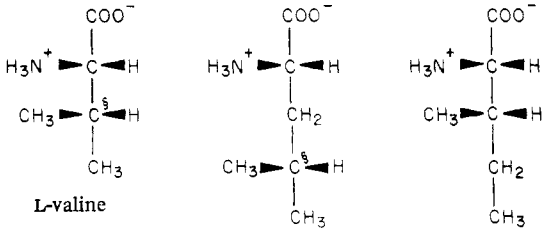
Stereospecificity of the Reductoisomerase-Catalyzed Step in the Pathway of Biosynthesis of Valine and Leucine†

Steven R. Sylvester and Carl M. Stevens*

ABSTRACT: Experiments are described which complete the determination of the stereochemistry of the intermediate steps in the biosynthesis of valine from pyruvate. Further, the preservation of stereospecificity on the pathway of biosynthesis of leucine from α-ketoisovalerate has been examined. (*RS*)-[2-$^{13}CH_3$]Acetolactate (2-hydroxy-2-[^{13}C]methyl-3-oxobutanoic acid) was added to cultures of *Escherichia coli* strain CU888 (blocked in the synthesis of acetolactate from pyruvate) on minimal medium supplemented with L-isoleucine. L-Valine isolated from the harvested cells was examined by carbon-13 nuclear magnetic resonance at 22.63 MHz and gave a single peak with a chemical shift of 17.8 ppm relative to tetramethylsilane. L-Leucine isolated from the same cells gave a single strong peak with a chemical shift of 21.4 ppm. These results establish the configuration of the isolated valine to be (2*S*,3*S*)-[4-^{13}C]valine, identical with the synthetic product [Kluender, H., Bradley, C. H., Sih, C. J., Fawcett, P., & Abraham, E. P. (1973) *J. Am. Chem. Soc. 95*, 6149–6150]. The stereochemistry of the reductoisomerase-catalyzed step is shown to be the conversion of (2*S*)-[2-$^{13}CH_3$]acetolactate to (2*R*,3*R*)-2,3-dihydroxy-3-[^{13}C]methylbutanoic acid [cf. Armstrong, F. B., Hedgecock, C. J. R., Reary, J. B., Whitehouse, D., & Crout, D. H. G. (1974) *J. Chem. Soc., Chem. Commun.*, 351–352, and Hill, R. K., & Yan, S-J. (1971) *Bioorg. Chem. 1*, 446–456]. The bioconversion of L-valine to L-leucine is proposed to occur without change in configuration of the terminal chiral center, yielding (2*S*,4*S*)-[5-^{13}C]leucine. The results suggest methods for the preparation of L-valine and L-leucine isotopically labeled in specific positions.

The pathways of biosynthesis of valine, leucine, and isoleucine have been the subject of intensive study [cf. Meister (1965) and Umbarger & Davis (1962)]. These branched-chain amino acids have close structural resemblances.[1]



L-valine L-leucine L-isoleucine

Even more striking is the fact that the three enzymes which catalyze the three key steps in assembly of the carbon skeleton of valine also catalyze the synthesis of the carbon structure of isoleucine. Of further note is the chain-lengthening mechanism by which the valine skeleton is converted to that of leucine, a sequence of reactions which is quite analogous to the conversion of oxaloacetate to α-ketoglutarate in the citric acid cycle.

Our particular interest has been in the identification and synthesis of intermediates in the biosynthetic paths and in the determination of the stereochemistry of the reactions [cf. Calvo et al. (1964) and Cole et al. (1973) and references cited therein].

Previous studies [see Hill & Yan (1971), Crout & Whitehouse (1972, 1977), and Armstrong et al. (1974) and references cited therein] have established the absolute configurations of compounds **3** and **4** in the isoleucine sequence (cf. Scheme I). In contrast, the corresponding intermediates in valine biosynthesis have a prochiral β center with two diastereotopic methyl groups. This is likewise a structural feature of all leucine intermediates which have prochiral γ centers. It is to the source and chirality of these centers that we direct our attention.

The ^{13}C NMR spectra of L-valine and L-leucine have been studied (Horsley et al., 1970; Tran-Dinh et al., 1974), with the conclusion that in each case the two methyl groups are nonequivalent and give rise to separate signals. In the case of valine, the conclusion has been confirmed by stereospecific organic syntheses. Kluender et al. (1973) synthesized (2*S*,3*S*)-[4-^{13}C]valine (signal at δ = 17.8 ppm). Synthetic (2*S*,3*R*)-[4-^{13}C]valine (Baldwin et al., 1973) exhibits one signal at δ = 18.7 ppm.

By specific labeling of one of the methyl groups in acetolactate (cf. Scheme I; **2**, R = CH_3) with ^{13}C, addition of the labeled compound to an appropriate mutant strain of *E. coli*, and isolation of valine from the cells, it should be possible to determine the absolute configuration of the chiral β center in compounds **3**–**5**. Furthermore, by isolation of leucine, the conservation of stereospecificity at the γ chiral center could be established and its configuration inferred.

Materials and Methods

Microorganisms. *E. coli* strain CU357 (Kline et al., 1974), auxotrophic for valine and leucine, and *E. coli* strain CU888, which shows no acetohydroxy acid synthetase activity but utilizes added acetolactate for the synthesis of valine and leucine, were kindly provided by Professor H. E. Umbarger, Purdue University, West Lafayette, IN.

Chemicals. A generous sample of (2*S*,3*S*)-[4-^{13}C]valine (~90 atom % ^{13}C) was provided by Professor C. J. Sih, University of Wisconsin, Madison, WI.

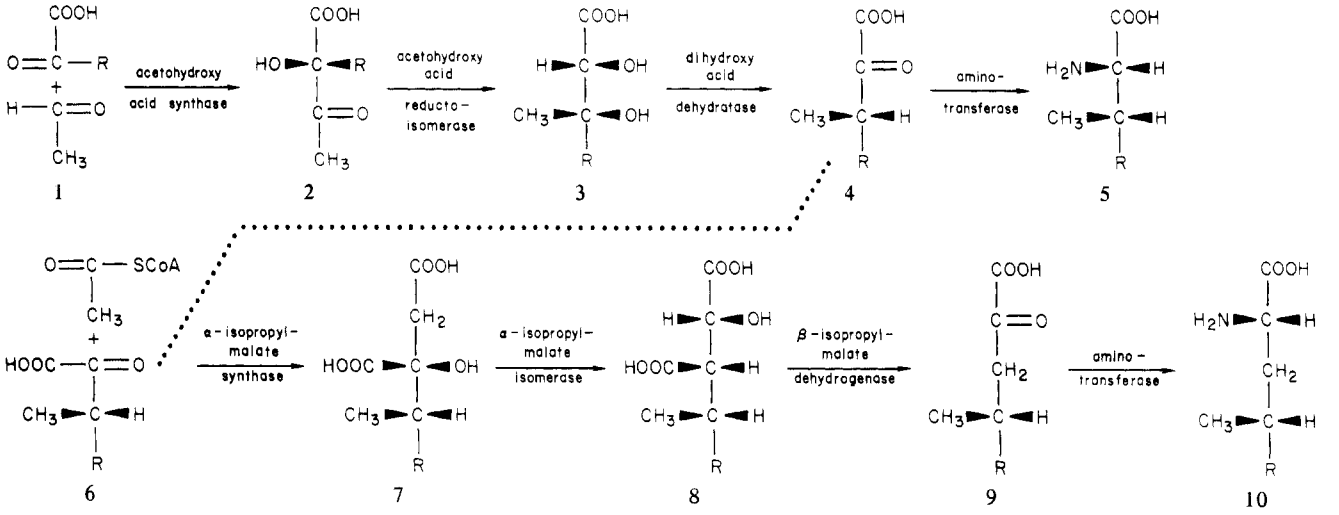
(*RS*)-[2-$^{13}CH_3$]Acetolactate was prepared by the method of Krampitz (1948) utilizing ^{13}C-labeled methyl iodide (70

† From the Program in Biochemistry and Biophysics, Department of Chemistry, Washington State University, Pullman, Washington 99164. *Received March 22, 1978; revised manuscript received May 20, 1979.* This investigation was supported in part by funds provided by the Washington State University Research and Arts Committee and in part by National Institutes of Health Grant GM 21996.

[1] The symbol § in the structures indicates prochiral centers with two diastereotopic methyl groups.

0006-2960/79/0418-4529$01.00/0

Scheme I: Diagram of Biosynthetic Pathways[a]



[a] For biosynthesis of valine, read upper row as R = CH_3; for biosynthesis of isoleucine, read upper row as R = CH_3CH_2; for biosynthesis of leucine, read both rows as R = CH_3.

atom % ^{13}C). In this procedure the final product is generated by saponification with 2 equiv of KOH in dilute aqueous solution at room temperature just prior to use. Silver picolinate was prepared by the method of Clarke et al. (1970) and silver(II) oxide according to the procedure of Bailar (1953).

Bioconversion of (2S,3S)-[4-^{13}C]Valine to [5-^{13}C]Leucine. *E. coli* strain CU357 was grown on minimal medium [Davis & Mingioli (1950), as modified by Kline et al. (1974)] and supplemented with L-isoleucine and (2S,3S)-[4-^{13}C]valine. The cultures were incubated at 37 °C in Erlenmeyer flasks on a rotary shaker. When growth approached maximum, cells were harvested, washed twice with minimal medium, and dried at 60 °C. In typical experiments, 100 mL of medium containing 10 mg of valine yielded 55–65 mg of dried cells.

The dried cells were hydrolyzed by refluxing in 6 M HCl for 20 h in an atmosphere of nitrogen. The acid was removed by evaporation, the residue oxidized with performic acid, excess performic acid destroyed by HBr, and the solution again evaporated to dryness. The resulting solids were extracted with 2 mL of 4 M HCl, and the extract was placed on a column of Dowex 50W-X8 (150 × 1 cm) in the hydrogen form (Hirs et al., 1954). It was found that satisfactory separation of leucine from isoleucine could be achieved by development of the column with 4 M HCl. The appropriate fractions were pooled and evaporated to dryness, and the samples were further purified by passage through a Dowex 1-X8 column (4 × 1 cm) in the acetate form and elution with dilute acetic acid (Hirs et al., 1954). Amino acids isolated by this method were identified and quantified by using an amino acid analyzer. Contamination of leucine and valine with other amino acids was usually negligible (1–4%) and caused no interference with the study. The yields of isolated L-valine and L-leucine were 2–4 mg/60 mg of dried *E. coli* cells.

Bioconversion of [2-$^{13}CH_3$]Acetolactate to (2S,3S)-[4-^{13}C]Valine and [5-^{13}C]Leucine by E. coli Strain CU888. The culture of *E. coli* strain CU888 was carried out essentially as in the previous experiment, the minimal medium being supplemented with L-isoleucine and (*RS*)-[2-$^{13}CH_3$]acetolactate. At the end of log phase the cells were harvested and the desired amino acids isolated by the procedure already described, except that the hydrolysate was placed on a column of Dowex 50W-X8 (45 × 1 cm) and developed with 0.5 M HCl to obtain a better separation of valine from proline.

Oxidation of Amino Acids. In anticipation of future studies of the stereochemistry of intermediates in the catabolism of leucine [cf. Aberhart & Tann (1979)], the ^{13}C-labeled amino acids were subjected to oxidation by silver picolinate followed by silver(II) oxide, according to the procedure of Clarke et al. (1970). Yields of isobutyric acid (from valine) and of isovaleric acid (from leucine) varied from 10 to 20% for isobutyric acid and from 25 to 50% for isovaleric acid, recovered as the potassium salts.

^{13}C NMR Spectra. ^{13}C NMR spectra [cf. Stothers (1972) and McInnes et al. (1976)] were obtained for the starting materials and final products. The spectra were recorded at 22.63 MHz in the Fourier transform mode of operation with proton broad-band decoupling on a Bruker HX-90E using 10-mm sample tubes. Ten percent D_2O served as the lock signal, and chemical shifts were determined relative to tetramethylsilane, using *p*-dioxane as the internal standard.

Results and Discussion

In Vivo Conversion of Valine to Leucine by E. coli Strain CU357. In the experiments which involved addition of (2S,3S)-[4-^{13}C]valine to cultures of *E. coli* strain CU357, valine isolated from the cells gave a single strong peak in the ^{13}C NMR spectrum (δ = 17.8 ppm), identical with that of the starting material and showing that no inversion or racemization had occurred. Leucine isolated from the cells showed a single strong peak (δ = 21.4 ppm). This demonstrates that stereospecificity at the chiral γ center had been preserved in the conversion of valine into leucine, but is equivocal as to the assignment of the configuration as *R* or *S*.

Conversion of Acetolactate to Valine and Leucine by E. coli Strain CU888. Addition of (*RS*)-[2-$^{13}CH_3$]acetolactate to cultures of *E. coli* strain CU888 in minimal medium supplemented only with L-isoleucine produced good growth. Valine isolated from the harvested cells exhibited one strong peak (δ = 17.8 ppm) identical with that of authentic (2S,3S)-[4-^{13}C]valine.[2] Leucine isolated from the cells also showed a single strong peak (δ = 21.4 ppm), confirming the results of the previous experiment.

Since the enzymes catalyzing the synthesis of compounds **2**–**5** (Scheme I) are apparently identical for both the valine and isoleucine intermediates, it could be predicted that the ^{13}C-labeled *methyl* group would appear in the position cor-

[2] Another very small peak was detected (δ = 18.7 ppm). This peak could arise from slight racemization (isomerization) of the ^{13}C-labeled acetolactate [cf. Armstrong et al. (1974)].

responding to the *ethyl* group of the isoleucine precursors. The results [cf. Crout & Whitehouse (1972, 1977)] confirm this prediction and support the conclusion that each of the compounds has the absolute configuration shown in Scheme I.

With respect to the stereochemistry of leucine biosynthesis, the data from both experiments demonstrate the retention of stereospecificity at the chiral center bearing the two methyl groups. Examination of the structural changes occurring in the stepwise conversion of α-ketoisovalerate to L-leucine (Scheme I) suggests no probable basis for change in the configuration of the chiral γ center. Thus, one would predict retention of configuration throughout the bioconversion of (2*S*,3*S*)-[4-^{13}C]valine to (2*S*,4*S*)-[5-^{13}C]leucine.

Degradation of ^{13}C-Labeled Valine and Leucine. The ^{13}C NMR spectrum of potassium isobutyrate derived from (3*S*)-[4-^{13}C]valine showed one strong peak (δ = 20.3 ppm). The spectrum of potassium isovalerate derived from the presumed (4*S*)-[5-^{13}C]leucine showed one strong peak (δ = 22.8 ppm). It seems probable that the configuration has been retained in each case and that these specifically labeled metabolites could be utilized in determining the stereochemistry of subsequent steps in the catabolism of the branched-chain amino acids.

Finally, it might be recalled that the in vivo experiments provide a method for synthesis of specifically labeled valine and leucine from suitably labeled acetolactate. Likewise, labeled acetohydroxybutyrate could produce specifically labeled isoleucine.

Acknowledgments

The authors express their thanks to Professor H. E. Umbarger for the gift of appropriate mutants, to Professor C. J. Sih and Professor J. A. Magnuson for the ^{13}C NMR spectra, and to Mayme Trumble and Suey Y. Lan for yeoman assistance. Amino acid analyses were performed by Dr. S. M. Gurusiddaiah.

References

Aberhart, D. J., & Tann, C-H. (1979) *J. Chem. Soc., Perkin Trans. 1*, 939–941.

Armstrong, F. B., Hedgecock, C. J. R., Reary, J. B., Whitehouse, D., & Crout, D. H. G. (1974) *J. Chem. Soc., Chem. Commun.*, 351–352.

Bailar, J. C., Jr. (1953) *Inorg. Syn. 4*, 12–14.

Baldwin, J. E., Löliger, J., Rastetter, W., Neuss, N., Huckstep, L. L., & De La Higuera, N. (1973) *J. Am. Chem. Soc. 95*, 3796–3797.

Calvo, J. M., Kalyanpur, M. G., Stevens, C. M., & Umbarger, H. E. (1964) *Biochemistry 3*, 2024–2027.

Clarke, T. G., Hampson, N. A., Lee, J. B., Morley, J. R., & Scanlon, B. (1970) *J. Chem. Soc. C*, 815–817.

Cole, F. E., Kalyanpur, M. G., & Stevens, C. M. (1973) *Biochemistry 12*, 3346–3350.

Crout, D. H. G., & Whitehouse, D. (1972) *J. Chem. Soc., Chem. Commun.*, 398–399.

Crout, D. H. G., & Whitehouse, D. (1977) *J. Chem. Soc., Perkin Trans. 1*, 544–549.

Davis, B. D., & Mingioli, E. S. (1950) *J. Bacteriol. 60*, 17–28.

Hill, R. K., & Yan, S-J. (1971) *Bioorg. Chem. 1*, 446–456.

Hirs, C. W. H., Moore, S., & Stein, W. H. (1954) *J. Am. Chem. Soc. 76*, 6063–6065.

Horsley, W., Sternlicht, H., & Cohen, J. S. (1970) *J. Am. Chem. Soc. 92*, 680–686.

Kline, E. L., Brown, C. S., Coleman, W. G., & Umbarger, H. E. (1974) *Biochem. Biophys. Res. Commun. 57*, 1144–1151.

Kluender, H., Bradley, C. H., Sih, C. J., Fawcett, P., & Abraham, E. P. (1973) *J. Am. Chem. Soc. 95*, 6149–6150.

Krampitz, L. O. (1948) *Arch. Biochem. Biophys. 17*, 81–85.

McInnes, A. G., Walter, J. A., Wright, J. L. C., & Vining, L. C. (1976) *Top. Carbon-13 NMR Spectrosc. 2*, Chapter 3.

Meister, A. (1965) *Biochemistry of the Amino Acids*, 2nd ed., Vol. 2, pp 729–742, Academic Press, New York.

Stothers, J. B. (1972) *Carbon-13 NMR Spectroscopy*, Academic Press, New York.

Tran-Dinh, S., Fermandjian, S., Sala, E., Mermet-Bouvier, R., Cohen, M., & Fromageot, P. (1974) *J. Am. Chem. Soc. 96*, 1484–1493.

Umbarger, H. E., & Davis, B. D. (1962) *Bacteria, 1962 3*, 208–216.