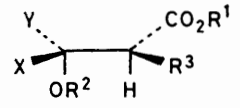
Stereospecific Hydrogen Loss in the Conversion of [$^2H_7$]Isobutyrate to β-Hydroxyisobutyrate in *Pseudomonas putida*. The Stereochemistry of β-Hydroxyisobutyrate Dehydrogenase

By **D. John Aberhart** * and **Chi-Tung Hsu**, The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, U.S.A.

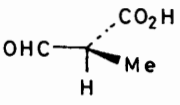
Ammonium [$^2H_7$]isobutyrate was converted by *Pseudomonas putida* (ATCC 21244) into a mixture of (2*S*)-β-hydroxy[3-$^2H_2$,2'-$^2H_3$]isobutyric acid (69%) and (2*S*,3*S*)-β-hydroxy[3-$^2H_1$,2'-$^2H_3$]isobutyric acid (31%). The latter is formed stereospecifically with loss of the 3-*pro*-*R*-hydrogen atom, probably *via* reversible formation of methylmalonic semialdehyde by the enzyme β-hydroxyisobutyrate dehydrogenase.

We recently reported our studies on the metabolism of isobutyrate by *Pseudomonas putida* (ATCC 21244) in which *S*(+)-β-hydroxyisobutyric acid (β-HIBA) (1a) accumulates.[1] It was suggested [1a] that the transformation of isobutyrate to β-HIBA involved isobutyryl-CoA, methacrylyl-CoA, and β-hydroxyisobutyryl-CoA as intermediates. Supporting evidence for the intermediacy of methacrylate (or methacrylyl-CoA) was reported.[1b] However, since we were working with an intact organism rather than purified enzymes, it was conceivable that alternative mechanisms for the interconversion might be operative. We now report the results of an investigation designed to determine whether five of the methyl hydrogens of isobutyrate were retained in the biosynthetic β-HIBA.† We also report on the stereochemistry of the observed loss of hydrogen from the resultant hydroxymethyl group of β-HIBA.

The precursor [$^2H_7$]isobutyric acid was synthesized by carboxylation of the Grignard reagent from 2-bromo-[$^2H_7$]propane. The mass spectrum of the corresponding *p*-phenylphenacyl ester indicated a deuterium content of *ca.* 97% $^2H_7$. The [$^2H_7$]isobutyric acid was then incubated with washed cells of *Ps. putida* as previously described,[1a] and the resultant β-HIBA was isolated as the methyl ester benzoate. The n.m.r. spectrum of the product showed the presence of a proton on C-2 rather than a deuterium atom indicating, as reported earlier,[1a] that the original C-2 deuterium of isobutyrate had undergone exchange with the medium. In addition, a small absorption (*ca.* 0.3 H) was visible at δ 4.48, due to one or both of the C-3 hydrogens. Since no absorption for the C-2 methyl group was visible, the absorption at δ 4.48 could not be attributed to (1b) formed from residual unlabelled β-HIBA, which might have been trapped in the cells during the preliminary growth of cells on unlabelled isobutyrate. This was supported by the fact that in the mass spectrum of the biosynthetic product, the molecular ion for [$^2H_5$]-β-HIBA methyl ester benzoate (1c), *m/e* 227, was accompanied by a peak at *m/e* 226 (45% of intensity of peak at *m/e* 227). However, no peak for unlabelled (1b) appeared at *m/e* 222. Also, in the mass spectrum of (1b), no peak appeared at $M - 1$, *m/e* 221. Thus, partial exchange of *one* of the C-3 hydrogens of β-HIBA had occurred at some stage in its biosynthesis.‡



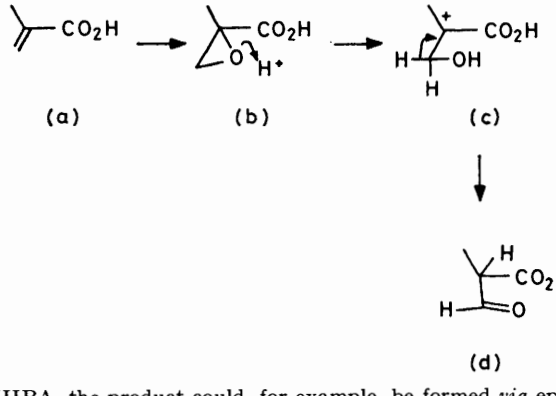
(1) a; $R^1 = R^2 = X = Y = {}^1H$, $R^3 = Me$
b; $R^1 = R^3 = Me$, $R^2 = Bz$, $X = Y = {}^1H$
c; $R^1 = Me$, $R^2 = Bz$, $X = Y = {}^2H$, $R^3 = C^2H_3$
d; $R^1 = Me$, $R^2 = Bz$, $X = {}^2H$, $Y = {}^1H$, $R^3 = C^2H_3$
e; $R^1 = R^2 = H$, $X = Y = {}^2H$, $R^3 = C^2H_3$



(2)

Since this hydrogen exchange comprised substantially less than 1.0 atom of hydrogen, it did not support mechanisms for the conversion of isobutyrate to β-HIBA involving, as obligatory intermediates, more highly oxidized substances such as methylmalonic semialdehyde. The most reasonable explanation of the observed results appeared to be that a portion of the original metabolite [$^2H_5$]-β-HIBA (1e) had undergone reversal of the next step in its catabolic pathway, namely oxidation by β-

† Although the enoyl-CoA hydratase- (or similar enzyme)-catalysed hydration of methacrylyl-CoA discussed in the preceding paper appears to be the most likely pathway for the formation of



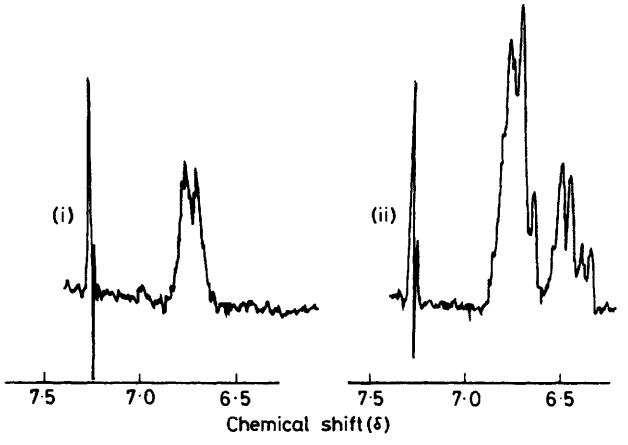
β-HIBA, the product could, for example, be formed *via* epoxide (b) which could transfer a hydrogen to C-2, or eliminate a proton (c) to form methylmalonate semialdehyde (d), reduction of which would give β-HIBA. We thank Dr. David Kupfer for this suggestion.

‡ Our observations parallel those of Mamer *et al.*[2,3] who observed the formation of mainly 2-ethyl [3-$^2H_1$]hydracylic acid. (67.8%) from 2-methyl [$^2H_3$]butyrate *via* the *R*-isoleucine pathway in rats. However, Tanaka *et al.* found no extra loss of C-3 deuterium in the conversion of [3-$^2H_6$]isobutyrate to β-HIBA in rats.[4]

hydroxyisobutyrate dehydrogenase[5] to [$^2H_4$]methylmalonaldehydic acid (2).*

In the reverse step, reduction of (2) back to β-HIBA had proceeded with the transfer of ^1H from unlabelled NADH to generate [$^2H_4$]-β-HIBA. Since β-aldehydo-acids such as (2) are well known to exist in solution predominantly in the enolic form,[6] the reversal of the enzymatic oxidation must either occur so rapidly on the nascent biosynthetic methylmalonaldehydic acid that insufficient time exists for loss of configurational purity at C-2, or the enzyme must accept as substrate only the 2*S*-enantiomer of methylmalonaldehydic acid; † otherwise optically pure *S*(+)-β-HIBA could not have been obtained from these cultures. That the isolated β-HIBA was indeed optically pure follows from the n.m.r. studies with biosynthetically labelled β-HIBA [1b] and the n.m.r. evidence which follows.

It appeared likely that the observed exchange of hydrogen would be stereospecific. In fact, if the forward and reverse directions of the exchange process did not involve the loss and recovery of a hydrogen at the same



Partial 100 MHz n.m.r. spectra in $CDCl_3$ solution with added Eu(fod)$_3$. (i) Biosynthetic product (1c) + (1d); (ii) (1c) + (1d) + (1b)

steric position of β-HIBA, it might be expected that a portion of the isolated metabolite would have exhibited the exchange of *two* hydrogen atoms through recycling. In the preceding paper, we reported an n.m.r. method for assigning the stereochemistry of β-HIBA methyl ester benzoate isotopically labelled at C-3. The biosynthetic product from [$^2H_7$]isobutyrate was also examined by this procedure. In the presence of Eu(fod)$_3$, the C-3 hydrogen doublet was shifted downfield *ca.* 6.9 p.p.m. After addition of a small amount of unlabelled (1b) and additional Eu(fod)$_3$, the spectrum was again recorded. From the Figure, it is clear that the exchanged hydrogen at C-3 of the biosynthetic product appears in the *downfield* half of the AB pattern exhibited by (1b). It therefore follows that this hydrogen is located in the 3-*pro*-*R*-position (1d). The fact that only a single doublet for the C-3 hydrogen was observed also proves that the metabolite was configurationally pure at C-2.

The observed stereochemistry of the process thus parallels that determined for the catalyzed oxidation of a wide variety of primary alcohols by yeast or liver alcohol dehydrogenase.[7]

EXPERIMENTAL

General procedures and instrumentation are given in the previous paper.[1b]

Ammonium [$^2H_7$]*Isobutyrate.*—A Grignard reagent was prepared in the usual manner from 2-bromo[$^2H_7$]propane (10 g; 99% $^2H_7$), and added to pulverized solid carbon dioxide. After addition of water, acidification with sulphuric acid, and steam distillation, the distillate was treated with an excess of ammonium hydroxide and evaporated to give the syrupy ammonium [$^2H_7$]isobutyrate (7.2 g). The n.m.r. spectrum of the product in ^2H_2O did not show any peaks except that of ^1H_2O. A portion of the product (100 mg) in methanol (10 ml) was treated with recrystallized α-bromo-*p*-phenylacetophenone (280 mg) at reflux for 3 h. After usual work-up and purification of the product by preparative t.l.c. (ethyl acetate-hexane 1 : 19 v/v), isobutyric acid *p*-phenylphenacyl ester (188 mg) was recrystallized from methanol as needles, m.p. 80—81 °C; δ($CDCl_3$) 5.32 (2 H, s), 7.2—8.0 (9 H, m); *m/e* ‡ 290 (22%), 289 (M^+, 100), 288 (5), 183 (46), 182 (99), 181 (100), 167 (27), 165 (15), 154 (32), 153 (100), 152 (100), 151 (63), 127 (22), 78 (57), and 50 (98). An unlabelled sample of this compound had *m/e* 283 (40), 282 (M^+, 100), 281 (2), 183 (42), 182 (100), 181 (100), 167 (44), 165 (20), 154 (42), 153 (100), 152 (100), 151 (81), 127 (28), 71 (61), and 43 (42).

Conversion of [$^2H_7$]*Isobutyrate to* β-*Hydroxyisobutyric Acid by* Pseudomonas putida.—Ammonium [$^2H_7$]isobutyrate (660 mg) was incubated with washed cells of *Ps. putida* (ATCC 21244) as previously described [1a] and the resultant β-hydroxyisobutyrate isolated as the methyl ester benzoate (24 mg); δ($CDCl_3$) 2.95 (1 H, br s, $W_{\frac{1}{2}}$ 13 Hz), 3.74 (3 H, s), 4.48 (0.3 H, br d, *J* 7 Hz), 7.4 (3 H, m), 8.0 (2 H, m); *m/e* 228 (13%), 227 (73), 226 (33), 209 (16), 208 (8), 196 (18), 195 (22), 194 (8), 167 (13), 124 (15), 123 (11), 122 (73), 106 (12), 105 (100), 104 (7), and 77 (8). An unlabelled sample of (1b) had *m/e* 223 (19%), 222 (100), 204 (22), 191 (28), 190 (23), 162 (14), 123 (22), 122 (53), 117 (12), 106 (11), 105 (92), 100 (12), and 77 (12).

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* When a mixture of [3-^{3}H, 1-^{14}C]isobutyrate, $^3H/^{14}C$ 3.91, was incubated with *Ps. putida*, β-HIBA having $^3H/^{14}C$ 4.71 was isolated. The recovered unmetabolized isobutyrate had the same $^3H/^{14}C$ value as the original precursor. Although it is quite difficult to interpret the significance of isotope effects encountered in a multistep process in an intact organism, the results may be indicative of a substantial hydrogen isotope effect in the conversion of β-HIBA to methylmalonate semialdehyde.

† A further possibility could be that the 2*S*-methylmalonaldehydic acid does not separate from the enzyme before reduction back to β-HIBA. If so, then at least the NAD^{2}H must separate and be replaced by NAD^{1}H; otherwise, only [$^2H_5$]-β-HIBA would have been obtained in this experiment.

‡ With the exception of the group of isotopic peaks comprising the molecular ion, only peaks with relative intensity ≥ 10% are reported.

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