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Production of a co-polyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from succinic acid by *Rhodococcus ruber*: biosynthetic considerations

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Abstract. The biosynthesis of the 3-hydroxyvalerate (3HV) monomer of polyhydroxyalkanoate by *Rhodococcus ruber* from succinic acid was investigated using nuclear magnetic resonance analysis. Polymer produced from [2,3-^{13}C]- and [1,4-^{13}C]succinate showed that the C-1-C-2 and C-4-C-5 fragments of 3HV were derived from carbons 2 and 3 of succinate, essentially without bond cleavage, and carbon 3 of 3HV was derived from a carboxyl carbon of succinate. Using [1,2-^{13}C]succinate it was demonstrated that the C-1-C-2 bond of succinate was cleaved during polymer biosynthesis. Methylmalonyl-coenzyme A (CoA) mutase activity was detected in cell-free extracts of *R. ruber* by enzyme assay and HPLC analysis of reaction products. A pathway, involving the known methylmalonyl-CoA pathway for propionate formation in Propionibacteria, followed by the established pathway for PHA biosynthesis from propionyl-CoA and acetyl-CoA, is proposed for the biosynthesis of 3HV from succinate by *R. ruber*.

Introduction

Poly(3-hydroxybutyrate) (PHB) is the most common poly(hydroxyalkanoate) (PHA) produced as a storage material by bacteria under restricted growth conditions. PHB biosynthesis usually occurs via the 3-ketothiolase-mediated condensation of two molecules of acetyl-coenzyme A (CoA) to yield acetoacetyl-CoA, followed by reduction to 3-hydroxybutyryl-CoA and polymerization (Dawes and Senior 1973; Steinbüchel 1991). PHB can also be synthesized from butyric acid, which is converted to 3-hydroxybutyryl-CoA without cleavage of the carbon chain (Doi 1990). Many bacteria produce PHA containing 3-hydroxybutyrate (3HB) and additional hydroxyacid monomer units. In most of the instances that have been studied to date, the carbon source is an obvious precursor of the additional hydroxyacid monomer (Doi 1990). For example, various bacteria including *Alcaligenes eutrophus* produce PHA containing 3-hydroxyvalerate (3HV) monomers when supplied with valeric acid or propionic acid (Holmes et al. 1985; Haywood et al. 1989). Although valeric acid is clearly a precursor of the 3HV monomer, the formation of 3HV from propionic acid may be explained (Ballard et al. 1987) by the condensation of acetyl-CoA with propionyl-CoA (derived from propionic acid) to yield 3-ketovaleryl-CoA, which is a precursor of 3HV.

Some bacteria accumulate a PHA containing 3HB and 3HV monomers from structurally-unrelated carbon sources. These include *Rhodococcus* species and related organisms (Anderson et al. 1990; Haywood et al. 1991), *Haloferax mediterranei* (Rodriguez-Valera and Lillo 1990), some non-sulphur purple bacteria (Liebergesell et al. 1991), *Alcaligenes* sp. SH-69 (Kim et al. 1992) and a mutant of *A. eutrophus* that is altered in the metabolism of branched-chain amino acids (Steinbüchel and Pieper 1992). In the last example, the mutant strain overproduces acetolactate synthase to compensate for a defective threonine dehydratase. The authors suggest that this leads to the overproduction of propionyl-CoA, which may condense with acetyl-CoA, as discussed above, leading to biosynthesis of the 3HV monomer.

The biosynthesis of poly(3HB-*co*-3HV) from unrelated carbon sources, in *Rhodococcus* sp. and bacteria from the related genera *Corynebacterium* and *Nocardia* (Anderson et al. 1990; Haywood et al. 1991), is of particular interest because 3HV is generally the principal monomer constituent of the PHA, in contrast to PHA from the other organisms described above, in which 3HV is not the main component. The composition of the PHA produced is dependent on the choice of carbon source. For example, *Rhodococcus* sp. NCIMB 40126 synthesizes PHA containing approximately 70 mol% 3HV from glucose and various other substrates, but if succinate is used as the carbon source a polymer containing an unusually high 3HV content (approximately 95 mol%) is produced. This suggests

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that succinate may be a precursor of the 3HV monomer unit. We have investigated the biosynthesis of PHA from succinate in this organism and report here a proposed biosynthetic pathway.

Part of this work was presented at the International Symposium on Bacterial Polyhydroxyalkanoates, Göttingen, Germany, 1–5 June, 1992 (Anderson et al. 1992).

Materials and methods

Materials. [1,2-^{13}C]Succinic acid was obtained from Isotec (Miamisburg, Ohio, USA). [1,4-^{13}C]Succinic acid was obtained from MSD Isotopes (Montreal, Canada). [2,3-^{13}C]Succinic acid was obtained from Cambridge Isotope Laboratories (Woburn, Mass., USA). The isotopic purity of each compound was 99 atom%.

Organism. The organism used in this study, *Rhodococcus* sp. NCIMB 40126, has been identified as *R. ruber* (J. Mergaert, University of Gent, personal communication).

Culture conditions. R. ruber was grown for 24 h at 30° C in shake-flask culture on nutrient broth, using a 1% inoculum grown in the same medium. Bacteria were harvested by centrifugation (6000 *g*) and resuspended in an equal volume of nitrogen-free defined medium (Haywood et al. 1991) containing sodium succinate (5 g l^{-1}) as carbon source. For nuclear magnetic resonance (NMR) studies, ^{13}C-labelled succinate was included at the required enrichment. The cultures were incubated as before for a further 48 h, washed with water and lyophilized for use in NMR studies; for enzyme work the bacteria were harvested after 24 h under nitrogen-free conditions and washed with 50 mM potassium phosphate buffer, pH 7.0.

Extraction of PHA. PHA was extracted from diethyl-ether-washed lyophilized bacteria by heating under reflux for 1 h with chloroform and the filtrate was evaporated to dryness. To remove contaminating lipids, PHA was dissolved in chloroform (50 g l^{-1}) and precipitated from solution with four volumes of hexane.

NMR analysis. Spectra were recorded on a JEOL GX 270 spectrometer operating at 67.9 MHz for ^{13}C and 270 MHz for ^{1}H. Spectra were recorded in $CDCl_3$ and ^{1}H spectra were referenced to tetramethylsilane and ^{13}C spectra to $CDCl_3$ at 77.05 ppm.

Preparation of cell-free extracts. All procedures were carried out at 4° C. Washed bacteria were suspended in 50 mM potassium phosphate buffer (pH 7.0, 2 vol.) and disrupted by two passages through a French press. Cell debris was removed by centrifugation (27000 *g*) for 20 min and the supernatant was clarified by centrifugation (100000 *g*) for 1 h. The supernatant from this centrifugation was used for enzyme assays and HPLC work and the pellet was resuspended in buffer to yield a membrane preparation.

Enzyme assays. Methylmalonyl-CoA mutase (EC 5.4.99.2) was assayed in a reaction mixture (3 ml) that contained 66.7 mM potassium phosphate buffer, pH 6.8, 3.3 mM sodium pyruvate, 100 μM NADH, 7.5 units (U) malate dehydrogenase, 3.3 μM coenzyme B_{12}, 0.03 U methylmalonyl-CoA:oxaloacetate carboxyltransferase (10 U mg^{-1} protein; purified 19-fold from crude cell-extracts of *Propionibacterium freudenreichii* by ion exchange chromatography on a DEAE Sepharose CL-6B column, followed by gel filtration on a Sephacryl S-300 column and FPLC using a Mono Q column), 66.7 μM succinyl-CoA and cell-free extract (0.1–0.4 mg protein). When a high endogenous NADH oxidase activity was observed, the reaction was performed in an anaerobic cuvette that was flushed with O_2-free N_2 for 5 min before assay. The reaction was initiated by the addition of succinyl-CoA.

Methylmalonyl-CoA:oxaloacetate carboxyltransferase (EC 2.1.3.1) was measured as described by Wood et al. (1969). Methylmalonyl-CoA decarboxylase (EC 4.1.1.41) was assayed as described by Denger and Schink (1990).

HPLC analysis of reaction products. The products of the methylmalonyl-CoA mutase reaction were analysed by HPLC using a 5 μm Spherisorb ODS2 column (4.6×100 mm, Jones Chromatography, UK) and a UV detector (260 nm). The mobile phase (flow rate 1 ml min^{-1}) used a linear gradient (9–30%, v/v) of methanol/chloroform (98:2 v/v) in 220 mM potassium phosphate buffer, pH 4.0, over a period of 20 min.

Reaction mixtures (1 ml) contained cell-free extract (100 μg protein), 10 μM coenzyme B_{12}, 100 μM succinyl-CoA and 45 mM potassium phosphate buffer, pH 6.8, and were incubated at 30° C for 60 min. Reactions were terminated by the addition of 100 μl of 40% (v/v) perchloric acid. After 10 min at 0° C the pH was adjusted to 4.0 with 5 M KOH and the precipitate removed by centrifugation. The supernatant was kept at 0° C and filtered (0.2 μm) prior to injection (20 μl) onto the HPLC column.

Results

^{13}C-NMR analysis of PHA produced from 1,4- and [2,3-^{13}C]succinic acid

The ^{13}C-NMR spectrum for PHA produced from succinic acid enriched with 10% [2,3-^{13}C]succinic acid shows that similar enrichment occurred at sites C-1, C-2, C-4 and C-5 of the 3HV monomer unit (Fig. 1A). Each of these signals comprised a doublet (d) due to one-bond coupling and a singlet (s) for uncoupled carbon; the intensity of the multiplet for C-3 was about five times smaller indicating only a small enrichment at this site. The chemical shift, coupling constant and d/s ratio for each signal are given in Table 1. The proton NMR spectrum for this polymer confirms the selective ^{13}C enrichment of C-1, C-2, C-4 and C-5 of the 3HV monomer unit, and the signals for protons attached to these carbons show ^{13}C side-bands in the same ratio (data not shown).

Table 1. ^{13}C-Nuclear magnetic resonance (NMR) analysis of poly(hydroxyalkanoates) (PHA) produced by *Rhodococcus ruber* from [2,3-^{13}C]succinate

Carbon atom[a]	Chemical shift (δ)	Doublet:singlet (d/s) ratio	Coupling constant (Hz)
V1	169.61	5.3	$J_{1,2}$ 58.6
V2	38.74	5.6	
V3	72.00	0.42	$J_{2,3}$ 39.0
V4	26.84	5.6	$J_{3,4}$ 34.1
V5	9.43	5.3	$J_{4,5}$ 34.2
B1	169.4	4.2	$J_{1,2}$ 58.0
B2	40.9	4.3	
B3	67.8	2.6	$J_{2,3}$ 39.0
B4	19.8	3.4	$J_{3,4}$ 39.0

[a] V1–V5 denote carbons 1 to 5 of the 3HV monomer unit. B1–B4 denote carbons 1 to 4 of the 3HB monomer unit

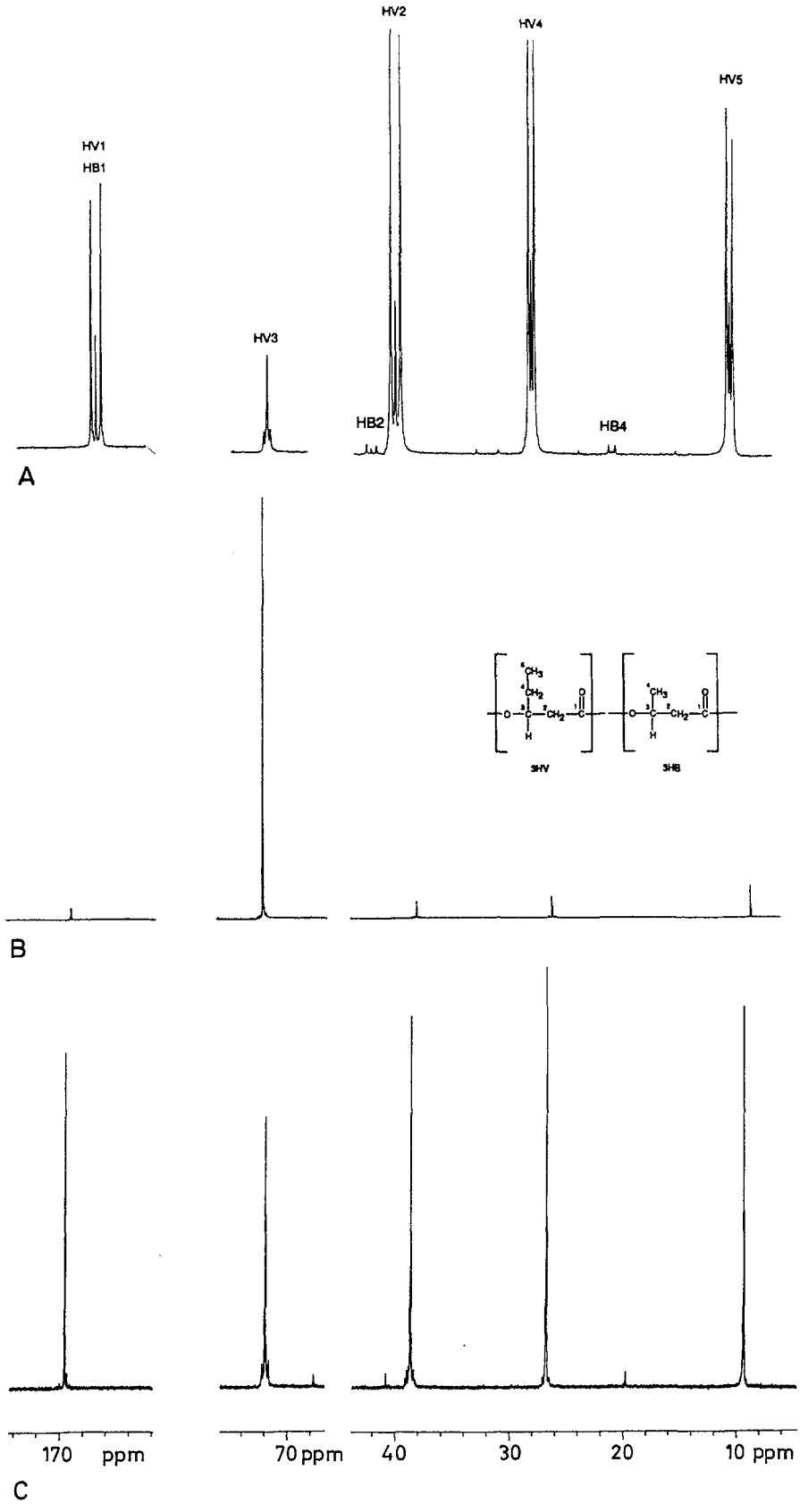

Fig. 1A–C. 67.9 MHz ^{13}C-Nuclear magnetic resonance (NMR) spectra of purified poly(hydroxyalkanoates) (PHA) produced from ^{13}C-enriched succinates by *Rhodococcus ruber*. **A** [2,3-^{13}C]Succinate (10% enrichment). **B** [1,4-^{13}C]Succinate (10% enrichment). **C** [1,2-^{13}C]Succinate (25% enrichment)

The ^{13}C-NMR spectrum for PHA synthesized from [1,4-^{13}C]succinic acid (10% enrichment) presents a complementary enrichment pattern, with C-3 being highly enriched, whereas the other carbons of the 3HV monomer unit yield essentially natural abundance signals (Fig. 1B). C-3 had small side-bands corresponding to a d/s ratio of 0.013. The signals representing C-2 and C-4 had a d/s ratio of 0.175 whereas those for C-1 and C-5 were singlets.

Since the PHA produced from succinate contains only a small proportion of 3HB monomer units (3–4 mol% in the above labelling experiments), quantitative analysis of the signals representing the 3HB monomer is difficult. It is clear, however, that all four carbons in 3HB monomer units produced from [2,3-^{13}C]succinate are substantially enriched, whereas these carbons showed little or no enrichment when [1,4-^{13}C]succinate was used as substrate.

^{13}C-NMR analysis of PHA produced from [1,2-^{13}C]succinic acid

The five signals representing the carbons of the 3HV monomer unit were of similar intensity. At 10% ^{13}C enrichment, each signal was a singlet with small side-bands in the range 2–4%. Increasing the enrichment to 25% yielded a spectrum (Fig. 1C) that permitted a more accurate analysis of the intensities of the multiplets representing each carbon (Table 2).

Enzyme activities in cell-free extracts

Methylmalonyl-CoA mutase activity was detected in cell-free extracts prepared from bacteria that had been allowed to accumulate polymer for 24 h with succinate as a carbon source. The enzyme had a specific activity of 0.07 U mg^{-1} protein and no activity was detected if transcarboxylase or coenzyme B_{12} were omitted from the assay mixture, or if the cell-free extract was heated at 100° C prior to the assay. Neither methylmalonyl-CoA:oxaloacetate transcarboxylase nor methylmalonyl-CoA decarboxylase activities were detected in cell-free extracts or membrane preparations.

Table 2. ^{13}C-NMR analysis of PHA produced by *R. ruber* from [1,2-^{13}C]succinate: distribution of intensity of signals for carbons of the 3HV monomer unit

Carbon atom[a]	Observed d/s ratio	Calculated d/s ratio[b]
V1	0.03	0.02
V2	0.19	0.17
V3	0.15	0.17
V4	0.05	0.02
V5	0.03	0.02

[a] V1–V5 denote carbons 1 to 5 of the 3HV monomer unit

[b] The calculated d/s ratio is based on the assumption that the pathway shown in Fig. 2 is used by *R. ruber* for the conversion of succinic acid to 3HV. Values were calculated on the basis of 25% ^{13}C enrichment at C-1 and C-2 of succinate and natural abundance (1% ^{13}C)

Products of incubation of a cell-free extract with succinyl-CoA

Incubation of succinyl-CoA and coenzyme B_{12} with cell-free extract at pH 6.8 yielded a product that co-chromatographed with authentic methylmalonyl-CoA. No methylmalonyl-CoA was detected if either succinyl-CoA or coenzyme B_{12} was omitted from the assay or if the cell-free extract was heated at 100° C prior to the incubation (results not shown). Inclusion of purified methylmalonyl-CoA:oxaloacetate transcarboxylase and sodium pyruvate in the assay mixture gave a product that co-chromatographed with propionyl-CoA instead of methylmalonyl-CoA.

Discussion

Under nitrogen-free conditions, *R. ruber* accumulates PHA containing both 3HV and 3HB monomer units from a wide range of carbon sources (Anderson et al. 1990; Haywood et al. 1991). A co-polymer containing up to 99 mol% 3HV is produced from valeric acid; this can be explained because valeric acid is a precursor of the 3HV monomer unit, as suggested for the synthesis of PHA copolymers containing a high proportion of 3HV monomers by *Alcaligenes eutrophus* (Doi et al. 1987). The accumulation of a PHA containing >95 mol% 3HV from succinate suggests that this substrate may also be a precursor of 3HV monomers and the data presented above is compatible with the biosynthetic pathway shown in Fig. 2.

^{13}C-NMR analyses of purified PHA produced from both [2,3-^{13}C]- and [1,4-^{13}C]succinic acid provide complementary evidence, showing that the methylene carbons (C-2 and C-3) of succinic acid are incorporated, essentially without cleavage, as C-1-C-2 and C-4-C-5 of the 3HV monomer unit. Also, C-3 of the 3HV monomer is derived from a carboxyl carbon (C-1 or C-4) of succinate. The ratio of the lines within the multiplets indicate that the contribution from mechanisms that involve the cleavage of the bond linking C-2 and C-3 of succinic acid cannot be more than approx. 10%. It might be expected, on the basis of the results for the [2,3-^{13}C]- and [1,4-^{13}C]labelling experiments, that C-3 in the 3HV monomer unit is introduced as part of an intact succinate unit. This would give rise to the incorporation of either the C-1-C-2-C-3 or the C-3-C-4-C-5 fragments as intact moieties. Examination of the results of the experiment with [1,2-^{13}C]succinate reveals that neither of these routes is possible for the incorporation of C-3. The d/s ratio for C-3 (Table 2) clearly indicates that the bond between C-1 and C-2 of succinate is cleaved during the synthesis of 3HV. The expected value for the d/s ratio for C-3 in 3HV if cleavage did not occur is approx. 30.

In order to account for these observations a pathway is proposed (Fig. 2), a key feature of which is the intramolecular rearrangement of succinyl-CoA to methylmalonyl-CoA. This rearrangement cleaves the C-1-C-2 succinate bond but preserves the C-2-C-3 suc-

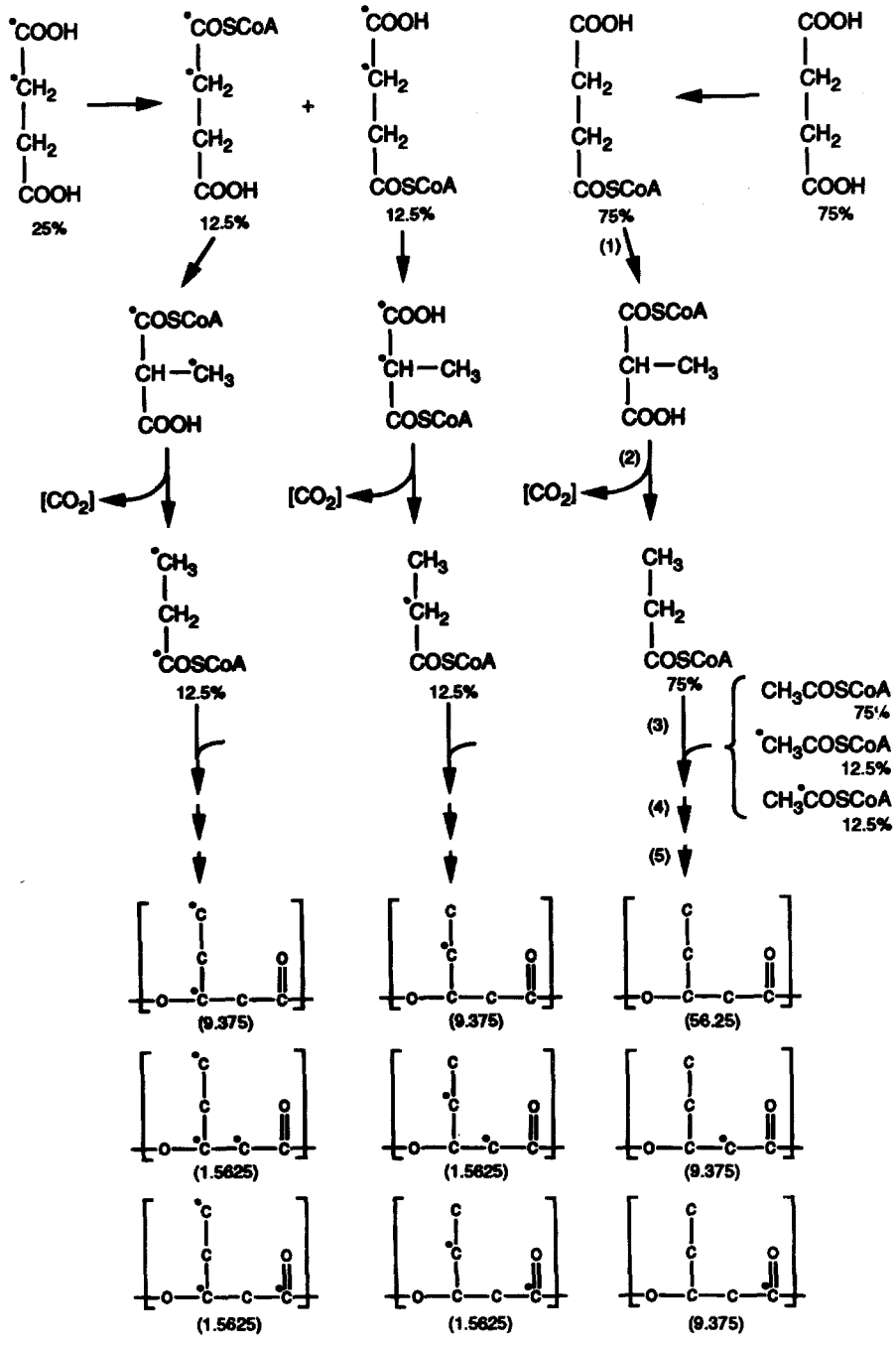

Fig. 2. Proposed pathway for the conversion of succinic acid to the 3-hydroxyvalerate monomer unit of PHA in *R. ruber*. The activities of methylmalonyl-CoA mutase (*1*), 3-ketothiolase (*3*), acetoacetyl-coenzyme A (CoA) reductase (*4*) and PHA synthase (*5*) have been demonstrated in *R. ruber*. The enzyme-catalysed conversion of methylmalonyl-CoA to propionyl-CoA (*2*) remains to be demonstrated (see text). *Values in parentheses* show the ^{13}C enrichment of metabolites produced from [1,2-^{13}C]succinate (25% enriched)

cinate bond, as required by the other labelling experiments. Thus the labels in [1,2-^{13}C]succinate are separated. The enrichment is equal at C-3, C-4 and C-5 of 3HV and the label is essentially isolated at each site; i.e. adjacent sites have ^{13}C at natural abundance only. The calculated d/s ratios (Table 2), based on the relative species populations shown in the scheme, show excellent agreement with the experimental results, given that minor pathways probably contribute about 10%.

The rearrangement of methylmalonyl-CoA to succinyl-CoA, catalysed by methylmalonyl-CoA mutase, is well established in mammalian systems. Various bacteria, of which *Propionibacterium* species are the best-known example, also use methylmalonyl-CoA mutase to catalyse the reverse reaction, i.e. the conversion of succinyl-CoA to methylmalonyl-CoA, in the formation of propionate (Halarnkar and Blomquist 1989). Methylmalonyl-CoA mutase activity was detected in cell-free extracts of *R. ruber* in the present study and HPLC analysis demonstrated the conversion of succinyl-CoA to methylmalonyl-CoA by a cell-free system.

The conversion of methylmalonyl-CoA to propionyl-CoA could be catalysed by either methylmalonyl-CoA decarboxylase (Denger and Schink 1990) or methylmalonyl-CoA:oxaloacetate transcarboxylase (Wood et al. 1969). Neither of these enzymes was detected in cell-free extracts or membrane preparations of *R. ruber*, despite repeated attempts, and it is possi-

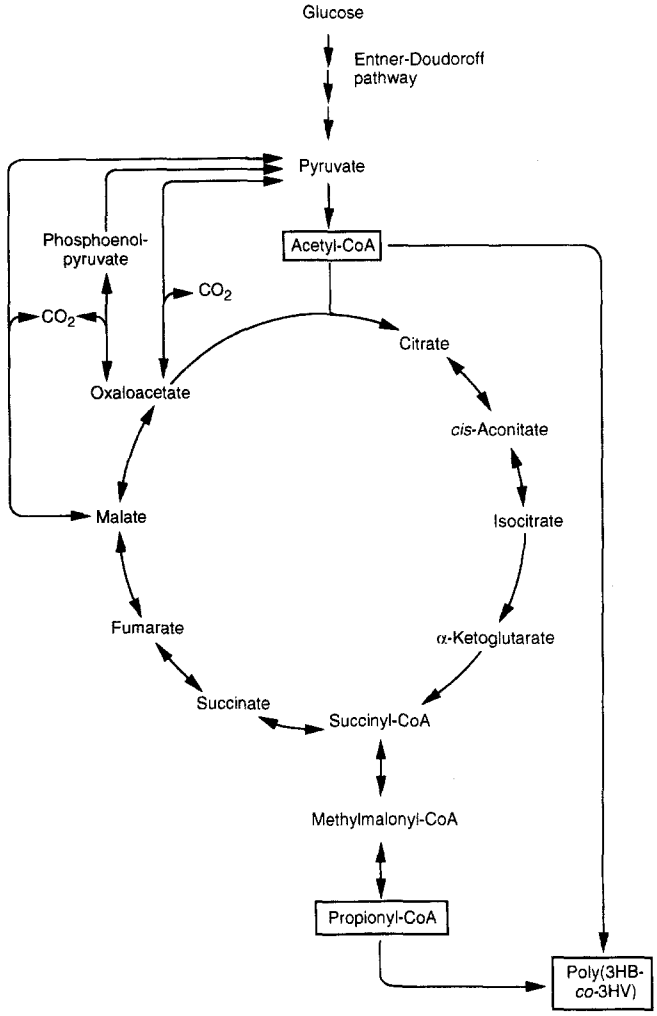

Fig. 3. Possible routes for the production of propionyl-CoA and acetyl-CoA for the synthesis of poly(3HB-*co*-3HV) in *R. ruber*: 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate

ble that the relevant enzyme produced by *R. ruber* is labile.

^{13}C-NMR analysis shows that the C-1-C-2 fragment of 3HV is synthesized from carbons 2 and 3 of succinate with no bond cleavage. This can be explained by the conversion of succinate to oxaloacetate via TCA-cycle intermediates followed by decarboxylation of oxaloacetate to pyruvate (Fig. 3). The subsequent decarboxylation of pyruvate yields acetyl-CoA, derived from the intact C2-C3 fragment of succinate, which is then incorporated into positions 1 and 2 of the 3HV monomer.

The proportion of 3HB in co-polymer produced by *R. ruber* from succinate is low (approx. 5 mol%), making quantitative ^{13}C-NMR analysis difficult. However, it is apparent that all four carbons of 3HB are derived from intact C-2-C-3 fragments of succinate. This data is compatible with the pathway described above for the conversion of succinate to acetyl-CoA, followed by condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, a precursor of 3HB.

We have previously suggested (Haywood et al. 1991) that biosynthesis of 3HV monomers by *R. ruber* could involve either the 3-ketothiolase-mediated condensation of propionyl-CoA with acetyl-CoA, as suggested for *A. eutrophus*, or some novel pathway that is independent of 3-ketothiolase. Despite our failure to demonstrate the conversion of methylmalonyl-CoA to propionyl-CoA, the NMR data and the presence of methylmalonyl-CoA mutase together offer good evidence that 3HV monomers are synthesized by the route shown in Fig. 2, which involves 3-ketothiolase. We have shown that the substrate specificities of the PHA biosynthetic enzymes of *R. ruber* (3-ketothiolase, NADPH-dependent acetoacyl-CoA reductase and PHA synthase) allow the synthesis of both 3HB and 3HV monomer units from acetyl-CoA and propionyl-CoA (Anderson et al. 1992).

We are now investigating the biosynthesis of the 3HV monomer of PHA from glucose by *R. ruber*. Abstraction of succinyl-CoA from the TCA cycle could provide a pathway for synthesis of the 3HV monomer from glucose (Fig. 3). Preliminary NMR studies (not shown) demonstrate that, even allowing for the scrambling of label through operation of the TCA cycle for maintenance metabolism, this could not represent the sole pathway. Branched-chain amino acid metabolism, which is involved in 3HV biosynthesis in a mutant strain of *A. eutrophus* (Steinbüchel and Pieper 1992), and a route that involves carboxylation of pyruvate or phosphoenolpyruvate, yielding TCA cycle intermediates (Fig. 3), may also be involved in 3HV formation. The complexity of the ^{13}C-NMR spectra of PHA produced from [2-^{13}C]- or [6-^{13}C]glucose supports our hypothesis that no single pathway can account for the biosynthesis of 3HV from glucose. The relative importance of the three possible routes described above remains to be established.

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