Biosynthesis of an Unusual Copolyester (10 mol % 3-Hydroxybutyrate and 90 mol % 3-Hydroxyvalerate Units) in *Alcaligenes eutrophus* from Pentanoic Acid

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Bacterial poly(3-hydroxybutyrate-co-3-hydroxyvalerate) esters with a wide range of compositions (0—90 mol % 3-hydroxyvalerate units) have been isolated from *Alcaligenes eutrophus* grown in nitrogen-free culture media containing pentanoic acid and glucose.

Optically active copolyesters of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) have recently been introduced by Imperial Chemical Industries as biotechnological products with thermoplastic properties.[1] The copolyesters were isolated from *Alcaligenes eutrophus* grown in culture media containing glucose and propionic acid,[2] and a range of product compositions was found varying from 0 to 47 mol % HV.[2,3] The copolyesters have been shown to have a statistically random distribution of HB and HV units.[4,5] Recently we investigated the biosynthetic pathway of the copolyester in *A. eutrophus* by using ^{13}C-labelled propionic acid, and concluded that the proportion of HV units is kept to less than *ca.* 50 mol % by a relatively fast metabolic pathway from propionyl- to acetyl-coenzyme A in the cells.[6,7] We now report a controlled fermentation for the production of copolyesters with unusually high proportions of HV units (up to 90 mol %), using pentanoic acid (valeric acid) as carbon source for *A. eutrophus*.

A. eutrophus (NCIB 11599) was grown at 30 °C in a nutrient-rich medium (100 cm^3) containing yeast extract, polypeptone, meat extract, and $(NH_4)_2SO_4$. After 24 h the cells were harvested by centrifugation and washed with water. Under these culture conditions, no accumulation of polyesters in the cells was observed. To promote polyester synthesis, 0.2—0.3 g quantities of the washed cells were transferred into a nitrogen-free mineral medium[8] containing pentanoic acid and glucose. The cells were cultivated in the nitrogen-free medium (100 cm^3, pH 7.0) for 48 h at 30 °C, harvested by centrifugation, washed with acetone, and finally dried *in vacuo* at room temperature. Polyesters were extracted from the dried cells with hot chloroform in a Soxhlet apparatus, and purified by reprecipitation with hexane. The ^1H and ^{13}C n.m.r. analyses of the polyester samples were carried out on a JEOL GX-500 spectrometer.

Table 1 lists the results on the biosynthesis of polyesters. The polyester content in the dried cells ranged between 17 and 54 wt %, depending on the mixture of carbon sources in the nitrogen-free culture media. The ^1H n.m.r. spectra of the

Table 1. Biosynthesis of polyesters in *Alcaligenes eutrophus* (NCIB 11599) at 30 °C.

Carbon source[a]/g		Polyester	Polyester composition[c]/mol %				
Bu^nCO_2H	Glucose	content[b]/wt %	F_B	F_V	$[\eta]$[d]/dl g^{-1}	M.p.[e]/°C	ΔH_m[f]/cal g^{-1}
2.0	0	36	10	90	4.0	108	13.8
2.0	0.05	24	27	73	2.7	106	9.9
2.0	0.10	17	39	61	2.0	104	6.9
0	2.0	54	100	0	4.4	178	21.7

[a] Carbon source in nitrogen-free culture media (100 cm^3). [b] Polyester content in dry cells. [c] Determined from ^1H n.m.r. spectra. B and V represent 3-hydroxybutyrate and 3-hydroxyvalerate units, respectively. [d] The intrinsic viscosity $[\eta]$ of the polyester, measured in chloroform at 30 °C. [e] Melting temperature was measured at 10 °C min^{-1}. [f] Enthalpy of fusion (1 cal = 4.184 J).

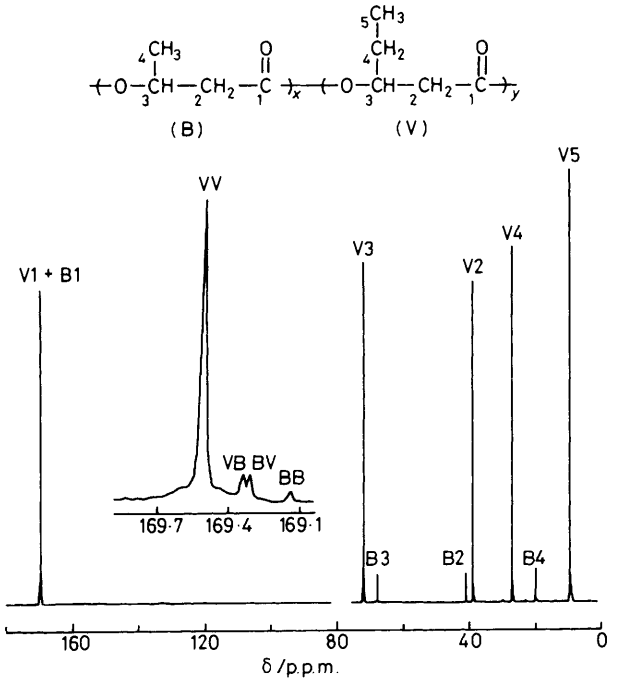
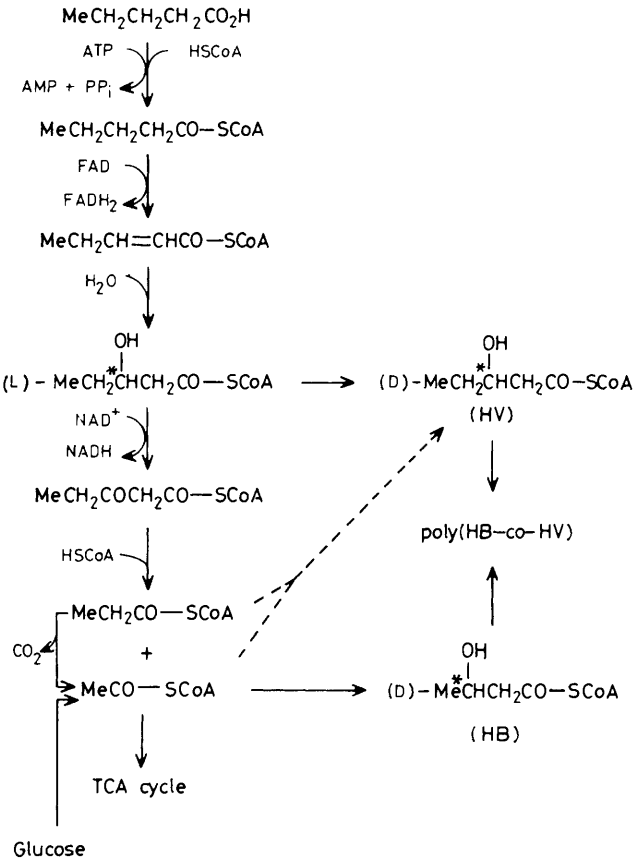


Figure 1. 125 MHz Proton-noise-decoupled ^{13}C n.m.r. spectrum of the copolyester derived biosynthetically from pentanoic acid, measured at 27 °C in $CDCl_3$. Spectral parameters: repetition time, 5 s; spectral width, 25 000 Hz; 64 K data points; 6300 accumulations.



Scheme 1. Biosynthetic pathway to the copolyester containing HB and HV units, in *Alcaligenes eutrophus* from pentanoic acid.

isolated polyesters showed that the polymers are composed of two monomeric units, HB and HV. The mole fractions of HB and HV units, F_B and F_V, in the copolyester samples were determined from the ^1H n.m.r. spectra, based on the intensity ratio of the methyl resonances in the side groups of the units.[3] When pentanoic acid was used as the sole carbon source, a copolyester with an unusually high proportion of HV units (90 mol %) was produced. Figure 1 shows the ^{13}C n.m.r. (125 MHz) spectrum of the copolyester in chloroform, together with the chemical shift assignments.[4,5] The carbonyl resonances (δ 169.1—169.6) were clearly resolved into four peaks, arising from the different diad sequences of the HB and HV units. The peak at δ 169.14 was assigned to the carbonyl resonance in the BB sequence, since its chemical shift is consistent with that of the carbonyl resonance in the poly(3-hydroxybutyrate), (δ 169.16). The other three peaks at δ 169.31, 169.34, and 169.51 were assigned to BV, VB, and VV diad sequences, respectively.[4] The diad sequence distribution of HB and HV units in the copolyester is F_{BB} 0.03, $F_{BV} + F_{VB}$ 0.12, and $F_{VV} = 0.85$, determined from the well resolved peaks of carbonyl resonance.

The addition of small amounts of glucose into the pentanoic acid culture solution results in a decrease in the fraction of HV units from 90 to 61 mol %. Only poly(3-hydroxybutyrate) (PHB) is produced in the culture containing glucose as the sole carbon source. Therefore, it is concluded that the copolyesters with a wide range of compositions up to 90 mol % HV units can be produced in *A. eutrophus* using pentanoic acid and glucose mixtures.

We propose the following biosynthetic pathway to the copolyester from pentanoic acid in *A. eutrophus* (as shown in Scheme 1). Pentanoic acid is transported in the cells and metabolised to L-3-hydroxyvaleryl-CoA (CoA = coenzyme A) in the β-oxidation cycle by the action of specific enzymes. Some of the L-3-hydroxyvaleryl-CoA is epimerized into D-3-hydroxyvaleryl-CoA which is then incorporated as HV units in copolyesters by the action of PHB polymerase.[9] The remaining L-3-hydroxyvaleryl-CoA is degraded into acetyl- and propionyl-CoA. When the concentration of propionyl-CoA is low, most is further metabolised to acetyl-CoA.[7] Some of the acetyl-CoA is metabolised as an energy source in the tricarboxylic acid (TCA) cycle, and the rest is used for the formation of D-3-hydroxybutyryl-CoA which is incorporated as HB units in the copolyesters. The addition of glucose leads to an increase in the proportion of HB units in the copolyesters.

In conclusion, the use of pentanoic acid as a carbon source is of practical importance in the production of bacterial copolyesters with high proportions of HV units.

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