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Substrate Stereochemistry of Isovaleryl-CoA Dehydrogenase Elimination of the 2-Pro-*R* Hydrogen in Biotin-Deficient Rats

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(2*R*)-[^{3}H]Isovaleric acid and (2*S*)-[^{3}H]isovaleric acid (ammonium salts) have been synthesized. These substances, mixed with [1-^{14}C]isovalerate, have been administered to biotin-deficient rats, which accumulate β-hydroxyisovaleric acid in their urine, the metabolite being formed via isovaleryl-CoA and β-methylcrotonyl-CoA. The results show that most of the tritium from (2*R*)-[^{3}H]isovalerate was lost, and most of the tritium from (2*S*)-[^{3}H]isovalerate retained in the conversion to β-hydroxyisovalerate. The stereochemistry of the isovaleryl-CoA dehydrogenase reaction is compared with the stereochemistry of other short-chain acyl-CoA dehydrogenase reactions.

INTRODUCTION

An early stage in the metabolism of the branched-chain amino acids, L-valine, L-isoleucine, and L-leucine, involves the dehydrogenation by a flavin-dependent enzyme (or enzymes) of the respective branched-chain acyl-CoA to the corresponding α,β-unsaturated analog (*1, 2*). Butyryl-CoA dehydrogenase (short-chain acyl-CoA dehydrogenase, EC 1.3.99.2) was thought to be responsible for the dehydrogenation of all acyl-CoA analogs derived from the branched-chain amino acids (*3*). However, more recently, Tanaka *et al.* (*4–6*) and Osmundsen *et al.* (*7*) have provided strong evidence for the existence in mammals of a specific dehydrogenase for isovaleryl-CoA.

In the continuation of our studies on the stereochemistry of reactions involved in branched-chain amino acid metabolism (*8–11*), we undertook an investigation of the stereochemistry of dehydrogenation of isovaleryl-CoA. We now report the determination of the stereochemistry of hydrogen elimination from C-2 in the conversion of isovaleryl-CoA to β-methylcrotonyl-CoA. (In this paper, this conversion will be referred to as the isovaleryl-CoA dehydrogenase reaction, although we do not have direct evidence that this enzyme exclusively dehydrogenates isovaleryl-CoA in biotin-deficient rats.)

RESULTS AND DISCUSSION

For the determination of the stereochemistry of the isovaleryl-CoA dehydrogenase reaction, we wished to avoid isolation of this enzyme and to work instead

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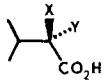
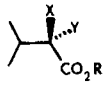
with an organism in which the dehydrogenation product (or a close analog of it) accumulates. In this regard, we were attracted to the report by Tanaka and Isselbacher (*15*) that biotin-deficient rats treated with large doses of leucine accumulated substantial amounts of β-hydroxyisovalerate in their urine. This metabolite undoubtedly arises from the action of enoyl-CoA hydratase on the intermediate β-methylcrotonyl-CoA, which in biotin-normal animals would be carboxylated by β-methylcrotonyl-CoA carboxylase (EC 6.4.1.4) (*16*). The hydration and subsequent deacylation steps would not be expected to involve hydrogen exchange at C-2. Thus, one could determine the stereochemistry of hydrogen elimination at C-2 of isovaleryl-CoA by administering stereospecifically C-2-tritiated isovalerate to biotin-deficient rats and assaying for the extent of tritium loss or retention in the β-hydroxyisovalerate.

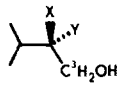
Thus, for this experiment, we required (2*R*)- and (2*S*)-[2-$^3H_1$]isovaleric acids (**1a**) and (**1b**). These were synthesized by a route closely analogous to the reported syntheses of (2*R*)- and (2*S*)-[2-$^3H_1$]palmitic acids (*17*). (2*S*)- and (2*R*)-2-Hydroxyisovaleric acid (**2a**) and (**2b**), obtained from L- and D-valine by a published route (*13*), were converted separately into the corresponding methyl ester tosylates (**2c**) and (**2d**). Treatment of each derivative with [^{3}H]$LiBH_4$ gave the corresponding isoamyl alcohol (**3a**) or (**3b**), with inversion of configuration at C-2 (*18*). Oxidation of the product with permanganate (*19*) gave the required isovaleric acid (**1a**) or (**1b**), isolated as the ammonium salt.



1a X=^{3}H, Y=H
1b X=H, Y=^{3}H



2a X=H, Y=OH, R=H
2b X=OH, Y=H, R=H
2c X=H, Y=OTs, R=Me
2d X=OTs, Y=H, R=Me



3a X=^{3}H, Y=H
3b X=H, Y=^{3}H

The individual ammonium [^{3}H]isovalerates were mixed with [1-^{14}C]isovalerate to give $^3H/^{14}C$ ratios of ca. 7–9. The exact ratios were determined on the recrystallized isovaleric acid *p*-phenylphenacyl ester. The [^{3}H]isovalerates (**1a**) and (**1b**) plus [^{14}C]isovalerate mixtures were then mixed with unlabeled sodium isovalerate and administered by intraperitoneal injection to biotin-deficient rats.

TABLE 1

$^3H/^{14}C$ RATIOS OF ISOVALERATE ADMINSTERED AND β-HYDROXYISOVALERATE RECOVERED[a]

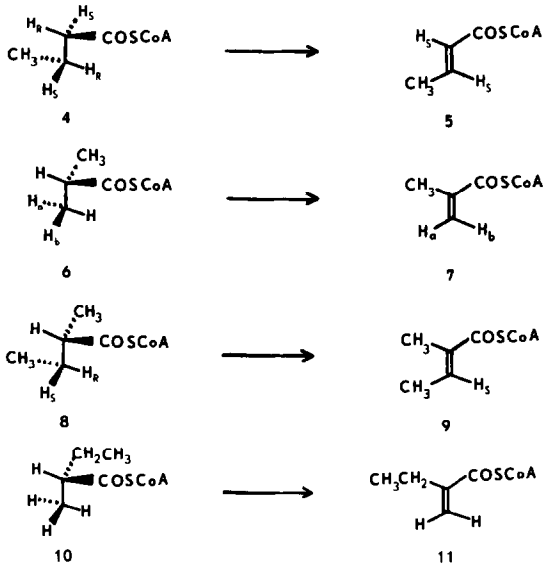
Isovalerate administered	β-Hydroxyisovalerate recovered	Percentage [^{3}H] retention
(**1a**), (2*R*-^{3}H)/(1-^{14}C) 8.50	1.43	17
(**1b**), (2*S*-^{3}H)/(1-^{14}C) 7.74	6.61	89

[a] All samples were counted as the four-times-recrystallized *p*-phenylphenacyl esters. No change in $^3H/^{14}C$ ratios occurred after the second recrystallization.

The resultant β-hydroxyisovaleric acid was recovered from the collected urine as the *p*-phenylphenacyl ester, which was recrystallized to constant $^{3}H/^{14}C$ ratio, as shown in Table 1.

It is apparent that most of the tritium from (2*R*)-[^{3}H]isovalerate (**1a**) was eliminated in the metabolite, whereas most of the tritium from (2*S*)-[^{3}H]isovalerate (**1b**) was retained. The small degree of nonstereospecificity observed in the results probably results from incomplete stereospecificity in the synthesis of the precursors, as previously observed in syntheses of similar compounds by this route (*17*). Alternatively, the observed nonstereospecificity may be the result of some minor alternative metabolism of isovalerate. In any case it is clear that loss of the (2*R*) hydrogen of isovalerate is substantially predominant.

It is satisfying to note that the C-2 hydrogen eliminated in the dehydrogenation of isovaleryl-CoA in rats corresponds sterically with the (2*R*) hydrogen eliminated in the dehydrogenation of *n*-butyryl-CoA by pig liver butyryl-CoA dehydrogenase (*20–22*), (**4**) → (**5**). It also corresponds with the C-2 hydrogens eliminated in the dehydrogenations of isobutyryl-CoA (*8, 23*), (**6**) → (**7**), (2*S*)-2-methylbutyryl-CoA (*24*), (**8**) → (**9**), and (2*R*)-2-methylbutyryl-CoA (*25*), (**10**) → (**11**), all except the last of which have now been shown to proceed by an antiperiplanar elimination of hydrogens at C-2 and C-3.[2]



[2] Since the stereochemistry of hydrogen elimination at C-3 of isovaleryl-CoA, relative to C-2, has not been determined in this study, the overall stereochemistry of dehydrogenation of isovaleryl-CoA remains unknown. We planned to administer to these rats isovalerate chirally labeled in the isopropyl group, and determine the chirality in the resultant β-hydroxyisovalerate. However, these plans were foiled by our discovery that, in biotin-deficient rats, β-methylcrotonyl-CoA undergoes nonstereospecific addition of hydroxyl at C-3 (*11*). We are now attempting to determine the relative stereochemistry of hydrogen elimination at C-3 using purified isovaleryl-CoA dehydrogenase.

MATERIALS AND METHODS

[^{3}H]$NaBH_4$ (100 mCi/mmol) and [^{14}C]$BaCO_3$ (5 mCi/mmol) were obtained from New England Nuclear. Silica gel HF-254 + 366 (E. Merck) was used to prepare thin-layer chromatography (tlc) plates of thickness 0.2 mm for analytical purposes or 1.5 mm for preparative separations. Precoated plates of MN300F cellulose were obtained from Analtech. Nuclear magnetic resonance (nmr) spectra were taken on a Varian EM-360 instrument with internal tetramethylsilane (TMS). Chemical shifts are expressed in ppm (δ) from TMS = 0. Radiochromatogram scanning was performed using a Nuclear Chicago Actigraph III instrument. Pyridine was dried by distillation from BaO. *p*-Toluenesulfonyl chloride was recrystallized from hexane. Diglyme was distilled from CaH_2, and then redistilled under reduced pressure (1 mm) from $LiAlH_4$. Liquid scintillation counting was performed using a Nuclear Chicago Mark III instrument. Samples were dissolved in 10 ml Aquasol (New England Nuclear). Microanalyses were performed by Galbraith Laboratories, Knoxville, Tennessee.

Ammonium [1-^{14}C]Isovalerate

Ammonium [1-^{14}C]isovalerate was prepared by reaction of isobutyl magnesium bromide with $^{14}CO_2$ (from $Ba^{14}CO_3$) following well-established methods (*12*). The product showed a single radioactive zone on radiochromatography on MN 300 cellulose, solvent *n*-BuOH saturated with 2 *N* NH_4OH.

Synthesis of Ammonium (2R) and (2S)-[2-^{3}H]Isovalerates, (1a) and (1b)

(2*S*)-2-Hydroxyisovaleric acid (**2a**) (200 mg, 1.69 mmol), $[\alpha]_D^{25}$ −15° (*c* 3.3, 1 *N* NaOH) (lit. (*13*) $[\alpha]_D$ −15.2°), prepared as described (*13*) from L-valine, was methylated with excess CH_2N_2–ether. After evaporation of the solvent, the residual oil was dissolved in pyridine (2 ml) and treated with *p*-toluenesulfonyl chloride (500 mg, 2.63 mmol) at 25°C for 24 hr. The product was isolated in a conventional manner, and was purified by preparative tlc (silica gel, solvent 25% ethyl acetate–hexane), yielding methyl (2*S*)-2-toluenesulfonyloxyisovalerate (**2c**), 325 mg, glass, $[\alpha]_D^{26}$ −34.2° (*c* 3.6, $CHCl_3$); nmr δ1.90 (6H, d, J = 5 Hz), 2.15 (1H, m), 2.48 (3H, s), 3.62 (3H, s), 4.65 (1H, d, J = 5 Hz), 7.37 and 7.84 (4H, AB, J_{AB} = 8 Hz).

A solution of [^{3}H]$NaBH_4$ (12.5 mCi) in diglyme (5 ml) containing unlabeled $NaBH_4$ (7–8 mg, 0.2 mmol) and lithium bromide (29 mg, 0.33 mmol) was refluxed under N_2 for 30 min. After cooling, tosylate (**2c**) (50 mg, 0.2 mmol) was added, and the mixture was stirred at 65°C for 18 hr. Aqueous sat Na_2SO_4 (0.1 ml) was added, followed by H_2O (5 ml), and the solution was extracted with ether. The ether extract was concentrated to a volume of 2 ml. Unlabeled isoamyl alcohol (50 mg, 0.5 mmol) was added, followed by a saturated solution of $KMnO_4$ in 0.01 *M* H_2SO_4 (10 ml). After 1.5 hr at 25°C, the mixture was treated dropwise with aqueous sat $NaHSO_3$ to decolorize, and was then filtered. The filtrate was made strongly alkaline with dil NaOH, and was extracted several times with ether (discarding the ether extract). The solution was acidified with dil H_2SO_4 to pH 2 and distilled to

near dryness. The distillate was treated with excess conc NH_4OH and evaporated under reduced pressure to a residue (ca. 200 μCi ^3H). A portion of the product was purified by preparative tlc (MN 300 cellulose, solvent, *n*-BuOH sat with 2 *N* NH_4OH). The zone corresponding to isovalerate was located by radiochromatogram scanning, and was scraped off and mixed with H_2O (5 ml). The mixture was acidified (ca. pH 2) with 0.01 *N* H_2SO_4 and distilled to near dryness. The distillate was treated with excess NH_4OH and evaporated under reduced pressure to yield (**1a**) (10 μCi from ca. 50 μCi crude product before chromatography). A radiochromatogram of (**1a**) showed a single radioactive zone corresponding to isovalerate.

Approximately one-half of the product (5 μCi) was mixed with ammonium [1-^{14}C]isovalerate to give a $^3H/^{14}C$ ratio of ca. 8. The solution was mixed with unlabeled isovaleric acid (200 mg, 2 mmol), diluted to a total volume of 10 ml with H_2O, and adjusted to pH 7.4 with conc NaOH. This solution was then administered to biotin-deficient rats by the procedure which follows.

A portion (0.4 ml) of the $^3H + ^{14}C$ isovalerate was diluted to 10 ml with methanol and treated with α-bromo-*p*-phenylacetophenone (300 mg) at reflux for 16 hr. The solvent was evaporated, and the residue treated with water (10 ml) and extracted with ether. The ether extract was dried (Na_2SO_4), and evaporated to a residue from which isovaleric acid *p*-phenylphenacyl ester (115 mg) was isolated by preparative tlc (silica gel, solvent 15% ethyl acetate–hexane). The product was recrystallized from methanol to constant $^3H/^{14}C$ ratio (no appreciable change occurred on recrystallization).

An identical procedure was used to prepare (2*S*)-[2-^{3}H]isovalerate (**1b**) from (**2b**) via (**2d**) and (**3b**). The yield of chromatographically pure (**1b**) was ca. 6–7 μCi ^3H.

Administration of Precursors to Biotin-Deficient Rats: Isolation of Metabolites

Female, weanling (35–45 g), Sprague–Dawley rats (Charles River Breeding Laboratories) were fed Biotin-Free Test Diet (ICN Life Sciences Group) over a period of 8–10 weeks. At the end of this period the rats showed symptoms typical of severe biotin deficiency (dermatitis). The rats were then housed in individual metabolic cages. The labeled isovaleric acids (50 mg/day/rat, 0.2 *M* in distilled H_2O, adjusted to pH 7.4 with NaOH) were administered by intraperitoneal injection, while the rats were under light ether anesthesia. Each precursor was administered to two rats in the morning of two successive days, and their urine was collected continuously for 48 hr. The urine was saturated with NaCl, acidified to pH 2 with HCl, and extracted with ether (4 × 20 ml), centrifuging at ca. 3000 rpm to break the resultant emulsions. The extract was dried (Na_2SO_4) and evaporated under reduced pressure to an oily residue (150–200 mg, ca. 1–2 μCi ^3H). This was dissolved in methanol (10 ml) and neutralized with KOH/MeOH (1 *M*, ca. 1 ml), to a methyl red end point. Then α-bromo-*p*-phenylacetophenone (300 mg) was added, and the mixture was refluxed for 14 hr. The solvent was evaporated, and the residue slurried with H_2O (20 ml) and extracted with $CHCl_3$. The extract was dried (Na_2SO_4) and evaporated to a residue which was separated by preparative tlc (silica gel, solvent 45% ethyl acetate–hexane). The band corresponding to β-

hydroxyisovaleric acid *p*-phenylphenacyl ester was isolated and extracted with ether. After evaporation of the ether, the residue was recrystallized from ether to constant $^{3}H/^{14}C$ ratio.

An unlabeled sample of β-hydroxyisovaleric acid was prepared by a literature method (*14*). The *p*-phenylphenacyl ester, prepared as above, gave needles from ether, mp 82–83°C (corr); nmr δ1.38 (6H, s), 2.67 (2H, s), 5.42 (2H, s), 7.5 (5H, m), 7.70 and 7.99 (4H, AB, J_{AB} = 8 Hz).

Anal. Calcd for $C_{19}H_{20}O_4$: C, 73.06; H, 6.45. Found: C, 72.88; H, 6.59.

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REFERENCES

1. V. W. Rodwell, "Metabolic Pathways" (D. M. Greenberg, Ed.), 3rd ed., Vol. 3, pp. 191–203. Academic Press, New York, 1961.
2. L. K. Massey, J. R. Sokatch, and R. S. Conrad, *Bacteriol. Rev.* **40**, 42 (1976).
3. H. Beinert, "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, Eds.), Vol. 5, pp. 546–557. Academic Press, New York, 1962.
4. K. Tanaka, M. A. Budd, M. L. Efron, and K. J. Isselbacher, *Proc. Nat. Acad. Sci. USA* **56**, 236 (1966).
5. K. Tanaka, E. M. Miller, and K. J. Isselbacher, *Proc. Nat. Acad. Sci. USA* **68**, 20 (1971).
6. W. J. Rhead and K. Tanaka, *Proc. Nat. Acad. Sci. USA* **77**, 580 (1980).
7. H. Osmundsen, D. Billington, and H. S. A. Sherratt, *Biochem. Soc. Trans.* **2**, 1286 (1974).
8. D. J. Aberhart, *Bioorg. Chem.* **6**, 191 (1977).
9. D. J. Aberhart and C. H. Tann, *J. Chem. Soc. Perkin I* 939 (1979).
10. D. J. Aberhart and C. T. Hsu, *J. Chem. Soc. Perkin I* 1404 (1979).
11. D. J. Aberhart, "Recent Advances in Phytochemistry" (T. Swain and G. R. Waller, Eds.), pp. 29–54. Plenum, New York, 1979.
12. A. Murray and D. L. Williams, "Organic Syntheses with Isotopes," pp. 34–37. Interscience, New York, 1958.
13. M. Winitz, L. Block-Frankenthal, N. Izumiya, S. M. Birnbaum, C. G. Baker, and J. P. Greenstein, *J. Amer. Chem. Soc.* **78**, 2423 (1956).
14. D. D. Coffman, R. Cramer, and W. E. Mochel, *J. Amer. Chem. Soc.* **80**, 2882 (1958).
15. K. Tanaka and K. J. Isselbacher, *Lancet* 930 (1970).
16. E. P. Lau, B. C. Cochran, L. Munson, and R. R. Fall, *Proc. Nat. Acad. Sci. USA* **76**, 214 (1978).
17. L. J. Morris and C. Hitchcock, *Eur. J. Biochem.* **4**, 146 (1968).
18. G. J. Schroepfer, Jr., and K. Bloch, *J. Biol. Chem.* **240**, 54 (1965).
19. D. J. Aberhart, *Tetrahedron Lett.* 4373 (1975).
20. L. Büchlers, A. Umani-Rochi, J. Rétey, and D. Arigoni, *Experientia* **26**, 931 (1970).
21. J. F. Biellman and C. G. Hirth, *FEBS Lett.* **8**, 55 (1970).
22. J. F. Biellman and C. G. Hirth, *FEBS Lett.* **8**, 335 (1970).
23. D. J. Aberhart and C. H. Tann, *J. Amer. Chem. Soc.* **102**, 6377 (1980).
24. R. K. Hill, S. Rhee, E. Leete, and B. A. McGaw, *J. Amer. Chem. Soc.* **102**, 7344 (1980).
25. O. A. Mamer and S. S. Tjoa, *Biomed. Mass Spectrom.* **2**, 133 (1975).