antibiotics in the body, for the greater activity of teicoplanin as compared with vancomycin.

Acknowledgment. We gratefully acknowledge the help of S. Santikarn in obtaining FAB mass spectra, Dr E. Houghton and P. Teale of Racecourse Securities Lab., Newmarket, for access to GCMS facilities and assistance in obtaining data, and SERC and Gruppo Lepetit for financial support. D.J.M.S. thanks the Royal Commission for the Exhibition of 1851 for an Overseas Research Scholarship.

Registry No. T-A2-1, 91032-34-7; T-A2-2, 91032-35-8; T-A2-3, 91032-36-9; T-A2-4, 91032-37-0; T-A2-5, 91032-38-1; T-A3-2, 91032-39-2; T-aglycone, 89139-42-4; teicoplanin, 61036-64-4.

Steric Course of Ketopantoate Hydroxymethyltransferase in *E. coli*†

D. John Aberhart* and David J. Russell

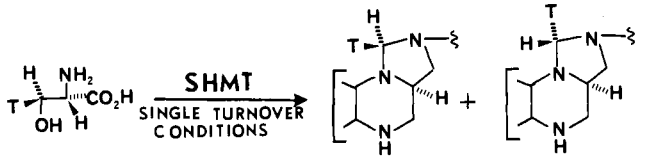
Contribution from the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. *Received December 12, 1983*

Abstract: The conversion of α-ketoisovaleric acid (α-KIVA) to ketopantoate by the 5,10-methylenetetrahydrofolate-dependent enzyme ketopantoate hydroxymethyltransferase (KHMT) in *E. coli* has been shown to proceed in a retention mode at the β-position of α-KIVA. 5,10-Methylenetetrahydrofolate formed in vivo by serine hydroxymethyltransferase (SHMT) from stereospecifically deuterated ($3S$-d_1) serine was converted by KHMT into an ca. 3:1 ratio of deuterated ketopantoates with the 4S isomer predominating. The results indicate that KHMT and SHMT have the same overall steric course in *E. coli*.

Ketopantoate hydroxymethyltransferase (5,10-methylenetetrahydrofolate: α-ketoisovalerate hydroxymethyltransferase; KHMT) catalyzes the reversible condensation of α-ketoisovaleric acid (α-KIVA) (**1**) with the biochemical equivalent of formaldehyde, N^5, N^{10}-methylenetetrahydrofolate (5,10-CH_2-H_4folate), to produce ketopantoate (**2**).[1,2] This reaction constitutes the first *committed* step in the biosynthesis of pantothenate and coenzyme A. KHMT obtained from *E. coli* K-12 was recently purified to apparent homogeneity by Snell et al.[3] They showed that it is this enzyme, and *not* a 5,10-CH_2-H_4folate-*independent* enzyme[4] in *E. coli*, capable of catalyzing the condensation of formaldehyde with α-KIVA, which is responsible for ketopantoate formation in vivo. Unlike the related 5,10-CH_2-H_4folate-dependent enzyme, serine hydroxymethyltransferase[5] (SHMT), KHMT is a class II aldolase and does not require pyridoxal phosphate as cofactor.

1 **2**

SHMT has been the subject of extensive stereochemical studies[6-13] (Scheme I). These studies have revealed that, in the conversion of glycine to (2S)-serine, the 2-H_{si}[14] atom is removed and the 2-H_{re} atom retained, **3** → **4**.[6-9] Thus the reaction proceeds stereospecifically with retention at C-2 of glycine. In contrast, studies by Biellmann et al.[10,11] of the steric course of formation of the hydroxymethyl group of serine revealed a most unusual partially stereospecific process. Incubation of tritiated formate (**5**) with rat liver slices led, via tritiated 5,10-methenyltetrahydrofolate (5,10-C^3H^+-H_4folate) (**6**) and tritiated 5,10-$C^3H_1{}^1H_1$-H_4folate (**7**), to serine having 72% of the tritium in the 3-H_{si} position (**8**) and 28% in the 3-H_{re} position (**9**). More detailed studies by Benkovic and Floss et al.[12] using purified SHMT also revealed a partially stereospecific course in the reverse process leading from serine (stereospecifically tritiated at C-3) to 5,10-CH_2-H_4folate. Under single-turnover conditions, from (2S,3S)-[3-$^3H_1$]serine (**8**) a ca. 76:24 ratio of tritiated 5,10-$C^3H_1{}^1H_1$-H_4folates (**10**) and (**11**), respectively[12b] (trapped by in situ dehydrogenation by 5,10-CH_2-H_4folate dehydrogenase to 5,10-CH^+-H_4folate), was formed. However, if the reversible SHMT reaction was allowed to proceed through many turnovers, essentially complete racemization of both the resultant 5,10-$C^3H_1{}^1H_1$-H_4folate at C-11 and the resultant serine at the C-3 prochiral center was observed.



8 **10** (76%) **11** (24%)

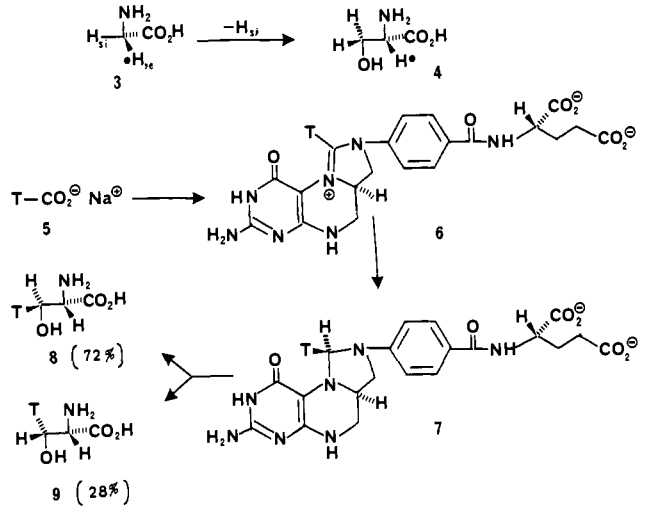
In view of the apparent similarity, at least in terms of reaction type, of the SHMT and KHMT reactions, it was of interest to compare the steric courses of the two reactions. We therefore undertook a stereochemical investigation of the KHMT-catalyzed reaction and now report the results of that investigation.

† This paper is dedicated to Dr. Eliahu Caspi on the occasion of his 71st birthday.

(1) Brown, G. M. "Comprehensive Biochemistry"; Florkin, M., Stotz, E. H., Eds.; Elsevier: New York, 1971; Vol. 21, pp 73-80.
(2) Abiko, Y. In "Metabolic Pathways"; Greenberg, D. M., Ed.; Academic Press: New York, 1975; Vol. 7, pp 1-25.
(3) (a) Teller, J. H.; Powers, S. G.; Snell, E. E. *J. Biol. Chem.* **1976**, *251*, 3780-3785. (b) Powers, S. G.; Snell, E. E. *J. Biol. Chem.* **1976**, *251*, 3786-3793. (c) Powers, S. G.; Snell, E. E. *Methods Enzymol. 62*, 204-209.
(4) McIntosh, E. N.; Purko, M.; Wood, W. A. *J. Biol. Chem.* **1957**, *228*, 499-510.
(5) Schirch, L. *Adv. Enzymol.* **1982**, *53*, 83-112.
(6) Besmer, P.; Arigoni, D. *Chimia* **1968**, *23*, 190.
(7) Besmer, P. Ph.D. Dissertation No. 4435, ETH, Zurich, 1970.
(8) Jordan, P. M.; Akhtar, M. *Biochem. J.* **1970**, *116*, 277-286.
(9) Akhtar, M.; El-Obeid, H. A.; Jordan, P. M. *Biochem. J.* **1975**, *145*, 159-168.
(10) Biellmann, J. F.; Schuber, F. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 517-522.
(11) Biellmann, J. F.; Schuber, F. J. *Bull. Soc. Chim. Biol.* **1970**, *52*, 211-227.
(12) (a) Tatum, C. M.; Benkovic, P. A.; Benkovic, S. J.; Potts, R.; Schleicher, E.; Floss, H. G. *Biochemistry* **1977**, *16*, 1093-1102. (b) Slieker, L.; Benkovic, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 1833-1838.
(13) Reviews on SHMT stereochemistry: (a) Rétey, J.; Robinson, J. A. "Stereospecificity in Organic Chemistry and Enzymology"; Verlag Chemie: Weinheim, 1982; pp 160-167; (b) Floss, H. G.; Vederas, J. C. In "Comprehensive Biochemistry"; Tamm, C. Ed.; Elsevier: Amsterdam, 1982; Vol. 3, pp 175-177.
(14) Nomenclature: Prelog, V.; Helmchen, G. *Helv. Chim. Acta* **1972**, *55*, 2581-2598. See also ref 13a, pp 1-16.

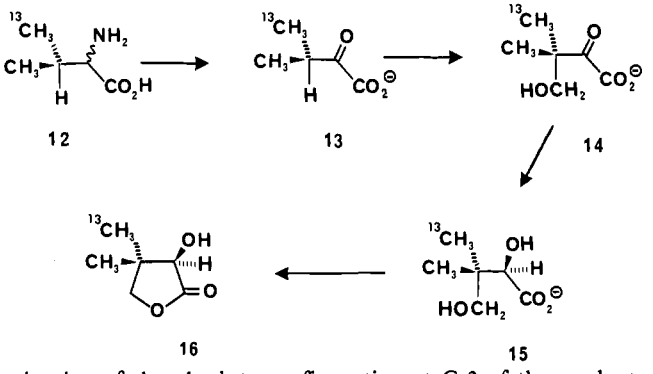
0002-7863/84/1506-4902$01.50/0

Scheme I. Steric Course of Serine Hydroxymethyltransferase



Results

Steric Course at C-3 of α-KIVA.[15] Our first objective was an examination of the steric course of replacement of the C-3 hydrogen of α-KIVA by the incoming hydroxymethyl group. In principle, this required conversion by KHMT of a chirally labeled sample of α-KIVA, e.g., **13**, to ketopantoate, followed by determination of the absolute configuration at C-3 of the product. However, since chirally labeled α-KIVA might be expected to be configurationally labile as a result of enolization, our approach was to generate chiral α-KIVA in vivo from chirally labeled valine (**12**) through the action of the branched-chain amino acid aminotransferase.[16,17] In such an in vivo experiment, the resultant ketopantoate would not be expected to accumulate but would be subsequently reduced by ketopantoate reductase[18] to (2*R*)-pantoate[19] (**15**), which in turn might (at least in part) be converted to subsequent metabolites (pantothenate, CoA, etc.). However, after acidic hydrolysis, pantoate and these subsequent metabolites would all be converted to (2*R*)-pantolactone (**16**) as shown by Teller.[20]

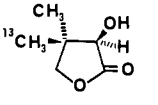


For maximum utilization of the synthetic exogenous valine without dilution by endogenous valine and/or α-KIVA, our studies were carried out by using a valine-isoleucine auxotroph of *E. coli* (ATCC 23783), following, with a few modifications, procedures developed by Teller.[20] The nature of the metabolic block in this organism (and in the *E. coli* V-4 valine–isoleucine auxotroph used by Teller) is unknown, but it is relevant to note that neither organism requires exogenous leucine. Since the normal pathway for leucine biosynthesis proceeds via α-KIVA derived from valine,[21] clearly the organism is able to produce adequate α-KIVA for leucine biosynthesis and growth from exogenous valine but is unable to biosynthesize α-KIVA from other endogenous precursors. Consequently the only source of α-KIVA for ketopantoate biosynthesis is also the exogenous valine. Teller[20] showed that the specific activity of ^{14}C-labeled pantolactone isolated after incubation of [1-^{14}C]valine with *E. coli* V-4 corresponded closely to that expected for biosynthesis without dilution with endogenous α-KIVA from sources other than the added valine.

Similarly, in preliminary incorporation experiments, we found that the specific activity of ^{14}C-labeled pantolactone, formed in 2.8% radiochemical yield after incubation of [4,4'-^{14}C]-DL-valine with *E. coli* ATCC 23783, was approximately equal to that expected for incorporation without dilution (prior to adding pantothenate in the workup). The radiochemical yield and the dilution factor after adding carrier (ca. 1:8) were quite adequate for use in tracing the fate of the C-3 prochiral center by ^{13}C NMR methods.

Thus, (2*RS*,3*S*)-[4-^{13}C]valine[22] (**12**) was incubated with *E. coli* (ATCC 23783), and after addition of calcium pantothenate and acidic hydrolysis, D-pantolactone (**16**) was isolated. The ^{13}C NMR spectrum ($CDCl_3$) of the biosynthetic product showed an eightfold enhancement of the downfield methyl signal (δ 22.94) as compared with the other methyl signal (δ 18.80) and other signals which were apparently unchanged in intensity. Thus, the condensation of α-KIVA with the C-1 donor takes place stereospecifically at C-3.

For assignment of the steric course of the reaction, it is necessary to know the assignment of ^{13}C signals to the diastereotopic methyls of pantolactone. In our earlier publication,[15] it was assumed by one of us (D.J.A.) that the downfield signal should be assigned to the methyl cis to the C-2 hydroxyl. Subsequently Wightman[23] reported that, in the Eu(fod)$_3$-shifted ^{13}C (and ^1H) NMR of pantolactone, the methyl signals underwent shifting at different rates, the upfield signal shifting faster than the downfield signal. Since the proton signal for the C-2 proton was shifted most rapidly, it appeared probable that the shift reagent was complexing mainly with the hydroxyl group, causing stronger shifting of the methyl group closest to the hydroxyl. The results suggested that the original signal assignment should be reversed. Assignment of the higher field signal at δ 18.8 to the methyl cis to the hydroxyl would also be consistent with shielding resulting from the "α substituent effect".[24] More recently, Wasmuth, Arigoni, and Seebach[25] have completed a stereospecific synthesis of **17** which firmly establishes



the revised signal assignment. It follows, therefore, that (3*S*)-[4-^{13}C]-α-KIVA (**13**) derived from chiral valine (**12**) is converted by KHMT to ketopantoate labeled as shown in **14** and that the condensation reaction proceeds in a *retention* mode at the carbon accepting the C-1 donor, as previously observed for the SHMT reaction.

Steric Course of Hydroxymethyl Group Transfer. We next turned our attention to a study of the stereochemistry of formation

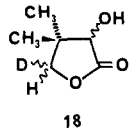
(15) Preliminary communication of some of the results in this section: Aberhart, D. J. *J. Am. Chem. Soc.* **1979**, *101*, 1354–1355.
(16) Martin, R. R.; Marshall, V. D.; Sokatch, J. R.; Unger, L. *J. Bacteriol.* **1973**, *115*, 198–204.
(17) For a similar enzymatic generation of chirally labeled α-KIVA from a configurationally stable precursor, see: Hill, R. K.; Yan, S.; Arfin, S. M. *J. Am. Chem. Soc.* **1973**, *95*, 7857–7859.
(18) (a) King, H. L.; Wilken, D. R. *J. Biol. Chem.* **1972**, *247*, 4096–4098. (b) Wilken, D. R.; Dyer, R. E. *Arch. Biochem. Biophys.* **1978**, *189*, 251–255.
(19) Absolute configuration: Hill, R. K.; Chan, T. H. *Biochem. Biophys. Res. Commun.* **1970**, *38*, 181–183.
(20) Teller, J. H. Ph.D. Dissertation, University of California, Berkeley, 1970.
(21) Bender, D. A. "Amino Acid Metabolism"; Wiley: New York, 1975; pp 135–136.
(22) Aberhart, D. J.; Lin, L. J. *J. Chem. Soc., Perkin Trans. 1* **1974**, 2320–2326.
(23) Wightman, R. H. *J. Chem. Soc., Chem. Commun.* **1979**, 818–819.
(24) Eggert, H.; VanAntwerp, C. L.; Bhacca, N. S.; Djerassi, C. *J. Org. Chem.* **1976**, *41*, 71–78. We thank Drs. David Cane and Haruo Seto for pointing this out to us.
(25) Wasmuth, D.; Arigoni, D.; Seebach, D. *Helv. Chim. Acta* **1982**, *65*, 344–352. We thank Professors Arigoni and Seebach for their courtesy in informing us of their results prior to publication.

of the hydroxymethyl group of ketopantoate (or of the oxymethyl group of pantolactone) by KHMT from a stereospecifically labeled 5,10-C*$H_1{}^1H_1$-H_4folate. We planned again to work with the intact *E. coli* in which ketopantoate would not accumulate, in order to avoid problems resulting from the reversibility of the KHMT reaction. Such reversibility might, a priori, be expected to cause racemization of the prochiral hydroxymethyl group, as observed by Benkovic et al.[12] in their studies of SHMT under multiple-turnover conditions.

We initially planned to prepare the required stereospecifically labeled 5,10-C*$H_1{}^1H_1$-H_4folate in vivo by the method used by Biellmann et al.[10,11], i.e., by enzymatic conversion of [^{2}H]- or [^{3}H]formate to 5,10-C*H^+-H_4folate, followed by reduction in vivo by methenyl-H_4folate reductase to 5,10-C*$H_1{}^1H_1$-H_4folate. Assuming that the steric course of this process in *E coli* is the same as in liver, the expected labeled 5,10-C*$H_1{}^1H_1$-H_4folate would be as shown in **7**. However, in preliminary incorporation experiments, we found that [^{14}C]formate (co-incubated with DL-valine) was poorly incorporated (ca. 0.005–0.02%) into pantolactone. This result was consistent with a report[26] which appeared during the course of this work. The authors concluded that, in *E. coli*, serine (C-3) and glycine (C-2) are the only significant sources of methylene groups for 5,10-CH_2-H_4folate biosynthesis and that formate does not enter the one-carbon pool through the folate pathway. The dilution of label (ca. 1:550–1:5000, depending on incubation and isolation conditions used) in our incorporations appeared to be too high for the use of deuterium labeling, although presumably tritium labeling could still have been used in this investigation.

As an alternative biosynthetic route to stereospecifically labeled 5,10-C*$H_1{}^1H_1$-H_4folate, we considered the use of stereospecifically labeled (3*R*)- or (3*S*)-3-*H_1-serines as precursors. These would be expected, through the agency of SHMT, to produce a mixture of **10** (ca. 75%) and **11** (ca. 25%) from (3*S*)-3-$^3H_1$-serine (**8**) or (**10**) (ca. 25%) and **11** (ca. 75%) from (3*R*)-3-$^3H_1$-serine (**9**), assuming again that the steric course and stereospecificity of the *E. coli* SHMT are the same as those of the liver enzyme. Presumably the same ratios of the deuterated analogues of **10** and **11** would result from the corresponding deuterated serines. After incubation of [3-^{14}C]-DL-serine with *E. coli*, [^{14}C]pantolactone was isolated in 0.5% radiochemical yield with a dilution (after adding carrier calcium pantothenate before acidic hydrolysis) of ca. 1:42. Thus, although serine was not as efficient a precursor of pantolactone as was valine, nevertheless the radiochemical yield and moderate dilution indicated that deuterated serines could be used as pantolactone precursors, in conjunction with the use of ^{2}H NMR for determination of the labeling stereochemistry in the resultant pantolactone.

In the ^{1}H NMR spectrum of pantolactone in $CDCl_3$,[27a] the C-4 proton signals are separated by only 0.073 ppm (δ_1 3.962, δ_2 4.035, J = 8.8 Hz). These signals were not appreciably further separated by adding Eu(fod)$_3$ to the solution. Similarly, in pyridine-d_5,[27b] the signal separation is only 0.070 ppm (δ_1 3.942, δ_2 4.012, J. = 8.7 Hz). However, in benzene-d_6 solution, these signals became separated by ca. 0.10–0.14 ppm. The separation was concentration dependent but became constant at concentrations ≤100 mg/mL (δ_1 3.177, δ_2 3.318, J = 8.8 Hz). Furthermore, the deuterium signals of (2*RS*,4*RS*)-pantolactone-4-d_1 (**18**), prepared by reduction of zinc aldopantoate with $NaBH_4$ followed by lactonization,[28] when recorded at 76.77 MHz, could be resolved nearly to base line (Figure 1a). Through a degradative sequence carried out on asymmetrically C-4-deuterated (2*R*)-pantolactone, described in detail in the accompanying paper,[29] it was shown that the signal due to 4-H_{si} appears to low field of the signal due to 4-H_{re} for (2*R*)-pantolactone.



18

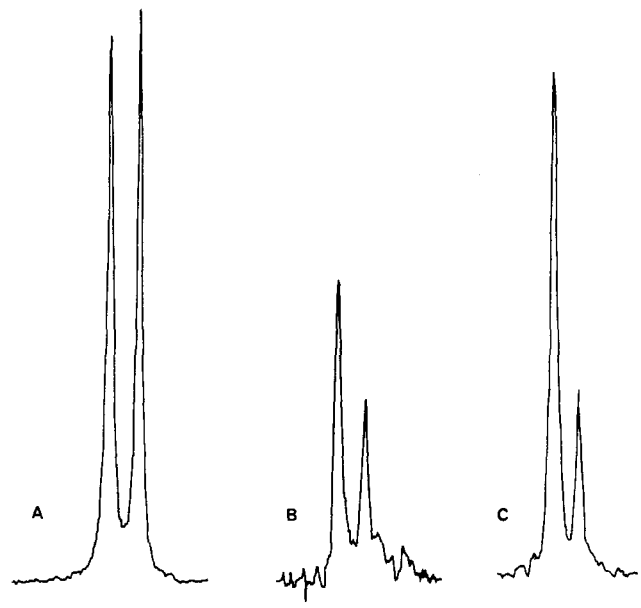
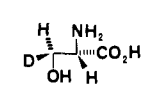


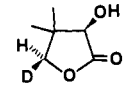
Figure 1. ^{2}H NMR Spectra: (A) Synthetic (2*RS*,4*RS*)-pantolactone-4-d_1; (B) biosynthetic pantolactone derived from 84% stereospecifically labeled (2*S*,3*S*)-serine-3-d_1; (C) biosynthetic pantolactone derived from ca. 95% stereospecifically labeled (2*S*,3*S*)-serine-3-d_1. Samples (80–100 mg/mL) were dissolved in C_6H_6 with internal C_6D_6, δ 7.15, as reference. Pantolactone-4-*d* signals appear at δ 3.20–3.24 and 3.32–3.34.

Before incubations with stereospecifically deuterated serines were carried out, (2*RS*)-serine-3,3-d_2 (plus DL-valine) was incubated with *E. coli*. The ^{2}H NMR of the resultant pantolactone showed two deuterium signals, δ 3.17 and 3.28 of equal intensity. This result establishes that no extraneous biochemical processes, unrelated to the formation of 5,10-CH_2-H_4folate via SHMT or subsequent transfer of the methylene group by KHMT to form ketopantoate, were taking place to any significant extent. The fact that deuterium signals only for deuterons at C-4 were observed also supports this conclusion.

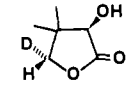
(2*S*,3*S*)-Serine-3-d_1 (**19**) was then synthesized by the method of Slieker and Benkovic.[30] The ^{2}H-decoupled 500-MHz ^{1}H NMR



19



20



21

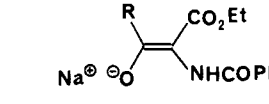
spectrum of this product indicated a diastereomeric purity of 84%, similar to that previously observed by others using this synthetic method.[30,31] This product, admixed with α-KIVA,[32] was then incubated with *E. coli*, and the resultant pantolactone isolated. The ^{2}H NMR spectrum (Figure 1b) showed two deuterium signals, δ 3.21 and 3.33 in a ratio of ca. 1:2, the downfield signal (4-H_{si}) being the more intense. Thus, two deuterium labeled pantolactones, **20** and **21**, were formed, with the major product (**20**) having the *same* absolute configuration, 4*S*, in the oxymethyl group as in the precursor serine. Therefore, the two hydroxymethyltransferase reactions, leading from serine to 5,10-CH_2-

(26) Dev, I. K.; Harvey, R. J. *J. Biol. Chem.* **1982**, *257*, 1980–1986.
(27) (a) Fritz, H.; Löwe, W. *Angew. Chem., Int. Ed. Engl.* **1962**, *1*, 592–593. (b) Lowry, J. B.; Riggs, N. V. *Tetrahedron Lett.* **1964**, 2911–2917. (The chemical shifts given in the text are from our work and were measured at 250 MHz.)
(28) Goodhue, C. T.; Snell, E. E. *Biochemistry* **1966**, *5*, 403–408.
(29) Aberhart, D. J.; Russell, D. J. *J. Am. Chem. Soc.*, following paper in this issue.
(30) Slieker, L.; Benkovic, S. J. *J. Labelled Comp. Radiopharm.* **1982**, *19*, 647–657.
(31) Townsend, C. A.; Brown, A. M.; Nguyen, L. T. *J. Am. Chem. Soc.* **1983**, *105*, 919–927.
(32) α-Ketoisovaleric acid (α-KIVA) was used in place of valine as coprecursor, since our studies described in the accompanying paper[29] indicated that, at least with glycine as precursor, higher incorporations into pantolactone could be obtained.

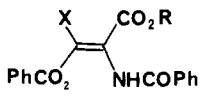
H_4folate (SHMT) and from 5,10-CH_2H_4folate to ketopantoate (KHMT) must have the same overall steric course.

Since a nonequal mixture of stereoisomeric deuterated pantolactones was formed, it is clear that at least one (or possibly both) of the transferase steps proceeded with only partial stereospecificity. If the *E. coli* SHMT proceeds to yield, from a stereospecifically labeled serine, the same 3:1 distribution of labeled 5,10-C*$H_1{}^1H_1$-H_4folates as found for the liver enzyme, it would be expected that the use of 84% stereospecifically labeled (3*S*)-serine-d_1 (**19**) would result in the formation of a ca. 2:1 mixture of **10** and **11** (D in place of T). Thus, it might be tentatively concluded that the subsequent KHMT-catalyzed reaction had proceeded nearly or completely stereospecifically.

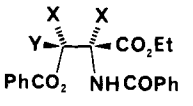
We wished to repeat this experiment using serine of higher labeling stereospecificity. We therefore considered alternative synthetic routes for its preparation. Besides the synthesis of Slieker and Benkovic,[30] several other syntheses of C-3 stereospecifically deuterated or tritiated serines have been published.[33] The method used by Kainosho and Ajisaka[33a] appeared to be quite convenient and was also highly stereospecific. Although few details were published about this synthesis, the authors generously provided us with further details.[34] Thus, crude **22a**[35] was converted into

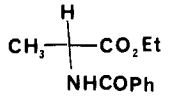


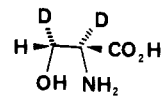
22 a R = H
b R = D



23 a R = Et X = H
b R = H X = H
c R = Et X = D







24 a X = Y = H
b X = D Y = H
c X = H Y = D

25

26

23a, which was easily purified by direct crystallization. The *Z* double bond stereochemistry of **23a** had been assigned[34] (in part) on the basis of the coupling between the C-3H and the carboxyl carbon of the corresponding acid (**23b**).

On attempted hydrogenation of **23a** over Pd(C) in ethyl acetate, a mixture of **24a** (38%) and **25** (62%) was obtained. The yield of **24a** was substantially improved (ca. 60%) by using Pd($CaCO_3$) or Lindlar catalyst, but we could not avoid partial hydrogenolysis under the conditions tried. Nevertheless, it was possible to isolate pure **24a** by direct crystallization of the mixture obtained by Pd($CaCO_3$) catalysis (or, with more difficulty, by preparative TLC). Thus, reduction of **23a** by using D_2 and Pd($CaCO_3$) gave, after crystallization, **24b** which was hydrolyzed to **26**. The ^1H NMR of **26** in strongly alkaline solution[36] indicated a relative stereochemical purity at C-3 of at least 95%. However, we did not use this serine-2,3-d_2 (**26**) for biosynthetic studies, because deuterated glycine formed in vivo by SHMT might, in turn, be used for ketopantoate biosynthesis via the glycine cleavage system,[29] in competition with the utilization of C-3 of serine. This synthetic route was then applied to the preparation of the desired (2*S*,3*S*)-serine-3-d_1 (**19**). Reaction of ethyl formate-*d* (or, in subsequent runs, methyl formate-*d*) with ethyl hippurate in EtOH/NaOEt gave the deuterated intermediate (**22b**) which was benzoylated to **23c**. Unexpectedly, however, **23c** showed a substantial vinyl H signal (ca. 0.25–0.5 H), and it was clear that some deuterium–hydrogen exchange had occurred, presumably in the condensation step.[37] This exchange was nearly completely suppressed by reacting deuterium-exchanged ethyl hippurate (N-deuterated) with methyl formate-*d* in EtOD/NaOEt. After benzoylation, the product (**23c**) showed ca. 0.05 H at δ 8.45. The synthesis was then completed by H_2 reduction of **23c** over Pd/$CaCO_3$ to give **24c** (and **25**), followed by acidic hydrolysis of **24c** to **19** (racemic). The desired (2*S*,3*S*)-serine-3-d_1 (**19**) was then obtained after resolution as before. The 500-MHz NMR of **19** showed a clean doublet for the C-2 proton at δ 3.33 (J = 4.2 Hz) and a doublet at δ 3.73 (J = 3.8 Hz) for the 3-H_{re} proton (0.91 H relative to the C-2 proton) accompanied by a minor multiplet at δ 3.67 (3-H_{si}, 0.09 H). At least half of the latter is due to the presence of unlabeled serine resulting from deuterium–hydrogen exchange of formate in the synthesis. Thus, the deuterated (2*S*,3*S*)-serine-3-d_1 was ca. 95% stereospecifically labeled. This product, admixed with α-KIVA, was incubated with *E. coli*, and after workup, the resultant pantolactone was examined by ^2H NMR. The spectrum, Figure 1c, showed a ca. 3:1 peak intensity ratio (downfield signal due to **20** more intense), consistent with expectation based on the prior experiment with 84% stereospecifically labeled serine.

Thus, in summary, our results show that the methylene group transfer reactions proceeding between 5,10-CH_2-H_4folate and the respective substrates of SHMT and KHMT in *E. coli* have the same overall steric courses. Although it is likely that the absolute steric course of SHMT in *E. coli* is the same as that previously established for the liver enzyme,[10-13] this point remains to be established and is under investigation in our laboratory. It is probable that KHMT has a higher (possibly absolute) stereospecificity than SHMT, but further studies with isolated enzymes will be necessary to firmly establish this point.

Experimental Section

General. ^1H NMR spectra were run on a Varian EM-360 instrument and on a Bruker WM-500 instrument. ^2H NMR spectra were run on a Bruker WM-500 instrument using 5-mm-diameter sample tubes.[38] ^{13}C NMR spectra were run on a Bruker SXP 22/100 and on a Bruker HX 270 instrument. Liquid scintillation counting was performed on a Nuclear Chicago Mark III instrument. Samples were dissolved in 10 mL of New England Nuclear Aquasol (if necessary with a little added H_2O). TLC was performed by using Analtech silica gel GF Uniplates or homemade plates prepared with E. Merck silica gel $HF_{254+366}$. Melting points were determined by using a hot stage apparatus and are uncorrected. Specific rotations were determined by using a Rudolph polarimeter. Hydrogenations were performed by using a Parr 450-mL pressure reactor. *E. coli* ATCC 23783 was obtained form the American Type Culture Collection, Rockville, Md 20852. DL-Serine-3,3-d_2 (98 atom % D) and ethyl formate-*d* were obtained from MSD Isotopes. [3-^{14}C]-DL-Serine was obtained from ICN. Sodium [^{14}C]formate was obtained from New England Nuclear. [4,4'-^{14}C]-DL-Valine was obtained from Schwarz-Mann. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN.

Conversion of Labeled Substrates into Pantolactone. The *E. coli* culture was maintained on Petrie plates prepared with *medium 1* (g/L): K_2HPO_4 (7), KH_2PO_4 (3), sodium citrate (1.5), $(NH_4)_2SO_4$ (1.0), $MgSO_4 \cdot 7H_2O$ (0.1), $CaCl_2 \cdot 2H_2O$ (0.01), $FeSO_4 \cdot 7H_2O$ (0.0005), tap H_2O (50 mL), glucose[39] (2), DL-valine[39] (0.10), L-isoleucine[39] (0.10), thiamine·HCl[39] (0.0001), and agar (20). Plates were incubated at 37 °C for 1–2 days and then kept at 4 °C. For growth of cells, the same medium, minus agar, was used. For biosynthetic runs, 0.10 mL of a recently grown culture (within a few days) was transferred to each of two 100-mL media (or 1 mL added to each of two 1000-mL media prewarmed to 37 °C), and the mixture was incubated on a gyrotory shaker at 37 °C for 24 h. The cells were rapidly collected by centrifugation and resuspended in 100 mL (or 1000 mL for larger scale experiments) of

(33) (a) Kainosho, M.; Ajisaka, K. *J. Am. Chem. Soc.* **1975**, *97*, 5630–5631. (b) Floss, H. J.; Scheicher, E.; Potts, R. *J. Biol. Chem.* **1976**, *251*, 5478–5482. (c) Cheung, Y.-F.; Walsh, C. *J. Am. Chem. Soc.* **1976**, *98*, 3397–3398. (d) Fuganti, C.; Ghiringhelli, D.; Grasselli, P. *Experientia* **1978**, *34*, 297–298. (e) Gani, D.; Young, D. W. *J. Chem. Soc., Perkin Trans. 1* **1983**, 2393–2398.

(34) We thank Prof. Kainosho for providing details about their synthetic route and for permission to publish information about their synthetic methods used to prepare stereospecically deuterated serine.

(35) Stoop, F.; Erlenmeyer, E. *Justus Liebigs Ann. Chem.* **1904**, *337*, 236–263.

(36) Ogura, H.; Arata, Y.; Fujiwara, S. *J. Mol. Spectrosc.* **1967**, *23*, 76–85.

(37) Elwyn, D.; Weissbach, A.; Henry, S. S.; Sprinson, D. B. *J. Biol. Chem.* **1955**, *213*, 281–285.

(38) ^1H-Decoupled ^2H NMR spectra, Figure 1, were run on a Bruker WM-500 instrument using 5-mm tubes, at 76.77 MHz, with 0–0.5-Hz line broadening, SW 2000 Hz, 0.488 Hz/pt, PW 30–55 μs, RD zero. Ca. 1000–2000 scans were taken on the biosynthetic samples (Figure 1b,c) with a repetition rate of 2.048 s.

(39) Sterilized separately.

prewarmed *medium 2*: same as medium 1 but with omission of $(NH_4)_2SO_4$ and inclusion of β-alanine (0.79 g/L). Unlabeled DL-valine was omitted in incorporation experiments with labeled valine, which was added at 20 mg/100 mL. For incorporations of sodium formate or serine, these substrates were added at 20–25 mg/100 mL or 200 mg/L, accompanied either by DL-valine (25 mg/100 mL or 250 mg/L) or, in the (2*S*,3*S*)-serine-3-d_1 runs, by sodium α-ketoisovalerate (α-KIVA, 200 mg/L). Incubations were continued for 24 h at 250 rpm at 37 °C. Calcium pantothenate (20 mg/100 mL or 200 mg/L) was then added and the mixture was then acidified to pH 1.5 with H_2SO_4 and further treated with 25% H_2SO_4 (20 mL/L) and autoclaved (121 °C, 15 min), then rapidly cooled and neutralized with concentrated NaOH. The 100-mL cultures were saturated with NaCl, whereas larger cultures were first freeze-dried, and the residues were resuspended in a smaller volume of H_2O (150–200 mL) and saturated with NaCl. After filtration and continuous ether extraction for 24–48 h, the extract was evaporated, and the pantolactone was isolated by preparative TLC (silica gel $HF_{254+366}$; solvent, 30% EtOAc/hexane, running several times). The isolated pantolactone was then rechromatographed once as before, giving ca. 50–70 mg chromatographically pure product/L culture.

(2*RS*,4*RS*)-Pantolactone-4-d_1 (18). Zinc aldopantoate[28] (2.5 g) was suspended in 95% EtOH (100 mL) and treated with $NaBD_4$ (1 g) at 25 °C for 80 min. The mixture was acidified with 2 N H_2SO_4 and filtered. The solvent was evaporated under reduced pressure, and the residue was extracted with $CHCl_3$. The extract was dried (Na_2SO_4) and evaporated under reduced pressure to yield **18** as an oil which rapidly crystallized; ^1H NMR (C_6D_6; concentration of **18** ca. 100 mg/mL) δ 0.66 (3 H, s), 0.78 (3 H, s), 3.23 (1 H, sl br s), 3.36 (1 H, sl br s), 3.85 (1 H, s), 4.40 (1 H, br s, $W_{1/2}$ = 12 Hz); ^2H NMR (C_6H_6) (Figure 1a) δ 3.20 and 3.34 (equal intensities).

(2*S*,3*R*)- and (2*R*,3*S*)-Serine-2,3-d_2[33a,40] (26). 2-Formylhippuric acid sodium salt (**22a**) was synthesized as previously described[35] but was not recrystallized. The crude salt (16 g) after vacuum drying to constant weight was dissolved in H_2O (200 mL) and treated with a solution of benzoyl chloride (13 g) in $CHCl_3$ (130 mL), with vigorous stirring for 42 h. The mixture was filtered, and the $CHCl_3$ phase was washed with 5% $NaHCO_3$ (2 × 100 mL) and saturated NaCl, dried ($NaSO_4$), and evaporated under reduced pressure to yield a solid. After recrystallization from $CHCl_3$ and absolute EtOH, ethyl **(*Z*)-3-benzoyloxy-2-benzamidoacrylate (23a)**, 12.4 g, was obtained as fine needles, mp 157–159 °C; NMR ($CDCl_3$) δ 1.37 (3 H, t, *J* = 7 Hz), 4.26 (2 H, q, *J* = 7 Hz), 7.2–8.2 (10 H, m), 8.45 (1 H, s).

Anal. Calcd for $C_{19}H_{17}NO_5$: C, 67.25; H, 5.05. Found: C, 67.15; H, 5.19.

The above product (**23a**) (2.0 g) was suspended in EtOAc (100 mL) and was stirred with 5% $Pd(CaCO_3)$ (100 mg) in a Parr pressure reactor at 25 °C with H_2 at 200 psi for 23 h. The mixture was filtered and the filtrate was evaporated under reduced pressure to yield a mixture of **24a** (ca. 60%) and **25** (40%). After recrystallization from MeOH, pure ***N*,*O*-dibenzoylserine ethyl ester (24a)**, 732 mg, was obtained as prisms, mp 91–93 °C; NMR ($CDCl_3$) δ 1.28 (3 H, t, *J* = 7 Hz), 4.23 (2 H, q, *J* = 7 Hz), 4.72 (2 H, d, *J* = 3 Hz), 5.10 (1 H, m), 6.9–8.2 (11 H, m).

Anal. Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61. Found: C, 66.91; H, 5.70.

(2*S*,3*R*)- and (2*R*,3*S*)-*N*,*O*-dibenzoylserine-2,3-d_2 ethyl ester (24b) was similarly prepared by using D_2 in place of H_2: NMR ($CDCl_3$) δ 1.28 (3 H, t, *J* = 7 Hz), 4.23 (2 H, q, *J* = 7 Hz), 4.70 (1 H, br s, $W_{1/2}$ = 3 Hz), 6.7–8.1 (11 H, m). The product (**24b**) (720 mg) was suspended in 1 N HCl (50 mL) and refluxed for 4 h. After cooling, the mixture was extracted twice with $CHCl_3$, and the aqueous extract was evaporated to dryness in vacuo. The serine was then isolated by absorption on a column of Dowex 50W-X8, 50-100 mesh, H^+ form, followed by elution with 2 N NH_4OH to give **26**, 149 mg.

(2*S*,3*S*)-Serine-3-d_1 (19). Potassium formate-*d* was prepared by a modification of the method of Sprinson et al.:[37] potassium cyanide (63 g, dried in vacuo at 100 °C) in D_2O (100 g) containing NaOD (3 g) was heated with stirring at 175 °C in a 450-mL Parr pressure reactor for 6 h. The pressure reached 445 psi during this period. The reactor was allowed to cool to room temperature while stirring was continued for 18 h. The solution was evaporated in vacuo and the residue was redissolved in H_2O (150 mL), acidified with 2 N H_2SO_4, filtered, and extracted continuously with ether overnight. The extract was concentrated to ca. 30-mL volume and mixed with H_2O, and the pH was adjusted to 7.0 with KOH/H_2O. The solution was then evaporated and the residue vacuum dried to give **potassium formate-*d***, 48 g. A portion of the product (30 g) was heated and stirred with dimethyl sulfate (70 mL) at 100–150 °C while distilling the resultant **methyl formate-*d*** through a 10-cm Vigreux column. The crude product, bp 30–60 °C, 21 g, was then redistilled, bp 30–32 °C, 20 g. NMR (neat) δ 3.70 (3 H, s), 8.08 (0.005 H, sl br s).

Ethyl hippurate (30 g) was dissolved in $CHCl_3$ (100 mL), and the solution was stirred with D_2O (100 mL). The $CHCl_3$ phase was separated and dried (Na_2SO_4), and the solvent was evaporated under reduced pressure. The product showed in the ^1H NMR ($CDCl_3$) a singlet, δ 4.13, for the CH_2 group in place of the doublet, *J* = 5 Hz, for this group in unlabeled ethyl hippurate. Methyl formate-*d* (20 g) was added to a solution of NaOEt/EtOD prepared by reaction of sodium spheres (6.2 g) with EtOD (120 mL). After 1 h at 25 °C, ethyl hippurate-N-*d* (53.9 g) was added, and the mixture was stirred at room temperature. After a few hours, the mixture set to a thick paste. After 42 h, the mixture was slurried with EtOH (50 mL), vacuum filtered, and washed with a little EtOH. After drying in vacuo to constant weight, 46.7 g of crude **22b** was obtained. This was benzoylated as described above to yield, after recrystallization from $CHCl_3$ and EtOH, **23c**, 26.3 g; NMR ($CDCl_3$) as for **23a** except δ 8.45 (0.05 H, s). The product was hydrogenated as described for **23a** → **24a** above, to yield, after recrystallization from MeOH, (2*S*,3*S*)- and (2*R*,3*R*)-*N*,*O*-dibenzoylserine-3-d_1 ethyl ester (**24c**), 10.7 g; NMR ($CDCl_3$) as for **24a** except δ 4.72 (1 H, br d, *J* = 4 Hz), 5.10 (1 H, dd, $J_1 = J_2$ = 3 Hz). Product (**24c**), 5.35 g, was then converted, as described for **24b**, to (2*S*,3*S*)- and (2*R*,3*R*)-serine-3-d_1 (**19**) (plus enantiomer), 1.29 g. The racemic product, 1.18 g, was then resolved by a literature procedure[41] to yield (2*S*,3*S*)-serine-3-d_1 (**19**), 0.44 g, $[\alpha]^{25}_D$ + 15° (lit. $[\alpha]^{25}_D$ + 15.1°) (*c* 2, 1 N HCl); NMR, see text.

Acknowledgment. We thank Peter Demou, Yale University, for the ^2H NMR spectra and the 500-MHz ^1H spectra, taken on the Bruker WM-500 instrument at the NSF Northeast Regional NMR Facility, funded by NSF Grant No. CHE-7916210. We also thank Frank Shea, Clark University, for some ^{13}C NMR spectra, run at the Worcester Consortium NMR Facility, supported in part by NSF Grant No. DMR-8108679. We thank Teresa Olefirowicz for some preliminary experiments. This work was supported by Grant No. GM 24420 from the National Institute of General Medical Sciences.

Registry No. 1, 759-05-7; **2**, 470-30-4; **12**, 42467-68-5; **13**, 78709-91-8; **14**, 90839-49-9; **15**, 90839-50-2; **16**, 90839-51-3; **17**, 90857-68-4; **18**, 90839-52-4; **19**, 83461-39-6; (±)-**19**, 83212-43-5; **20**, 90940-33-3; **21**, 90940-34-4; **22a**, 90839-53-5; **22b**, 90839-54-6; **23a**, 90839-55-7; **23b**, 90839-56-8; **23c**, 90839-57-9; **24a**, 90839-58-0; **24b**, 90839-59-1; **24c**, 90839-60-4; **25**, 5446-46-8; **26**, 80612-42-6; KHMT, 56093-17-5; SHMT, 9029-83-8.

(40) This procedure is based on information provided to us by Prof. M. Kainosho.[33a]

(41) Greenstein, J.; Winitz, M. "Chemistry of the Amino Acids"; Wiley: New York, 1961; p 2229.