# Biosynthesis of Polyesters by *Alcaligenes eutrophus:* Incorporation of $^{13}C$-Labelled Acetate and Propionate

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Polyesters specifically-labelled using $^{13}C$ have been isolated from *Alcaligenes eutrophus* H16 grown in nitrogen-free culture media containing the sodium salt of [2-$^{13}C$]acetate or [1-$^{13}C$]propionate as carbon source; the biosynthetic pathway is discussed.

Optically active copolyesters of 3-hydroxybutyrate and 3-hydroxyvalerate are of industrial importance as biotechnological products with thermoplastic properties.[1,2] The copolyesters derived from acetate and propionate in *Alcaligenes eutrophus* have been shown to have a statistically random distribution of comonomer units,[3] and the biosynthetic pathway of these copolymers is still open to discussion. The present work describes the use of $^{13}C$-labelled acetate and propionate to determine the origin of the carbon skeleton of polyesters formed in *A. eutrophus*.

*A. eutrophus* H16 (ATCC 17699) was first grown at 30 °C in a nutrient-rich medium (50 $cm^3$) containing yeast extract, polypeptone, and meat extract. The cells were harvested by centrifugation after 48 h and washed with water. Under these culture conditions the accumulation of polyesters in the cells was not observed. To promote polyester synthesis, the washed cells were transferred into a nitrogen-free mineral medium[4] containing the sodium salt of [2-$^{13}C$]acetate or [1-$^{13}C$]-propionate as carbon source: medium A; 2.0 g of sodium [2-$^{13}C$]acetate (6.0% $^{13}C$ per C atom), and medium B; 1.0 g of sodium [1-$^{13}C$]propionate (10% $^{13}C$ per C atom). The cells were cultivated in the nitrogen-free media for 48 h at 30 °C, harvested, washed with acetone, and finally dried under vacuum. Polyesters were extracted from the dried cells with hot chloroform, and purified by reprecipitation with hexane. Polyester contents in dried cells were in the range of 15—20 wt%.

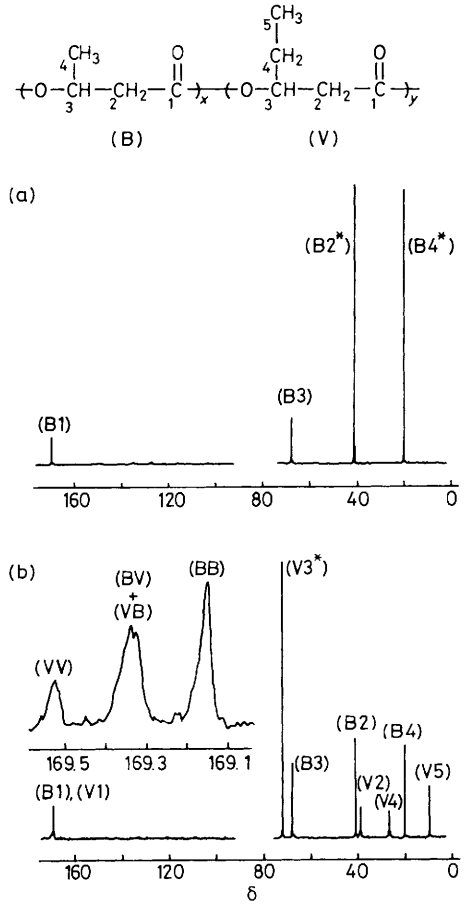
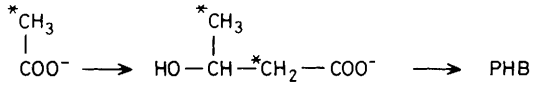


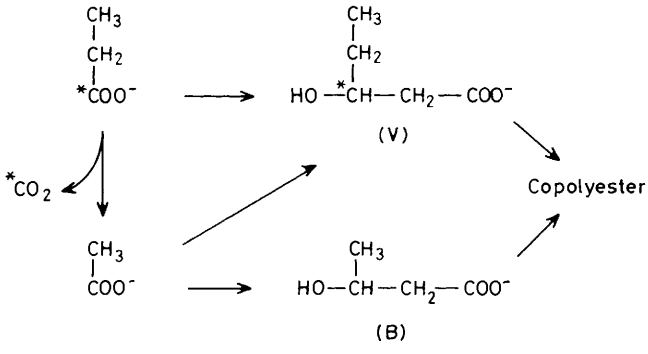

**Figure 1.** 125 MHz Proton-noise-decoupled $^{13}C$ n.m.r. spectra of polyesters in $CDCl_3$ at 27 °C, derived biosynthetically from (a) [2-$^{13}C$]acetate (6% $^{13}C$) and (b) [1-$^{13}C$]propionate (10% $^{13}C$) Spectral parameters; repetition time 5 s, 25 000 Hz, spectral width, 32K data points, and 2000 accumulations.

The 125 MHz $^{13}C$ n.m.r. spectra of chloroform solutions of the isolated polyesters were recorded on a JEOL GX-500 spectrometer and the chemical shift assignments[3] are shown in Figure 1. Poly(3-hydroxybutyrate) (PHB) was isolated from the cells grown in medium A, and the copolyester containing both 3-hydroxybutyrate (B) and 3-hydroxyvalerate (V) units was obtained from the cells grown in medium B. The dyad sequence distribution of (B) and (V) units in the copolyester sample is $F_{(BB)} = 0.40$, $F_{(BV)} + F_{(VB)} = 0.48$, and $F_{(VV)} = 0.12$, determined from the three well-resolved peaks due to carbonyl resonance in Figure 1.

The $^{13}C$ n.m.r. spectrum shown in Figure 1(a) of the sample of PHB displays specific enhancements in the intensities of (B2) and (B4) resonances. The intensity ratios of (B2) and (B4) to the (B3) resonance were 5.5 ± 0.5, indicating that the $^{13}C$-labelled methyl carbon (6.0% $^{13}C$) of acetate is selectively




**Scheme 1**




**Scheme 2**

introduced into the specific sites (B2) and (B4) of PHB without scrambling to other carbons. The selective incorporation of the $^{13}C$-labelled carbon was also confirmed by the 500 MHz $^{1}H$ n.m.r. spectrum which showed a fine structure due to the $^{13}C$–H splitting in both methylene (B2) and methyl (B4) proton resonances. It was determined from the $^{1}H$ n.m.r. spectrum that $^{13}C$ populations in (B2) and (B4) were about 6%. These results support the biosynthetic pathway of PHB from acetate *via* 3-hydroxybutyrate,[5,6] as represented by Scheme 1.

Addition of sodium [1-$^{13}C$]propionate (10% $^{13}C$) to *A. eutrophus* produces a random copolyester whose $^{13}C$ n.m.r. spectrum in Figure 1(b) shows a specific enhancement for the resonance of (V3) in (V) units. The relative intensity of the (V3) to (V4) resonance was $9 \pm 1$, indicative of the selective incorporation of the $^{13}C$-labelled carbonyl carbon of propionate into the (V3) site of the copolyester. The 500 MHz $^{1}H$ n.m.r. spectra of the copolyester sample showed that there was no significant labelling on other carbons except for (V3). When propionate is used as the sole carbon source, 3-hydroxybutyrate is formed from two molecules of acetate which is generated by the elimination of $^{13}C$-labelled carbonyl from propionate, and 3-hydroxyvalerate is formed by the reaction of propionate with the acetate, resulting in the formation of a random copolyester under the action of PHB polymerase (see Scheme 2).

The use of $^{13}C$-labelled acetate and propionate as carbon sources is of practical importance in the synthesis of polyesters specifically-labelled using $^{13}C$, as well as in determining the biosynthetic pathway.

*Received, 28th July 1986; Com. 1059*

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