Nuclear Magnetic Resonance Studies on Poly(β-hydroxybutyrate) and a Copolyester of β-Hydroxybutyrate and β-Hydroxyvalerate Isolated from *Alcaligenes eutrophus* H16

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ABSTRACT: Poly(β-hydroxybutyrate) (PHB) and a copolyester of β-hydroxybutyrate and β-hydroxyvalerate are isolated from *Alcaligenes eutrophus* H16 grown in nitrogen-free culture media containing CH_3COONa and CH_3CH_2COONa as carbon sources. The chain dynamics of PHB ($\bar{M}_w$ = 420 000) in chloroform are studied by ^{13}C NMR measurement at 25 MHz. The carbon-13 spin–lattice relaxation times (T_1) and nuclear Overhauser enhancements (NOE) indicate that the average correlation times (τ_c) for segmental motion of the PHB molecule are in the range (6–8) × 10^{-11} s at 27 °C, as expected for flexible linear polymers. The conformational structure generated by rotation about CH_2–CH bonds of PHB is determined in chloroform and dimethylformamide over a temperature range of 20–100 °C by analysis of vicinal coupling in the 500-MHz ^1H NMR spectra. In both solvents the gauche (G) and trans (T) conformers are found to exist predominantly, the $\bar{G}$ conformer being suppressed. The sequence distributions of the monomeric units in the copolyester of β-hydroxybutyrate and β-hydroxyvalerate are determined by analysis of 125-MHz ^{13}C NMR spectra. The diad and triad sequence distributions are accounted for in terms of first-order Markov statistics.

Introduction

Poly(β-hydroxybutyrate) (PHB) is a highly crystalline and optically active polyester, which is synthesized by many bacteria.[1] Recently, this thermoplastic biopolymer has attracted industrial attention as a possible candidate for large-scale biotechnological production,[2,3] since PHB has a high tensile strength, comparable to that of isotactic polypropylene, and is completely biodegradable to an innocuous compound, D-(−)-3-hydroxybutyric acid. In addition, it has recently been found that some bacteria produce a copolyester of β-hydroxybutyrate and β-hydroxyvalerate.[4,5]

The crystal structure of optically active PHB in the solid state was studied by Cornibert and Marchessault[6] and Yokouchi et al.,[7] who indicated a left-handed 2_1 helix for the PHB molecule in crystalline regions. The conformational structure of the PHB molecule in solution was investigated by Marchessault et al.[8,9] and then by Akita et al.[10] by means of viscometry, light scattering, and optical rotatory dispersion. Marchessault et al.[8] found a sharp helix–coil transition for PHB in solution by varying the temperature or the solvent composition. They concluded that the helical conformation of PHB is retained in chloroform and ethylene dichloride but that the conformation of PHB becomes randomly coiled in solvents such as dimethylformamide and dichloroacetic acid.[8,9] However, no appreciable helix–coil transition was observed by Akita et al.,[10] who questioned whether the helical structure of PHB is retained in solution. Recently, we analyzed the conformational structure of PHB in chloroform on the basis of vicinal coupling in the 500-MHz ^1H NMR spectra and showed the predominance of the gauche (G) and trans (T) conformers of the CH_2–CH bonds of the backbone.[11]

In this paper, we have measured the carbon-13 spin–lattice relaxation times and nuclear Overhauser enhancements of each carbon in PHB to study the chain dynamics. Secondly, the conformational preferences of the PHB molecule have been determined in chloroform and dimethylformamide by analysis of the 500-MHz ^1H NMR spectra. Finally, we have isolated an interesting copolyester of β-hydroxybutyrate and β-hydroxyvalerate from *Alcaligenes eutrophus* H16 and have determined the sequence distribution of monomeric units by analysis of the 125-MHz ^{13}C NMR spectra.

Experimental Section

Biopolymer Synthesis. The PHB and copolyester samples were isolated from *Alcaligenes eutrophus* H16 (ATCC 17699). The strain H16 was maintained on nutrient agar slants at 4 °C by monthly subculture. The bacteria were first grown at 30 °C in the nutrient-rich medium (100 cm^3) containing 10 g/dm^3 of yeast extract, 10 g/dm^3 of polypeptone, 5 g/dm^3 of meat extract, 10 g/dm^3 of fructose, and 5 g/dm^3 of NaCl. The cells were harvested by centrifugation after 24 h, corresponding to the end of exponential growth, and washed with water. Under these culture conditions accumulation of PHB in the cells was not observed. To promote PHB synthesis, 0.20–0.25-g quantities of the washed cells were transferred into a nitrogen-free mineral medium[12] (100 cm^3) containing organic acids as carbon sources. Three different compositions of organic acids were used: medium A, 22 g/dm^3 of CH_3COONa; medium B, 22 g/dm^3 of CH_3COONa and 10 g/dm^3 of CH_3CH_2COONa; medium C, 22 g/dm^3 of CH_3CH_2COONa. The cells were cultivated in the nitrogen-free media for 48 h at 30 °C, harvested by centrifugation, washed with acetone, and finally dried under vacuum at room temperature.

PHB and copolyester were extracted from the dried cells with hot chloroform in a Soxhlet apparatus and precipitated by the slow addition of diethyl ether to the chloroform extract. The precipitate was redissolved in chloroform and the polymers were again precipitated with ether and then dried under vacuum. Table I shows the weight of dry cells and polyester content, together with the composition and melting temperature of isolated polyester. PHB (sample 1) was isolated from cells grown in nitrogen-free medium A, and copolyesters (samples 2 and 3) of β-hydroxybutyrate and β-hydroxyvalerate were isolated from cells grown in media B and C.

Analytical Procedures. The ^1H and ^{13}C NMR analyses of the polymer samples were carried out on a JEOL GX-500 spectrometer in the pulse Fourier trnansform (FT) mode. The 500-MHz ^1H NMR spectra were recorded at 20–100 °C using $CDCl_3$ or $DCON(CD_3)_2$ solutions of polyester at a concentration of 0.01 g/cm^3 with 6.0-s pulse repetition, 5000-Hz spectral width, 32K data points, and 100 accumulations. The 125-MHz ^{13}C NMR spectra were recorded at 27 °C on a $CDCl_3$ solution of the polyester (0.05 g/cm^3) with 5.0-s pulse repetition, 25000-Hz spectral width, 64K data points, and 10 000 accumulations. The ^{13}C NMR T_1 measurements were made on a JEOL FX-100 spectrometer operating at 25 MHz using the standard inversion–recovery (π–τ–$\pi/2$–T) pulse sequence. Each T_1 value was obtained at 27 °C by using eight τ values and the peak areas. The value of T was greater than $5T_1$ for either carbon. The ^{13}C–^1H NOE value of each ^{13}C resonance was determined by direct comparison of peak areas obtained with complete ^1H decoupling to the corresponding areas

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Table I
Culture Conditions and Properties of Polyesters Isolated from *Alcaligenes eutrophus* H16

sample	carbon source[a]	cell dry wt, g	polyester content,[b] wt %	polyester compn,[c] mol % F_B	F_V	T_m,[d] °C
1	CH_3COONa (A)	0.44	53	100	0	179
2	CH_3COONa + CH_3CH_2COONa (B)	0.49	51	81	19	149
3	CH_3CH_2COONa (C)	0.38	35	57	43	79

[a] Carbon source in nitrogen-free culture media A, B, and C (see Experimental Section). [b] Polyester content in dry cells. [d] Determined from ^1H NMR spectra. B and V represent β-hydroxybutyrate and β-hydroxyvalerate units, respectively. [d] Melting temperature (T_m) measured at 10 °C/min.

Table II
T_1 Values, Nuclear Overhauser Enhancements (NOE), Average Correlation Times (τ_c), and Chemical Shift Assignments for ^{13}C Resonances of PHB at 25 MHz in Chloroform at 27 °C

^{13}C resonance	δ ^{13}C[a]	T_1, s	NOE	τ_c, s
CH_3	19.79	0.65	2.5	2.5×10^{-11}
CH_2	40.82	0.37	2.9	6.6×10^{-11}
CH	67.64	0.63	3.0	7.8×10^{-11}
C=O	169.16	n.d.	n.d.	

[a] Chemical shifts are in ppm downfield from Me_4Si.

obtained with gated ^1H decoupling. Each spectrum for T_1 and NOE measurements was recorded with a 5000-Hz spectral width, 8K data points, and 1200 accumulations.

The melting temperatures (T_m) of polymer samples were recorded o a Shimadzu DSC-30. Polymer samples of 5–10 mg were encapsulated in aluminum pans and heated at 10 °C/min to 200 °C.

The weight-average molecular weight $\bar{M}_w$ of the PHB sample was determined by the relation[8]

$$[\eta] = 7.7 \times 10^{-5} \bar{M}_w^{0.82} \quad (1)$$

where $[\eta]$ is the intrinsic viscosity in chloroform at 30 °C.

Results and Discussion

Chain Dynamics of PHB. The ^{13}C NMR spectrum of PHB (sample 1, $\bar{M}_w$ = 420 000) was recorded at 125 MHz and 27 °C in chloroform. Each peak of PHB was very sharp and only four lines were observed (see Table II). The spectrum was identical with the spectrum of PHB ($\bar{M}_w$ = 120 000) isolated from *Bacillus megaterium* and reported in a previous paper.[11] Here, it is noted that the structure of PHB is independent of the source of bacteria and is represented by an isotactic polyester with a regular head-to-tail sequence of D-(−)-3-hydroxybutyrate units:

$$-(OCH(CH_3)CH_2C(O))_n- \quad (2)$$

The ^{13}C NMR spin–lattice relaxation times (T_1's) for methyl, methylene, and methine carbons of PHB were determined at 25 MHz in chloroform. The results are given in Table II, together with the NOE values. Maximum values of NOE are observed for methine and methylene carbons at 27 °C. The NT_1 values of the methine and methylene carbons are not equal in PHB, suggesting that segmental motion is anisotropic. However, if we assume conditions of extreme narrowing and isotropic motion, the average correlation time (τ_c) and NOE can be represented by[13]

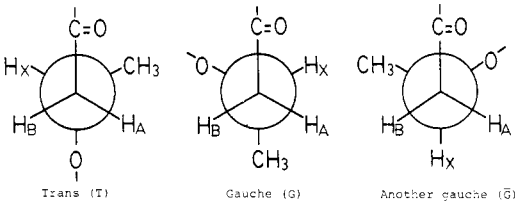


Figure 1. Newman projections of the three rotational isomeric states.

$$\tau_c = \frac{\gamma_H^2 \gamma_C^2 h^2}{N T_1 r_{CH}^6} = \frac{4.92 \times 10^{-11}}{N T_1} \quad (3)$$

and

$$\text{NOE} = 1 + \gamma_H / 2\gamma_C = 2.988 \quad (4)$$

where γ_C and γ_H are the gyromagnetic ratios for ^{13}C and ^1H, respectively, r_{CH} is the C–H bond length (taken as 0.110 nm), and N is the number of directly bonded protons. The T_1 and NOE data in Table II indicate that the values of τ_c for segmental motion are in the range of (6–8) × 10^{-11} s at 27 °C. The τ_c value is similar to that for isotactic polypropylene under comparable conditions,[14] i.e., the value expected for a flexible linear polymer. These results indicate that the PHB molecule is not rigid but rather flexible in chloroform.

Conformational Structure of PHB. The conformational structure generated by rotation about CH_2–CH bonds was determined by analysis of the methylene (CH_2) proton resonance of PHB (sample 1) in the ^1H NMR spectra at 500 MHz. As reported in a previous paper,[11] the methylene proton resonance at 2.45–2.65 ppm was associated with the methine proton (H_X) and was analyzed as ABX type with vicinal coupling of H_A and H_B protons. The resulting parameters at 20–100 °C are listed in Table III for PHB in chloroform and dimethylformamide. PHB was dissolved in dimethylformamide at temperatures above 70 °C where ^1H NMR spectra could be recorded. The observed vicinal couplings are averaged over the three possible conformers: trans (T), gauche (G), and another gauche (Ḡ), as shown in Figure 1. Then the observed coupling constants J_{AX} and J_{BX} are represented by average

Table III
Parameters of Methylene Proton Resonance in 500-MHz ^1H NMR Spectra of PHB and Conformational Structure of CH_2–CH Bonds

solvent	temp, °C	δ ^1H[a] H_A	H_B	coupling constant, Hz J_{AB}	J_{AX}	J_{BX}	$(J_{AX} + J_{BX})$	conformer fraction P_T	P_G	$P_{\bar{G}}$
$CDCl_3$	20	2.474	2.607	−15.6	5.8	7.3	13.1	0.41	0.59	0.00
$CDCl_3$	40	2.466	2.600	−15.6	5.8	7.3	13.1	0.41	0.59	0.00
$CDCl_3$	60	2.460	2.596	−15.6	6.1	7.0	13.1	0.45	0.55	0.00
DMF[b]	80	2.577	2.636	−15.6	6.1	7.0	13.1	0.45	0.55	0.00
DMF[b]	100	2.572	2.622	−15.6	6.1	7.0	13.1	0.45	0.55	0.00

[a] Chemical shifts are in ppm downfield from Me_4Si. [b] *N,N*-Dimethylformamide-d_7 ($DCON(CD_3)_2$).

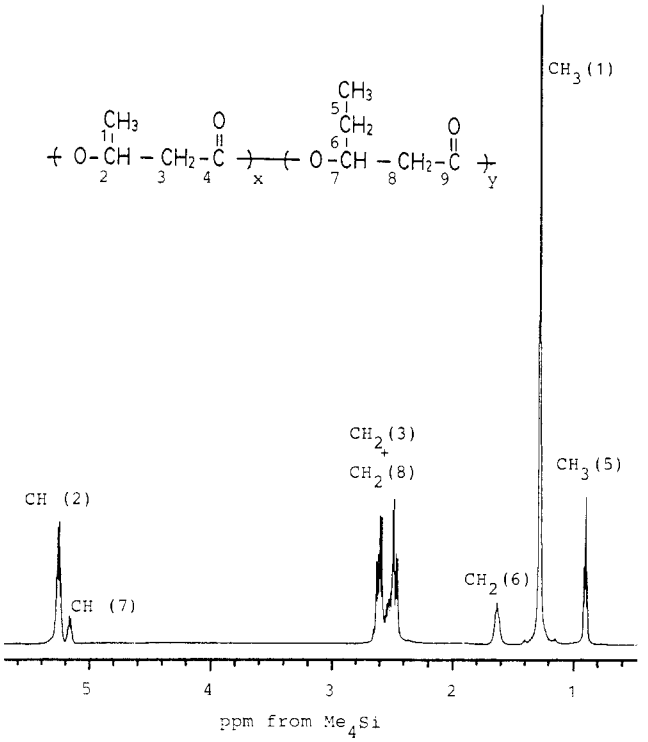


Figure 2. 500-MHz ^{1}H NMR spectrum of a copolyester (sample 2) containing β-hydroxybutyrate and β-hydroxyvalerate units at 27 °C in chloroform. Chemical shifts are in ppm downfield from Me_4Si.

values of the component coupling constants in the three conformers weighted by their fractional populations P_T, P_G, and $P_{\bar{G}}$:

$$J_{AX} = P_T J_t + P_G J_g + P_{\bar{G}} J_g \quad (5)$$

$$J_{BX} = P_T J_g + P_G J_t + P_{\bar{G}} J_g \quad (6)$$

$$1 = P_T + P_G + P_{\bar{G}} \quad (7)$$

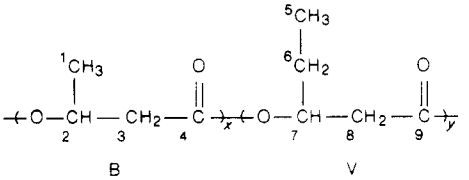
J_g and J_t are the gauche and trans vicinal coupling constants. The fractional populations can be determined by the relations

$$P_G - P_T = \frac{J_{AX} - J_{BX}}{J_g - J_t} \quad (8)$$

$$P_{\bar{G}} = \frac{(J_{AX} + J_{BX}) - (J_g + J_t)}{J_g - J_t} \quad (9)$$

The observed values, $J_{AX} + J_{BX} = 13.1$ Hz, are independent of both temperature and solvent, suggesting that the $\bar{G}$ conformer is strongly disfavored by energy and that the fraction $P_{\bar{G}}$ is very small. The parameters J_g and J_t for the fragment studied are not known. Here, we estimated the fractional populations P_T, P_G, and $P_{\bar{G}}$, assuming the reasonable values of $J_g = 2.1$ Hz and $J_t = 11.0$ Hz.[15] The result is given in Table III. It can be concluded that the G and T conformers predominate in both chloroform and dimethylformamide. This conclusion is in good agreement with the result by energy calculation,[6,7] which indicated a left-handed 2_1 helix of G conformer units for the solid state of PHB.

Sequence Distribution of Copolyester. Copolyesters of β-hydroxybutyrate and β-hydroxyvalerate were isolated from *Alcaligenes eutrophus* H16 grown in nitrogen-free culture media containing CH_3CH_2COONa as a carbon source (samples 2 and 3 in Table I). Figure 2 shows the 500-MHz ^{1}H NMR spectrum of sample 2 in chloroform, which indicates that the polymer contains two monomeric units of B and V:



(10)

For simplification, β-hydroxybutyrate and β-hydroxyvalerate units are represented by B and V, respectively. The mole fractions of the two monomeric units were determined from the intensity ratio of the doublet CH_3-proton resonance 1 at 1.274 ppm to the triplet CH_3-proton resonance 5 at 0.894 ppm. The result is given in Table I. The mole fraction of V units increased with an increase in the fraction of CH_3CH_2COONa to CH_3COONa in the nitrogen-free culture media.

Figure 3 shows the proton-noise-decoupled 125-MHz ^{13}C NMR spectrum of sample 2 in chloroform, together with the chemical shift assignment for each ^{13}C resonance. As expanded in Figure 4, carbonyl and methylene carbon

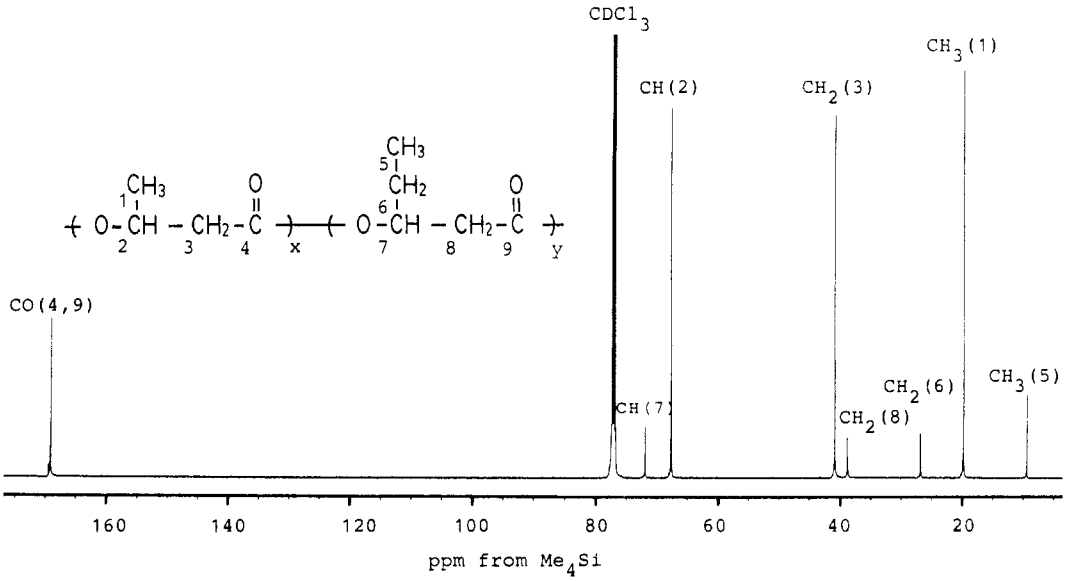


Figure 3. Proton-noise-decoupled 125-MHz ^{13}C NMR spectrum of sample 2 in chloroform at 27 °C. Chemical shifts are in ppm downfield from Me_4Si.

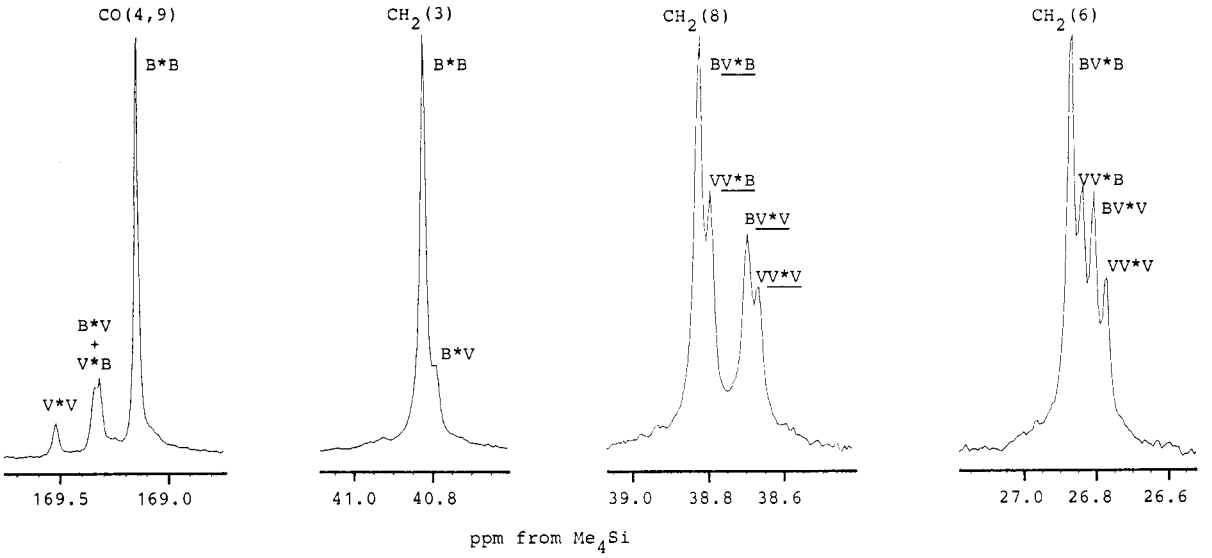


Figure 4. Expanded spectra for carbonyl and methylene carbon resonances of sample 2. The ^{13}C chemical shift assignments are given in Table IV.

Table IV
^{13}C Chemical Shifts for Carbonyl and Methylene Carbons in Copolyester of β-Hydroxybutyrate and β-Hydroxyvalerate

species	^{13}C chem shift,[a] ppm	rel intensity[b] sample 2	rel intensity[b] sample 3	sequence[c]
$CH_2(6)$	26.77	0.11	0.19	VV*V
	26.81	0.23	0.27	BV*V
	26.84	0.21	0.27	VV*B
	26.87	0.45	0.27	BV*B
$CH_2(8)$	38.67	0.12	0.22	(V)V*V
	38.70	0.22	0.25	(B)V*V
	38.80	0.20	0.25	(V)V*B
	38.83	0.46	0.28	(B)V*B
$CH_2(3)$	40.80	0.15	0.43	B*V
	40.83	0.85	0.57	B*B
CO(4,9)	169.14	0.67	0.33	B*B
	169.32	0.26	0.46	B*V+V*B
	169.52	0.07	0.21	V*V

[a] Downfield from internal Me_4Si at 27 °C in chloroform. [b] Determined from peak areas. [c] B and V represent β-hydroxybutyrate and β-hydroxyvalerate units, respectively.

resonances split into several peaks, which reflects the sensitivity of the carbon nuclei to different sequences of B and V units. In addition, methyl and methine carbon resonances split into two or three peaks which were not well resolved. In this study, we have made the ^{13}C chemical shift assignment for the well-resolved carbonyl and methylene carbon resonances. The result is summarized in Table IV.

The carbonyl resonances in the ^{13}C NMR spectra of samples 2 and 3 were clearly resolved into three peaks, arising from different diad sequences of connecting B and V units. The peak at 169.14 ppm is assignable to the carbonyl resonance in the BB sequence, since its chemical shift is consistent with that (169.16 ppm) of the carbonyl resonance in the PHB homopolymer. Previously, Iida at al.[16] reported ^{13}C NMR spectra of PHB and PHV homopolymers and found a difference in the chemical shifts of the carbonyl resonances of 0.38 ppm for the homopolymers. Therefore, the peak at 169.52 ppm is assigned to the carbonyl resonance in the VV sequence, and the other peak at 169.32 ppm is assigned to carbonyl resonances in the BV and VB sequences. The resonance of methylene carbon 3 in the B units splits into two peaks at 40.80 and 40.83 ppm, which are respectively assignable to methylene resonances in the BV and BB diad sequences. In contrast, the resonance of the main-chain methylene carbon 8 in the V units is composed of four peaks. The relative areas of the four peaks are in good agreement with those of the four peaks for the resonance of side-chain methylene carbon 6 in the V units. Those four peaks in the methylene resonances may be assigned to triad sequences of VVV, BVV, VVB, and BVB, as shown in Table IV. The relative intensities for each ^{13}C resonance are

Table V
Diad and Triad Sequence Distributions of B and V Units in Copolyesters

	sample 2						sample 3					
	^1H NMR	^{13}C NMR					^1H NMR	^{13}C NMR				
	CH_3	CO	$CH_2(3)$	$CH_2(6)$	$CH_2(8)$	calcd[a]	CH_3	CO	$CH_2(3)$	$CH_2(6)$	$CH_2(8)$	calcd[a]
F_B	0.81		0.81			0.81	0.57		0.56			0.56
F_V	0.19			(0.19)	0.19	0.19	0.43			(0.44)	0.44	0.44
F_{BB}		0.67	0.69			0.67		0.33	0.32			0.32
F_{BV}		0.26	0.12			0.14		0.46	0.24			0.24
F_{VB}					0.13	0.14					0.23	0.24
F_{VV}		0.07			0.06	0.05		0.21			0.21	0.20
F_{VVV}				0.02	0.02	0.01				0.08	0.10	0.09
F_{BVV}				0.04	0.04	0.04				0.12	0.11	0.11
F_{VVB}				0.04	0.04	0.04				0.12	0.11	0.11
F_{BVB}				0.09	0.09	0.10				0.12	0.12	0.13

[a] Calculated with the values of p_{ij} in Table VI.

Table VI
Four Conditional Probabilities p_{ij} and Reactivity Ratio Product r_1r_2

sample	p_{11}[a]	p_{12}[a]	p_{21}[a]	p_{22}[a]	r_1r_2[b]
2	0.83	0.17	0.75	0.25	1.6
3	0.57	0.43	0.55	0.45	1.1

[a] The estimated errors in the values of p_{ij} are <0.03. [b] $r_1 = k_{11}/k_{12}$ and $r_2 = k_{22}/k_{21}$.

determined from the peak areas, which are listed in Table IV. Table V gives the diad and triad sequence distributions of B and V units in samples 2 and 3, determined from the relative intensities of carbonyl and methylene carbon resonances.

The observed sequence distributions of monomeric units may be interpreted in terms of the statistics of a binary copolymerization involving the following propagation steps:

$$\text{---B}^* + \text{B–CoA} \xrightarrow{k_{11}} \text{---BB}^* + \text{CoA} \quad (11)$$

$$\text{---B}^* + \text{V–CoA} \xrightarrow{k_{12}} \text{---BV}^* + \text{CoA} \quad (12)$$

$$\text{---V}^* + \text{B–CoA} \xrightarrow{k_{21}} \text{---VB}^* + \text{CoA} \quad (13)$$

$$\text{---V}^* + \text{V–CoA} \xrightarrow{k_{22}} \text{---VV}^* + \text{CoA} \quad (14)$$

It has been suggested that the biosynthesis of PHB takes place by the condensation of D-(−)-3-hydroxybutyryl coenzyme A with PHB primer in the presence of PHB synthetase.[17-20] In the above scheme, CoA is coenzyme A, k_{ij} is the rate constant of step ij, and the subscripts 1 and 2 refer to B and V units, respectively. Since the monomer concentrations of B–CoA and V–CoA in a cell are not known, we use here the conditional probability p_{ij} of step ij with the relation that $p_{11} + p_{12} = 1$ and $p_{21} + p_{22} = 1$, as represented by

$$p_{11} = k_{11}[\text{B–CoA}]/(k_{11}[\text{B–CoA}] + k_{12}[\text{V–CoA}]) \quad (15)$$

$$p_{22} = k_{22}[\text{V–CoA}]/(k_{21}[\text{B–CoA}] + k_{22}[\text{V–CoA}]) \quad (16)$$

Assuming a first-order Markovian process for the above copolymerization, one can express lifferent diad and triad fractions of B and V units with four conditional probabilities:[21]

$$F_{BB} = p_{21}p_{11}/(p_{12} + p_{21}) \quad (17)$$

$$F_{BV} = F_{VB} = p_{12}p_{21}/(p_{12} + p_{21}) \quad (18)$$

$$F_{VV} = p_{12}p_{22}/(p_{12} + p_{21}) \quad (19)$$

$$F_{VVV} = p_{12}p_{22}p_{22}/(p_{12} + p_{21}) \quad (20)$$

$$F_{VVB} = F_{BVV} = p_{12}p_{21}p_{22}/(p_{12} + p_{21}) \quad (21)$$

$$F_{BVB} = p_{21}p_{12}p_{21}/(p_{12} + p_{21}) \quad (22)$$

Combining the above relations with the observed sequence distributions, we determined the four conditional probabilities p_{ij}. The values of p_{ij} are listed in Table VI, together with the reactivity ratio product, r_1r_2. For comparison, the diad and triad distributions calculated with the values of p_{ij} are given in Table V. The calculated diad and triad distributions are in agreement with the observed distributions for both samples 2 and 3, confirming that the present copolymerization is accounted for in terms of first-order Markov statistics. The observed values of the reactivity ratio product r_1r_2 are close to unity, as expected for an ideal random copolymerization.

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Registry No. PHB (SRU), 26744-04-7; PHB (homopolymer), 26063-00-3; (B)(V) (copolymer), 80181-31-3; B, 300-85-6; V, 10237-77-1.

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