statistically corrected due to use of [U-^{14}C]serine. [d] See text for discussion. Based on $R + S$ % retention = 100%.

TABLE V: Control Reactions with Coupling Enzyme.

Conditions	(3*R*)-3-[^{3}H]Ser $^3H/^{14}C$	*d*-5,10-CH^+-H_4-folate[d] $^3H/^{14}C$	% ^{3}H Retained (±5%)
Coupled reaction[a]	3.78	0.91	24
5-min sequential (with coupling enzyme)[b]	3.78	1.04	27
5-min sequential (with coupling enzyme)[c]	3.78	0.77	21

[a] Reaction mixture (25 °C) contained complete complement of both enzymes, serine transhydroxymethylase (0.34 unit/mL) and 5,10-methylenetetrahydrofolate dehydrogenase (0.52 unit/mL). [b] Serine transhydroxymethylase reaction (0.17 unit/mL) at 25 °C in the presence of 5,10-methylenetetrahydrofolate dehydrogenase (0.49 unit/mL) run for 5 min followed by addition of NADP. [c] NADP, serine hydroxymethylase (0.33 unit/mL), and 5,10-methylenetetrahydrofolate dehydrogenase (0.53 unit/mL) initially present run at 25 °C for 5 min followed by addition of *d,l*-H_4folate. [d] $^3H/^{14}C$ statistically corrected due to use of [U-^{14}C]serine.

TABLE VI: Stereochemistry of Serine Produced at Equilibrium from Chiral *d,l*-[^{3}H]-5,10-CH_2-H_4folate.[a]

[^{3}H]/[^{14}C]-L-Ser	[^{3}H]/[^{14}C] Trp[b]	[^{3}H]/[^{14}C]-Indolmycin[b]	% ^{3}H Retained
14.83	14.86	7.50	50.6

[a] Isomeric distribution of 76/24. Reaction, incubated for 6 min at 25 °C, contained 0.51 unit of serine transhydroxymethylase, 0.68 μmol of [U-^{14}C]glycine at 25 °C, and 0.32 μmol of *d,l*-[^{3}H]-5,10-CH_2-H_4folate in a total volume of 100 μL. [b] Assayed by method of Skye et al. (1974).

of asymmetry occurs at this center. This classification also includes those intermediates possessing an alternating axis of symmetry, i.e. torsio-symmetric (Hanson, 1975), with the same stereochemical consequences. A specific example in this case would be the involvement of a discrete, freely rotating transient formaldehyde which is formed along with the glycine anion following C-2–C-3 bond cleavage of the serine. A model study employing serine and pyridoxal in the presence of Al^{3+} has demonstrated the operation of this pathway (Metzler et al., 1953, 1954). The second category includes intermediates bound and acted upon at the active site stereospecifically so that net retention or inversion of configuration occurs. The great majority of enzyme-catalyzed processes fall under this heading (Hanson and Rose, 1975). The specific example cited above would appear in this category provided interaction with the protein prevented rotation of formaldehyde prior to transfer.

Although it has been generally assumed (Biellmann and Schuber, 1967, 1970; Blakley, 1969) that 5,10-methylenetetrahydrofolate dehydrogenase stereospecifically removes one imidazolidine hydrogen from *d*-5,10-CH_2-H_4folate, this has not previously been rigorously demonstrated. Using racemic *d,l*-[^{3}H]-5,10-CH_2-H_4folate (Table I) synthesized under identical conditions to the deuterio analogue, which was shown to be racemic at the methylene group by integration of the ^{1}H NMR resonances, one would expect to obtain 50% tritium in each of the products NADPH and *d*-5,10-CH^+-H_4folate *only* if the reaction were stereospecific. Results of up to 90% retention would be obtained if the reaction were completely random (Battersby, 1972) owing to discrimination between ^{3}H and ^{1}H in the transfer step. That the latter transfer contributes significantly to the rate-determining step is in accord with the twofold slower oxidation of the D,D relative to the H,H methylene isomer by the enzyme (Farina et al., 1973). Therefore, since 50% of the tritium from racemic *d,l*-[^{3}H]-5,10-CH_2-H_4folate is isolated in each of the two products, the 5,10-methylenetetrahydrofolate dehydrogenase is stereospecific for removal of hydride from *d*-5,10-CH_2-H_4folate.

Chemically synthesized *d,l*-[^{3}H]-5,10-CH_2-H_4folate which assayed for an isomeric distribution of 76/24 was of sufficient optical purity to study the nonenzymic racemization rates. As can be seen in Figure 2, the racemization rate measured as a decrease in the tritium assayed as *retained label* is buffer catalyzed. Extrapolation of k', the pseudo-first-order rate constant for racemization, to zero buffer concentration suggests the rate of racemization is negligible in the absence of buffer. Although further verification of this observation was not attempted, it would seem to indicate that ring opening of 5,10-CH_2-H_4folate to the iminium cation is stereospecific, and that racemization is due to trapping of this species by buffer, followed by rotation, and the reclosure to starting material. The conditions chosen preclude racemization due to the formation of the carbinolamine followed by return to 5,10-CH_2-H_4folate since at this pH the rate-determining step is hydration of the iminium cation with the carbinolamine decomposing rapidly to aldehyde. Furthermore, racemization due to equilibration with free formaldehyde is obviated by the presence of sufficient mercaptoethanol to trap any formaldehyde liberated (Kallen and Jencks, 1966). Examination of both Corey–Pauling–Koltun (CPK) models and the ^{1}H NMR suggests that the polycyclic structure of 5,10-CH_2-H_4folate constrains the N-5

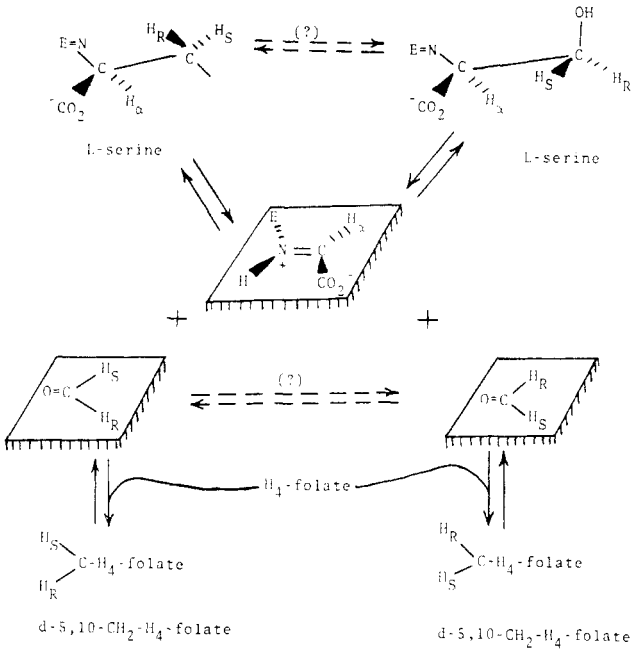


FIGURE 4: Possible binding and reaction modes for serine cleavage.

from inversion. If the favored mode of ring opening requires the leaving group to be anti-periplanar to the N-5 lone pair as in the case of E2 elimination reactions (Saunders, 1976) then the above result requires that ring opening/ring closing occur from either one conformation exclusively or, less likely, from two nonequilibrating conformations in which the N-10 amino function lies above and below the plane of the incipient iminium cation, respectively.

The results of the enzyme-catalyzed hydroxymethylase transfer, shown in Table III, are in excellent agreement with those obtained with rat liver tissue by Biellmann and Schuber (1967, 1970). Starting with optically pure (3*S*)-3-[^{3}H]serine, using the purified rabbit liver enzyme, one obtains *d*-5,10-CH^+-H_4folate containing an average of 76% of the label whereas with (3*R*)-3-[^{3}H]serine, one obtains *d*-5,10-CH^+-H_4folate containing 24% of the label. Consequently, there is about 24% crossover of label from each serine isomer into the *d*-5,10-CH_2-H_4folate isomer with the methylene unit of opposite stereochemistry.

In order to exclude extraneous factors as being responsible for the partial stereospecificity, several control experiments were performed. The lack of response of the isomeric distribution of product to the amount of 5,10-methylenetetrahydrofolate dehydrogenase (Table III) rules out nonenzymic racemization of the 5,10-CH_2-H_4folate as a significant competing process in accord with direct measurements of the nonenzymic racemization rates. Secondly, data obtained upon preincubation (Table V) do not indicate that the racemization of serine is catalyzed by the coupling enzyme, 5,10-methylenetetrahydrofolate dehydrogenase, or impurities within this preparation. Thirdly, the racemization of serine does not occur in the presence of transhydroxymethylase enzyme within this time frame if the tetrahydrofolate cofactor is absent (Table V). However, the degree of racemization is dependent on the extent of equilibration, increasing with increasing enzyme concentration, and consequently is a function of the reaction itself (Table IV). Additional support for this view is seen in Table VI, where L-serine racemic at C-3 is obtained at equilibrium from partially optically pure 5,10-CH_2-H_4folate. Fourthly, the dehydrogenase preparation does not contribute to the racemization of the intermediate *d*-5,10-CH_2-H_4folate since the enzyme removes only 50% of the total tritium from a nonstereospecifically labeled substrate (Table I). Finally, the rate of racemization of *d,l*-5,10-CH_2-H_4folate is not accelerated by the presence of serine transhydroxymethylase.

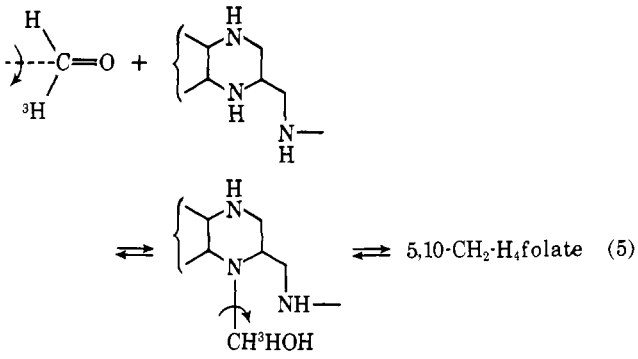
The major conclusion that may be drawn is that one pass of the substrate through the reaction sequence does not lead to complete racemization. At some point in the conversion, therefore, the two diastereotopic hydrogens of the hydroxymethyl residue become equivalent, but factors which cause this equivalence are not absolute; therefore, complete racemization is not observed. Nonenzymic processes are ruled out as significant contributors to this racemization. Since one observes only partial racemization in one turnover, i.e. 75% of the initial isomeric distribution is retained,[4] the enzyme exerts incomplete control over the steric course of the reaction it catalyzes.

The mechanistic consequences of this finding can be examined in terms of Figure 4 where the enzyme catalyzes the cleavage of serine (or in the reverse reaction, the condensation of glycine with 5,10-CH_2-H_4folate) by two parallel pathways. Normally these are indistinguishable because of the symmetry of the hydroxymethyl and methylene moieties. However, when heavy isotopes are employed so that one can distinguish between the two hydrogens, crossover between the pathways may be observed. It is plausible, for reasons discussed below, that crossover may occur in the binding step of serine or in a catalytic step involving formaldehyde or its carbinolamine as a discrete species. Equilibration, i.e. many turnovers, in either case would lead to the gradual racemization of 5,10-CH_2-H_4folate commencing from the (3*R*)- or (3*S*)-3-[^{3}H]serine. While in principle these two possibilities are experimentally distinguishable, provided the rate-limiting step in serine synthesis is not its release from the enzyme, the present experiments are incapable of such a distinction.

Previous studies (Schirch and Diller, 1971; Palekar et al., 1973) have shown that the enzyme catalyzes the cleavage of threonine and allothreonine and *erythro-* or *threo-*β-phenylserines to their corresponding aldehydes plus glycine and the nonstereospecific cleavage of aminomalonate to glycine and carbon dioxide. This indicates that there is considerable latitude in the steric requirements of the binding site with regard to substituents on C-3 and that the configuration at the β carbon is not critical for binding *or* substrate activity. Thus, the distribution of [^{3}H]-5,10-CH_2-H_4folate isomers may reflect the initial distribution of two bound serine conformations. It is assumed in Figure 4 that the transfer of the one carbon unit to H_4folate from serine (depicted as formaldehyde) occurs only from the *si* face of the glycine anion since extensive studies (Jordan and Akhtar, 1970) have shown that H^+ is exchanged only on this face, implying that the glycine binds one way. An analogous example of this type of phenomenon has recently been discussed by Meloche et al. (1975) where two bound forms of substrate led to the formation of two diastereomeric products in the reversible reaction catalyzed by 2-keto-4-hydroxyglutarate aldolase of *Escherichia coli*.

Alternatively, the crossover may occur at the intermediate formaldehyde stage. Expanding in eq 5 this aspect of Figure 4, two additional plausible points of equilibration may be identified, namely rotation of the formaldehyde prior to condensation with H_4folate or rotation at the level of carbi-

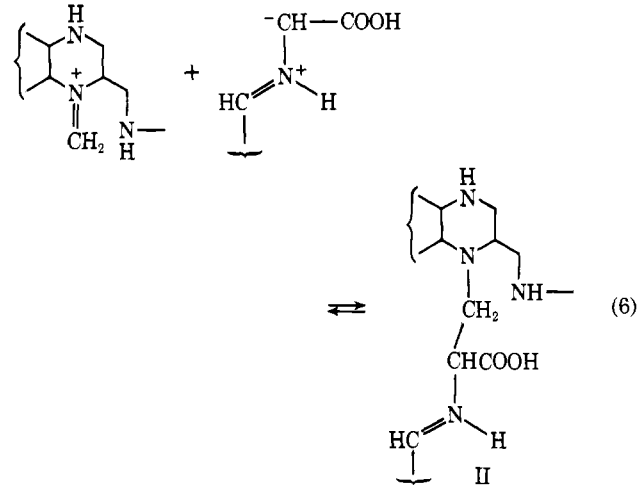
[4] One can interpret this result in terms of one pathway partitioning in the rate ratio 75:25 or two parallel routes, completely stereospecific and nonstereospecific, respectively, in the rate ratio 50:50. In any case, the enzyme is partially stereospecific.



(5)

nolamine.[5] In the case of threonine and allothreonine cleavage, this would involve the intermediacy of acetaldehyde bound on either the *re* or *si* face following cleavage.

It should be noted that an argument in favor of the intermediacy of formaldehyde has been advanced on chemical grounds (Jordan and Akhtar, 1970). A mechanism proceeding via II, the product of the capture of the N-5 iminium cation[6] by glycine anion, which has been previously discounted on the tenuous basis that hydrolytic cleavage of II is a difficult process, is now less likely in view of the stereochemical results. Ring opening/ring closing via the N-5 iminium cation apparently is stereospecific. Since cleavage of II most likely would require general acid–base catalysis it is highly probable that the serine–glycine interconversion via this mechanism would be stereospecific.



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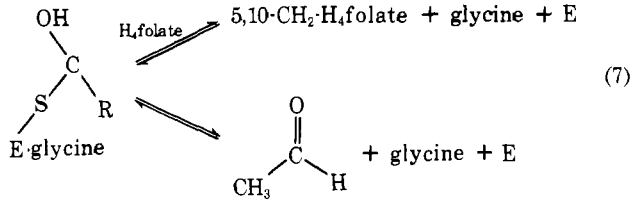
The scheme depicted in Figure 4, however, is subject to the following criticism. As illustrated it would appear possible to demonstrate either serine or 5,10-CH_2-H_4folate racemization independently of cosubstrate in the presence of serine transhydroxymethylase. The failure of such experiments may be due to an unfavorable equilibrium between free substrate and the requisite bound intermediate in the absence of cosubstrate necessitating the use of larger concentrations of enzyme or longer reaction times than employed here. Secondly, the scheme offers no obvious rationale for catalysis by H_4folate of serine cleavage but not that of allothreonine or threonine. Recent experiments (Jordan et al., 1976) have demonstrated that the cleavage of threonine containing ^{18}O at the hydroxyl function proceeds with complete transfer of label to acetaldehyde seemingly ruling out an enzyme–imine intermediate.[7] It is plausible, however, that the aldehyde may be bound noncovalently or as a thiol (eq 7) or hemiacetal and its mode of release from the enzyme may be dependent on the identity of the aldehyde. The decreased stability of imidazolidine adducts of H_4folate when R = CH_3 or C_6H_5 vs. R = H (Tatum, 1975) offers some support for this hypothesis.



(7)

Finally, it is of interest that the enzyme can catalyze the condensation of glycine with formaldehyde in the absence of H_4folate (Chen and Schirch, 1973a). The rate of this reaction is about 0.006 that in the presence of H_4folate. Since under the conditions of the reaction, >99% of the formaldehyde would exist as the hydrated form (Le Hénaff, 1963), and since high levels of H_2CO (and hence the hydrate) are known to inhibit the enzyme, one of the catalytic roles of H_4folate probably is to maintain H_2CO in a catalytically active and noninhibitory form, a condition not required for the other less hydrated aldehydes. Acetaldehyde, for example, is ca. 50% hydrated under these conditions (Bell and Clunie, 1952). In the presence of excess H_4folate, virtually all of the H_2CO would exist as 5,10-CH_2-H_4folate ($K_{eq} = 10^4$); therefore, the effective concentration of the active methylene group would be a factor of about 100 times greater in the presence of H_4folate than in its absence.

[5] Given the likely assumption that N-5 may invert in the N_5-hydroxymethyltetrahydrofolate intermediate, antielimination of H_2O by the nitrogen lone-pair electrons to form the N-5 iminium cation may take place with the -OH functionality either syn or anti to the hydrogen at C-6. This leads to crossover of the two possible diastereomeric [^{3}H]-5,10-CH_2-H_4folates, although it requires that attack or departure of the hydroxyl moiety be possible on either face of the iminium cation.

[6] That the iminium cation is at N-5 rather than at N-10 has been discussed by several authors (Kallen and Jencks, 1966; Benkovic et al., 1969a,b; Barrows et al., 1976).

[7] A mechanism involving group transfer of the aldehyde to the enzyme possibly stabilized as an imine has been postulated as an intermediate step between cleavage of the C-2–C-3 bond of serine and formation of 5,10-CH_2-H_4folate (Chen and Schirch, 1973b).

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Enzymic Hydrolysis of Phosphonate Esters. Reaction Mechanism of Intestinal 5′-Nucleotide Phosphodiesterase†

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ABSTRACT: The mechanism of bovine intestinal 5′-nucleotide phosphodiesterase was investigated by determining kinetic constants of systematically varied substrates, with emphasis on esters of phosphonic acids (which have much higher V_{max} values than conventional phosphodiester substrates), and by pre-steady-state kinetics using bis(4-nitrophenyl) phosphate as substrate. The results suggest a ping-pong type mechanism, with participation of a covalent enzyme intermediate.

We have previously demonstrated that enzymes which hydrolyze phosphonate esters[1] are widely distributed in nature (Kelly and Butler, 1975) and that this activity is due to and characteristic of 5′-nucleotide phosphodiesterase enzymes (Kelly et al., 1975). These substrates thus provide a convenient and economical means of assaying 5′-nucleotide phosphodiesterases and of distinguishing them from phosphodiesterases which produce 3′-nucleoside monophosphates and do not hydrolyze phosphonate monoesters (Kelly et al., 1975). Several other names have been applied to enzymes having properties similar to the enzyme which we refer to as 5′-nucleotide phosphodiesterase (Kelly and Butler, 1977).

We have investigated the mechanism of bovine intestinal 5′-nucleotide phosphodiesterase by several techniques. This paper describes steady-state kinetic parameters obtained using a group of substrates with systematically varied structures, and pre-steady-state kinetics using one of these substrates. The results suggest a "ping-pong" type mechanism (Cleland, 1967) involving an intermediate consisting of a phosphonyl (phos-

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[1] As used here, the term "phosphonate esters" refers only to *monoesters* of phosphonic acids.