Biosynthesis and Characterization of Poly(β-hydroxyalkanoates) Produced by *Pseudomonas oleovorans*

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ABSTRACT: The biosynthesis of poly(β-hydroxyalkanoates) (PHA's) by *P. oleovorans* was carried out by using the sodium salt of *n*-alkanoic acids as carbon sources. The PHA's produced contained at least two major (>5 mol %) monomer units. That is, depending on the carbon source used, the PHA's can have *n*-alkyl pendant groups with chain lengths from methyl to nonyl. The maximum cellular yield and polymer content (in percent of the cellular dry weight) obtained were 1.5 g/L and 49%, respectively, using nonanoate as the carbon source. The $\bar{M}_w$ values of these poly-β-esters measured by GPC ranged from 160 000 to 360 000. Using the carbon sources sodium caproate and sodium heptanoate, *P. oleovorans* produced PHA's with approximately twice the degree of polymerization relative to the polymers biosynthesized from sodium octanoate and sodium nonanoate, even though the latter carbon sources resulted in significantly higher cell yields and cellular PHA content. ^{1}H and ^{13}C NMR spectra of the isolated PHA's were in agreement with the expected structure of these poly-β-esters. Studies by differential scanning calorimetry and X-ray diffraction showed that the PHA's containing predominantly longer side chains (*n*-pentyl or greater) were crystalline polymers (T_m from 45 to 61 °C, ΔH_m up to 8.3 cal/g), whereas those which contained mostly *n*-propyl or *n*-butyl side chains showed little tendency to crystallize. The X-ray diffraction patterns of the longer side chain PHA's suggest that they crystallize with participation of both the main and side chains in a layered packing order.

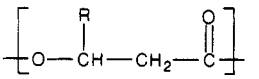
Introduction

Poly(β-hydroxyalkanoates) (PHA's) are a class of β-monoalkyl-substituted poly-β-esters which are naturally occurring in a wide variety of bacterial microorganisms throughout nature.[1-3] Bacterial PHA's function as intracellular carbon and energy storage materials.[4] Some types of bacteria, when grown under nutrient-limiting conditions, and in the presence of a sufficient supply of a carbon source, may accumulate this bacterial storage product so that up to 70% of the cellular dry weight is PHA.[5] The chain chiral centers have only the *R* stereochemical configuration[2,4] so that these polymers are isotactic and optically active.[6]

A great deal of research recently has been carried out on the microbial production and characterization of PHA copolymers which contain β-hydroxybutyrate (HB) and β-hydroxyvalerate (HV) monomer units.[7-14] These copolyesters, P(HB-*co*-HV), have been synthesized by using *Alcaligenes eutrophus*[7] and were shown by Bluhm et al.[9] to be statistically random and of high crystallinity (>60%) throughout a range of compositions varying from 0 to 47 mol % HV.

The isolation and compositional analyses of PHA from marine sediment,[1] freshwater algal mats,[2] and activated sewage sludge[3] have shown that PHA which accumulated in natural environments consists of mainly HB and HV monomer units, but smaller quantities of other monomer units with pendant groups as long as pentyl were reported in marine sediment.[1]

Work by Witholt and co-workers using *Pseudomonas oleovorans* showed that this microorganism is capable of growth and subsequent metabolism of *n*-alkanes to PHA's in two-phase liquid/liquid fermentations.[15,16] When *P. oleovorans* was grown under ammonium-limiting conditions on the substrates hexane through dodecane, PHA's were formed which, depending on the growth substrate used, contained variable amounts of the monomer units shown below:[16]



R = propyl, β-hydroxycaproate (HC)
R = butyl, β-hydroxyheptanoate (HH)
R = pentyl, β-hydroxyoctanoate (HO)
R = hexyl, β-hydroxynonanoate (HN)
R = heptyl, β-hydroxydecanoate (HD)
R = octyl, β-hydroxyundecanoate (HUD)
R = nonyl, β-hydroxydodecanoate (HDD)

Witholt and co-workers reported that when the organism was grown on octane and nonane, PHA's were formed which contained predominantly HO and HN, respectively.[15,16] It was, therefore, demonstrated by these workers that *P. oleovorans* is capable of producing very unusual PHA's which contain relatively long *n*-alkyl pendant groups.

In an attempt to overcome the technical difficulties and improve the efficiency of a biphasic alkane–water medium for PHA production by *P. oleovorans*, it seemed reasonable to study a homogeneous aqueous system which used the sodium salt of *n*-alkanoic acid carbon sources. In an earlier study, therefore, we established growth and culture conditions for PHA production by *P. oleovorans* using the sodium salts caproate, heptanoate, octanoate, nonanoate, and decanoate as carbon sources.[17] The monomer composition and molecular weight of the PHA's produced were determined and the metabolism of the *n*-alkanoate carbon sources to the various monomer units found in the product PHA's was discussed.

In this report results are presented, which were obtained by using *P. oleovorans*, on (1) further improved culture conditions for PHA production and (2) on characterization of the isolated PHA's by using gel permeation chromatography (GPC), ^{1}H and ^{13}C nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), and X-ray diffraction.

Experimental Section

Biosynthesis of PHA. The cells were grown under aerobic

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conditions as batch cultures using 10 mM concentrations (with the exception of sodium caproate, where a 20 mM concentration was used) of a sodium *n*-alkanoate carbon source in a 12-L temperature-controlled fermentor (New Brunswick; 30 °C, 2000 rpm, 2 L of air/min). A culture pregrown on 10 mM octanoate, 200 mL, was added aseptically as an inoculum. Samples were periodically withdrawn from the fermentor and the optical density was measured at 660 nm. The PHA content was determined qualitatively by observing the presence of visible, intracellular granules using a light microscope at a magnification of approximately 1200. Usually, these batch cultures were harvested after 24 h (method A).[18]

In order to increase the final yield of dry cells and the polymer content, ammonium phosphate (1.1 g/L), trace elements, and the growth substrate (same quantity as used initially) were added from sterile stock solutions. The cultures were grown for an additional 24 h (method B).

After reaching the early stationary growth phase, the cells were harvested by centrifugation (Sorvall RC2-B; 4 °C, 12,000xg). The pellets were resuspended and washed once with 10 mM Tris-HCl buffer (pH 7.5). The harvested cells were lyophilized and transferred to screw-cap glass vials which were stored in the freezer at −18 °C until further processing.

The following carboxylic acid sodium salt carbon sources were used in this study: caproate, heptanoate, octanoate, nonanoate, and decanoate. These compounds were purchased from Aldrich Chemical Co., in the highest purity available, as their corresponding carboxylic acids and used without any additional purification. These carboxylic acids were added to the medium followed by the addition of the appropriate quantity of 10 M sodium hydroxide, so that the final pH of the medium was 7.0.

PHA Isolation. Intracellular PHA was extracted from lyophilized cells into chloroform by using a Soxhlet extractor.[19] After a reflux period of 6 h, the chloroform solution was concentrated by using a rotary evaporator. The solution was then passed through a hot glass funnel containing a cotton plug into rapidly stirred methanol. The ratio of methanol to chloroform was 10:1. The precipitated polymer was separated by centrifugation (Sorvall RC2-B; 4 °C, 10000*g*), washed twice with methanol, and dried in vacuo (1 mmHg) for 16 h at room temperature. The resultant material was then dissolved in acetone, filtered again, and subsequently added dropwise into rapidly stirred methanol. The polymer was again separated by centrifugation and finally dried as described above. The total amount of PHA was determined gravimetrically and calculated as the percentage of cellular dry weight.

GC Assay. The polymer content of lyophilized whole cells (expressed as the percent of the cellular dry weight which contains PHA) and the repeating unit composition of the isolated PHA's were determined by the acid-catalyzed methanolysis of the PHA's to their constituent β-hydroxyalkanoic acid methyl esters. The methanolysis reaction was carried out in chloroform/methanol/sulfuric acid (1 mL/0.85 mL/0.15 mL) at 100 °C for 140 min following a procedure identical with that described previously.[16,17] The methyl esters were assayed by gas chromatography using a Perkin-Elmer 8500 GC equipped with a Durabond-Carbowax-M15 megabore capillary column (15 m × 0.54 mm; J&W Scientific) and a flame ionization detector.[20]

Identification and quantification of the β-hydroxyalkanoic acid methyl esters formed by methanolysis was possible after the synthesis of the corresponding β-hydroxyalkanoic acid methyl esters as described below. Standard curves were constructed for the synthesized methyl esters following the identical procedure which was carried out when analyzing lyophilized cells or isolated PHA samples, with the exception that known concentrations of the standard methyl esters are added to the chloroform/methanol/sulfuric acid solution and reacted for approximately zero time at ambient temperature.

Preparation of β-Hydroxyalkanoic Acids. The procedure used here for the reaction of the dilithio salt of acetic salt with *n*-alkyl aldehydes was similar to those found in the literature for the synthesis of other β-hydroxycarboxylic acids.[21] The reactions were carried out in flame-dried glassware under an argon atmosphere. All chemicals used in this preparation were obtained from the Aldrich Chemical Co. To a three-neck flask was transferred dry THF (distilled from sodium metal) followed by 14.0 mL (0.100 mol) of anhydrous diisopropylamine (distilled from CaH_2). This solution was cooled to 0 °C and 40 mL (0.100 mol) of 2.5 M *n*-butyllithium in hexanes was added dropwise with stirring over 0.5 h to the three-neck flask at 0 °C. Then 2.9 mL (0.050 mol) of glacial acetic acid (obtained in 99.8% purity and used as received) in 10 mL of dry THF was added dropwise with stirring over 0.5 h to the three-neck flask cooled to 0 °C. The three-neck flask was then heated with stirring to 45–50 °C for 2 h, producing a milky white suspension. After this, 0.050 mol of an *n*-alkyl aldehyde (the series from propionaldehyde to decanal was purchased in the highest purity available and used as received) in 15 mL of dry THF was added dropwise over 1 h followed by an additional 2.5-h reaction period with stirring at 40 °C. To this mixture, 5 mL of distilled H_2O was added and the solvent was removed giving a white solid. The minimum quantity of distilled water was used to dissolve the white solid and this solution was extracted with ether. The aqueous phase was acidified to a pH between 2.0 and 2.5 with 6 N HCl, and sodium chloride was added to saturation. This was then extracted with ether and the ether was removed yielding yellow oils. The yield of these crude products was generally greater than 70%.

Preparation of β-Hydroxyalkanoic Acid Methyl Esters. Diazomethane in ether was prepared by a literature procedure.[22] To 0.050 mol of a β-hydroxyalkanoic acid dissolved in 100 mL of ether at 0 °C was added the yellow diazomethane solution until no additional gas is evolved and the color of the methyl ester solution stays slightly yellow. The solvent was removed and the crude methyl esters were purified by short-path fractional distillation under reduced pressure giving the series of unbranched β-hydroxyalkanoic acid methyl esters from methyl β-hydroxyvalerate to methyl β-hydroxydodecanoate in overall yields greater than 50%. The ^1H and ^{13}C NMR spectra, IR spectra, and elemental analyses were consistent with the expected structures. GC showed the methyl esters to be in excess of 98% purity. Methyl β-hydroxybutyrate was obtained commercially (99% purity, Aldrich Chemical Co.).

GC–MS Analyses. The volatile methyl esters synthesized by the methanolysis reaction (see Experimental Section GC assay) were identified by comparison of both their retention times and mass spectra with those of the standard methyl β-hydroxyalkanoates (the synthesis of which was described above). These analyses were carried out by using a Hewlett-Packard 5890A GC equipped with a Carbowax-20M capillary column (50 m × 0.32 mm; Alltech) and a Hewlett-Packard 5970 series mass-selective detector. A 0.1-μL splitless injection was analyzed with helium as the carrier gas (2 mL/min). The temperatures of the injector and detector were 230 and 275 °C, respectively. A temperature program was used which efficiently separated the different methyl β-hydroxyalkanoates (80 °C for 5 min, temperature ramp of 7 °C per min, 130 °C for 14 min). The data were processed with a Hewlett-Packard laboratory data system. The mass spectrometer was autotuned with perfluorotributylamine. The major fragments which were consistently seen for all of the synthesized methyl β-hydroxyalkanoates had the following *m/e* values: 103, 74, and 71.

Molecular Weight Measurements. All molecular weights reported in this study were determined by GPC. The instruments used included a Waters Model 6000A solvent delivery system, Model 401 refractive index detector, and Model 730 data module with 2 Ultrastyragel linear columns in series. Tetrahydrofuran was used as eluant at a flow rate of 1.0 mL/min. Sample concentrations of 0.3% wt/v and injection volumes of 300 μL were used. Polystyrene standards with a low polydispersity were purchased from Polysciences and used to generate a calibration curve.

NMR Spectroscopy. Solution ^{13}C NMR measurements were recorded at 75.4 MHz on a Varian XL-300 NMR spectrometer. The solution ^1H NMR measurements were recorded either at 200 or 300 MHz on Varian XL-200 and XL-300 NMR spectrometers, respectively. The ^{13}C and ^1H NMR spectra of the PHA's were recorded at 20 °C in chloroform-*d* (40 mg/mL) and benzene-d_6 (10 mg/mL), respectively. The solvent chloroform-*d* and tetramethylsilane (TMS) were used as internal chemical shift references for ^{13}C and ^1H NMR spectra, respectively. The delay time between sampling pulses for both ^{13}C and ^1H NMR measurements was 3.0 s. The ^{13}C NMR spectra taken were proton-decoupled

with a 16500-Hz spectral width, 30K data points, and a 56° pulse (10 μs) and typically 10000–15000 transients were accumulated. Peak areas for both ^1H and ^{13}C NMR spectra were determined by spectrometer integration from unenhanced spectra, unless otherwise indicated.

Thermal Analysis. The glass transition (T_g), melting temperature (T_m), and the heat of fusion (ΔH_m) were measured for the PHA samples biosynthesized by using a Perkin-Elmer Model DSC-4. The PHA samples studied had been precipitated twice into methanol and the solvent was removed, in vacuo, at ambient temperature. The weight of each sample was typically 4–8 mg. PHA samples were heated at a rate of 20 °C/min from −73 to 127 °C, quickly cooled, and then scanned a second time using the same heating rate and temperature range as the first scan. Data used for T_g, T_m, and ΔH_m were reported from the first scan. T_g was taken as the onset temperature and T_m as the peak of the melting endotherm. When multiple endotherms were observed, the T_m from the higher temperature endotherm is reported.

X-ray Diffraction. X-ray diffraction measurements were made under reduced pressure by using a Statton camera and a Siemens K710H generator operating at 40 kV and 30 mA. The X-ray beam was pinhole collimated. Nickel-filtered Cu Kα radiation (λ = 0.1542 nm) was used. PHA films were cast from acetone solution onto Teflon-coated glass. These films (0.3–0.8 mm thick) were allowed to crystallize for a minimum of 3 weeks at ambient temperature before they were examined. The films of PHA samples from *P. oleovorans* grown on octanoate, nonanoate, and decanoate were easily removed from the Teflon-coated glass, maintaining their respective shapes. The films of PHA samples from *P. oleovorans* grown on hexanoate and heptanoate were still weak and sticky after standing at ambient temperature for a minimum of 3 weeks and were only capable of maintaining their shapes upon removal from the Teflon-coated glass if these samples were brought below their respective T_g's by using dry ice. Once these films were removed from the dry ice, they would immediately become weak and sticky once again. A partially oriented film of the PHA sample from *P. oleovorans* grown on nonanoate (method B) was prepared by using a solution-cast film which had been allowed to crystallize for a minimum of 3 weeks at ambient temperature. This film was manually stretched to approximately two times its original length by holding the ends of the film securely with hemostatic forceps (with positive-lock ratchets) and stretching the film to the break point. For wide-angle (2θ greater than 30°), moderate-angle (2θ from 10° to 30°), and small-angle (2θ less than 10°) measurements the sample to film distances used were 17.75 mm (position 1), 74.7 mm (position 3), and 194.5 mm (position 4), respectively. The sample and film exposure times for measurements in positions 1 and 3 were from 6 to 8 h, while measurements made when using position 4 had exposure times of 12–14 h.

Routine Procedures. Infrared spectra were recorded on a Perkin-Elmer Model 283 spectrometer.

Elemental analyses were carried out by the Microanalysis Laboratory at the University of Massachusetts, Amherst, MA.

Results and Discussion

PHA Production and Composition. To achieve good PHA yields it is essential to obtain both high cell yields and polymer content (expressed as the percent of the cellular dry weight which contains PHA). If *P. oleovorans* is grown on *n*-alkanoic acid sodium salts and the organism is fed a total of two times (the second time after 24 h growth) with the carbon source along with other nutrients (described as method B in the Experimental Section), cell yields of up to 1.5 g/L were obtained with a polymer content as high as 49% (see Table I). This represents an improvement from our earlier work with this microorganism.[23]

The analysis of the repeating unit composition involved the acid-catalyzed degradation (methanolysis) of the PHA samples to their corresponding methyl β-hydroxyalkanoates.[24] Identification and quantitation of the various monomers from which the PHA samples are composed was accomplished via the synthesis of methyl β-hydroxyalkanoate standards (see Experimental Section). Using GC-MS analysis,[24] a positive identification of the methyl β-hydroxyalkanoates produced by the acid-catalyzed PHA methanolysis was carried out by a comparison of the retention times and mass spectra of the PHA degradation products to those of the synthesized standard methyl β-hydroxyalkanoates.

Table I
Production of PHA by *P. oleovorans* Grown on *n*-Alkanoate Sodium Salts[a]

carbon source	cell yield,[b] g/L	polymer content[c,d] of cells, %	PHA yield,[e] g/L
caproate	0.9	5	<0.1
heptanoate	0.9	22	0.2
octanoate	1.5	41	0.6
nonanoate	1.5	49	0.7
decanoate	1.2	37	0.4

[a] The biosynthesis was carried out by using method B (see Experimental Section). [b] The quantity of harvested cells after they were washed with buffer and lyophilized (see Experimental Section). [c] Expressed as the percent of the cellular dry weight which contains PHA. [d] These values were obtained gravimetrically from the isolation of PHA samples by the solvent extraction of lyophilized cells (see Experimental Section). [e] PHA yield = (cell yield) × (fraction of the cellular dry weight which contains PHA).

Table II
Composition of PHA Samples Produced by *P. oleovorans* Grown on *n*-Alkanoates[a]

carbon source	repeating units found in PHA, mol %								
	HB	HV	HC	HH	HO	HN	HD	HUD	HDD
caproate[c]	3	<1	72		22		3		
heptanoate[c]		7	<1	86	<1	7			
octanoate[c]	<1	1	6		75		17		
octanoate[b]		2	7		85		6		
nonanoate[c]		3	<1	20	5	72			
nonanoate[b]		3	<1	27	6	61	2	1	
decanoate[c]	<1	1	7		44		47		<1

[a] Analyses were performed on isolated PHA samples by GC (see Experimental Section). [b] The microorganism was fed once with the respective carbon source (method A, see Experimental Section). [c] The microorganism was fed twice with the respective carbon source (method B, see Experimental Section).

The polyesters produced were all heteropolymers, where the PHA composition varied with the sodium *n*-alkanoate used for growth (see Table II). In every case, the major monomer unit found in the polymer had the same number of carbon atoms as the *n*-alkanoate carbon source used. In earlier work, the metabolism by *P. oleovorans* of the carbon source (whether it is an *n*-alkanoate[17] or an *n*-alkane[15,16]) to form the various monomer units identified in these storage polyesters has been discussed.[16,17] A very apparent metabolite is the conversion of the carbon source, presumably by fatty acid degradation (β-oxidation), to a monomer unit which had two carbons less than the *n*-alkanoate used for growth.[16,17] In the case of *P. oleovorans* grown on nonanoate and decanoate, the PHA samples produced contain a relatively large mole percent of the above metabolite (see Table II). This result, along with the high polymer content of cells grown on heptanoate through decanoate, suggests that the enzymes which carry our the metabolism into PHA repeating units of the substrates butanoyl-CoA through hexanoyl-CoA and heptanoyl-CoA through decanoyl-CoA show a combined higher specificity for the latter group of longer chain length substrates. The above is in sharp contrast to the preferential synthesis of PHA's which contains mainly HB and HV by other microorganisms capable of synthesizing this polyester.[1-4,14,25]

Table III
Percent Carbon and Percent Hydrogen in PHA Samples Measured by Routine Microanalysis and Calculated from GC Repeating Unit Composition Results

carbon source	microanal. % C	microanal. % H	GC anal. % C	GC anal. % H
caproate[b]	63.92	9.07	64.09	9.06
heptanoate[b]	65.72	9.75	65.45	9.42
octanoate[a]	66.88	9.94	67.24	9.84
nonanoate[a]	67.97	10.13	67.88	9.99
decanoate[b]	69.09	10.38	68.59	10.17

[a] The biosynthesis was carried out by using method A (see Experimental Section). [b] The biosynthesis was carried out by using method B (see Experimental Section).

Table IV
Molecular Weight Measurements[a] of PHA Samples[b] Produced by *P. oleovorans* Grown on *n*-Alkanoates

carbon source	$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	DP[c]
caproate	330 000	140 000	2.4	970
heptanoate	360 000	120 000	3.0	940
octanoate	160 000	54 000	3.0	380
nonanoate	190 000	70 000	2.7	480
decanoate	190 000	70 000	2.7	460

[a] Determined by GPC (see Experimental Section). [b] The biosynthesis was carried out by using method B (see Experimental Section). [c] DP is the degree of polymerization which was calculated by using the values for $\bar{M}_n$ given in this table along with knowledge of the repeating unit composition (see Table II).

In earlier work[17] it was shown that the yield of isolated PHA, determined gravimetrically by the complete solvent extraction of intracellular PHA from lyophilized cells (see Experimental Section), was almost identical with the value estimated by GC analysis after the methanolysis of whole cells (see Experimental Section).[17] In addition, if the results from elemental analysis of PHA samples as measured by routine microanalysis (see Experimental Section) are compared with the percent carbon and percent hydrogen calculated by using the repeating unit composition determined by GC analyses (see Table II), we see that these independent methods are in excellent agreement (see Table III). The results above, therefore, verify the reliability of the GC assay values obtained.

The molecular weights determined by GPC of PHA samples which were synthesized in this work are shown in Table IV. The degree of polymerization (DP) for PHA's synthesized by *P. oleovorans* when grown on caproate and heptanoate are approximately twice as large as the DP for PHA's which were produced when using octanoate, nonanoate, and decanoate as carbon sources. This unexpected result cannot be simply explained when hydrodynamic volume arguments are considered.[26] These results and what they suggest about the polymerization mechanism will be discussed later in this report.

Studies by NMR. The ^{1}H NMR (at either 200 or 300 MHz) spectra of the PHA samples produced in this work were consistent with the structure proposed for these poly-β-esters.[4,15,16] The ^{1}H NMR (200 MHz) spectrum of the PHA produced by method A (which involves feeding the organism just once with the carbon source and nutrients), using nonanoate as the carbon source, is shown in Figure 1. The integration values shown above the peaks agree reasonably well with the proposed polymer structure, where peak d has a value of 5.9 H as compared to a value of 6.6 H calculated from the repeating unit composition determined by GC analysis (see Table II). The complex multiplet and unsymmetrical triplet observed in the expansions of regions a and e, respectively, are consistent with the overlap of peaks from the major monomer units

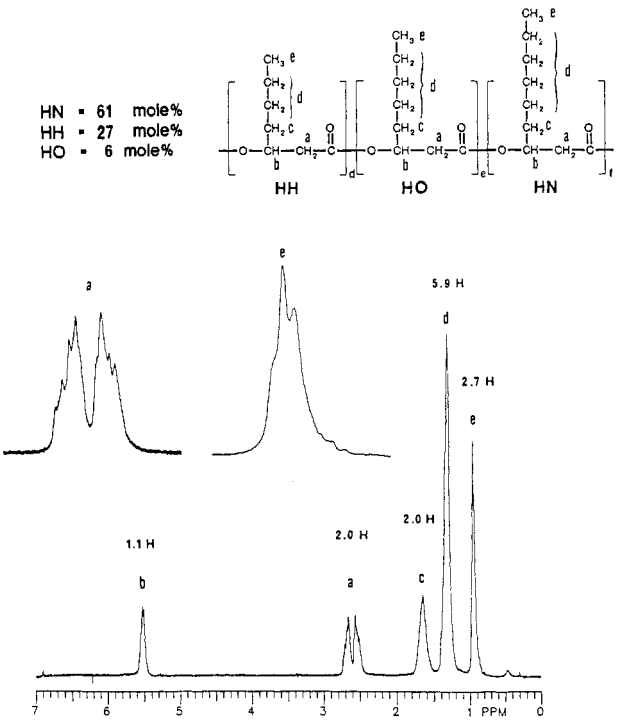

Figure 1. 200-MHz ^{1}H NMR spectrum, recorded at 20 °C in benzene-d_6, of the PHA biosynthesized by method A (see Experimental Section) with nonanoate as the carbon source, along with expanded regions from the spectrum.

Table V
Chemical Shift Data (in ppm) from the ^{13}C NMR Spectra (75.4 MHz) of Isolated PHA Samples

carbon[a]	repeating units identified in PHA samples: HB[b]	HV[b]	HC	HH	HO	HN	HD
1	169.13	169.51	169.41	169.38	169.40	169.40	169.39
2	40.80	38.80	39.12	39.11	39.08	39.10	39.12
3	67.61	71.88	70.61	70.82	70.83	70.84	70.86
4	19.77	26.85	35.91	33.49	33.74	33.81	33.82
5		9.37	18.31	27.14	24.70	25.01	25.07
6			13.78	22.40	31.51	29.04	29.35
7				13.89	22.49	31.69	29.17
8					13.97	22.56	31.78
9						14.04	22.62
10							14.06

[a] The number assignments for the carbons of the repeating units HB, HV, HC, HH, HO, HN, and HD are shown in Figures 2–7. [b] These chemical shifts were obtained as described in ref 27.

found within the PHA sample (HH, HO, and HN). The ^{1}H NMR spectra of the other PHA samples produced in this work show peaks which appear at almost the identical chemical shifts and have integration values that agree quite well with those shown in Figure 1, with the exception of peak d for which the integration varied according to the carbon source utilized in the biosynthesis of the sample. Changes in the detailed appearance of regions a–e (see Figure 1) recorded for the ^{1}H NMR spectra of the other PHA samples with different repeating unit compositions are expected and of course were observed.

The PHA samples produced in this work were also studied by using ^{13}C NMR (75.4 MHz). The ^{13}C NMR spectra of the PHA's produced when caproate, octanoate, and nonanoate were used as carbon sources are shown in Figures 2, 4, and 6, respectively. Expansions of selected regions in Figures 2, 4, and 6 are displayed in Figures 3, 5, and 7, respectively. Method A (see Experimental Section) was used to synthesize the PHA's for which NMR spectra are shown in Figures 4–7 (octanoate and nonanoate

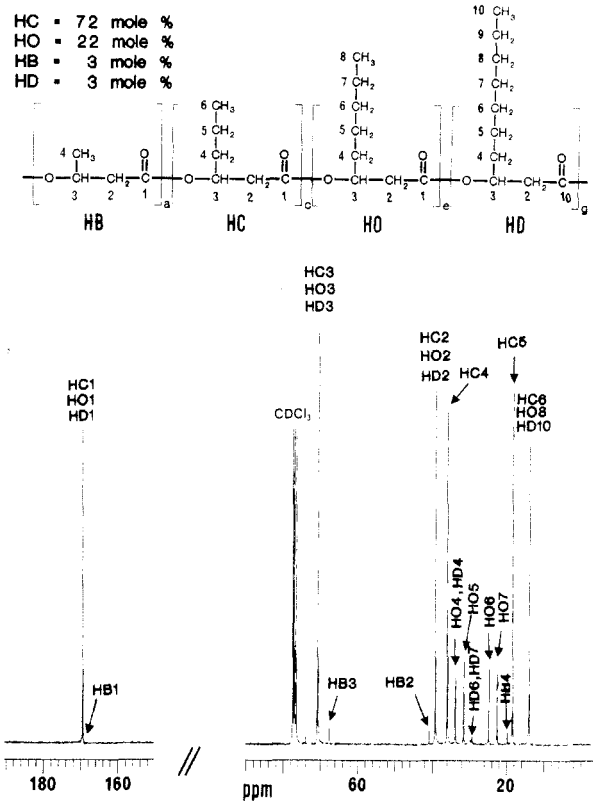

Figure 2. 75.4-MHz ^{13}C NMR spectrum, recorded at 20 °C in chloroform-*d*, of the PHA biosynthesized with caproate as the carbon source.

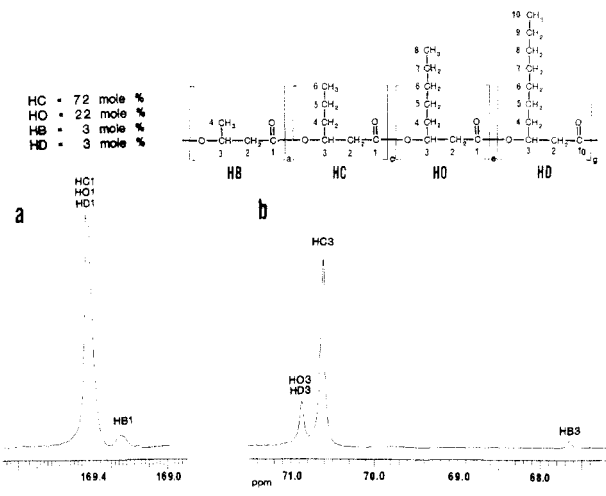

Figure 3. Expanded regions from the ^{13}C NMR spectrum shown in Figure 2 of (a) the carbonyl carbon region and (b) the methine carbon region.

as the carbon sources). Assignment of the chemical shifts for the carbons in HC, HH, HO, and HN (see Table V) were made by observing the ^{13}C NMR spectra of the PHA samples produced by using caproate, heptanoate, octanoate, and nonanoate as carbon sources, identifying the major peaks, and assigning them as being due to the major monomer unit which is present in the sample as determined by GC analysis (see Table II). For the case of the PHA which was synthesized by *P. oleovorans* when the microorganism was grown on deconoate, the polymer is composed of approximately equal quantities of HO and HD (see Table II). Assignment of the peaks from the carbons of HD was possible after the peaks which were due to HO were determined (see above).

The main chain carbons (1–3, see Figures 2–7) of the above repeating units were assigned by comparison with

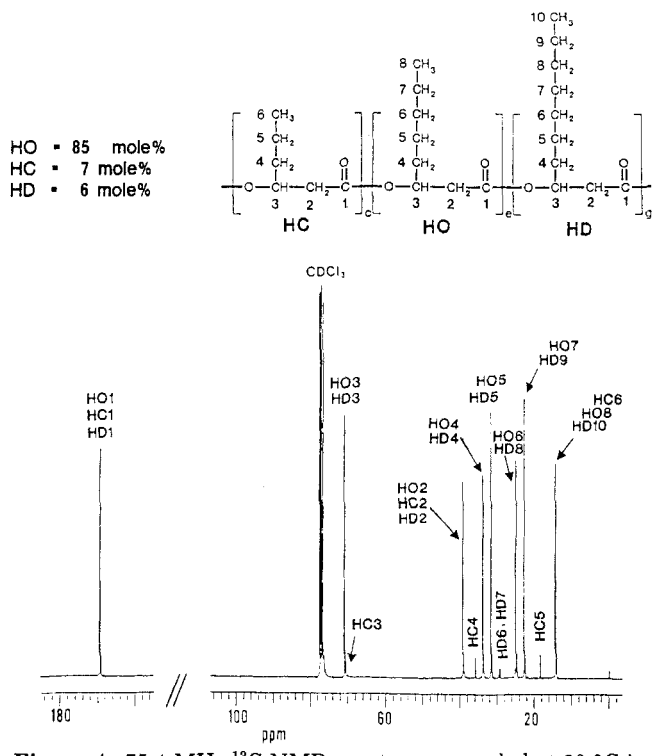

Figure 4. 75.4-MHz ^{13}C NMR spectrum, recorded at 20 °C in chloroform-*d*, of the PHA biosynthesized by method A (see Experimental Section) with octanoate as the carbon source.

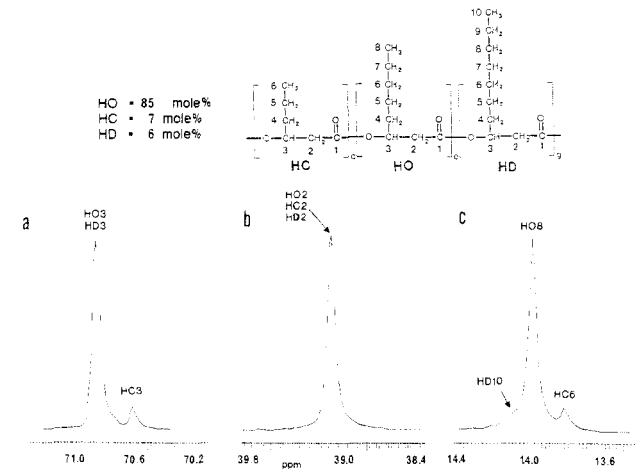

Figure 5. Expanded regions from the ^{13}C NMR spectrum shown in Figure 4 of (a) the methine carbon region, (b) the main-chain methylene carbon region, and (c) the methyl carbon region.

the chemical shifts for carbons 1–3 of HB and HV[27] (values given in Table V) which have been previously reported in the literature.[28] The carbons in the *n*-alkyl pendant groups were assigned from the known additive shift parameters for hydrocarbons.[29] In this way, the chemical shifts were compiled for the carbons of HB, HV, HC, HH, HO, HN, and HD, which are shown in Table V.

In the spectra shown in Figures 2–7, many of the minor peaks agreed within the experimental error of the spectrometer (±0.05 ppm) with the chemical shifts given in Table V for either HC, HH, HO, HN, or HD. For the repeating units HB and HV, which were found by GC analysis to be minor components of the PHA samples biosynthesized by *P. oleovorans* (see Table II), the ^{13}C NMR peaks which are believed to be due to the carbons of these repeating units had chemical shifts which agreed within ±0.10 ppm with the values given in Table V, accounting for the remaining minor peaks which were seen

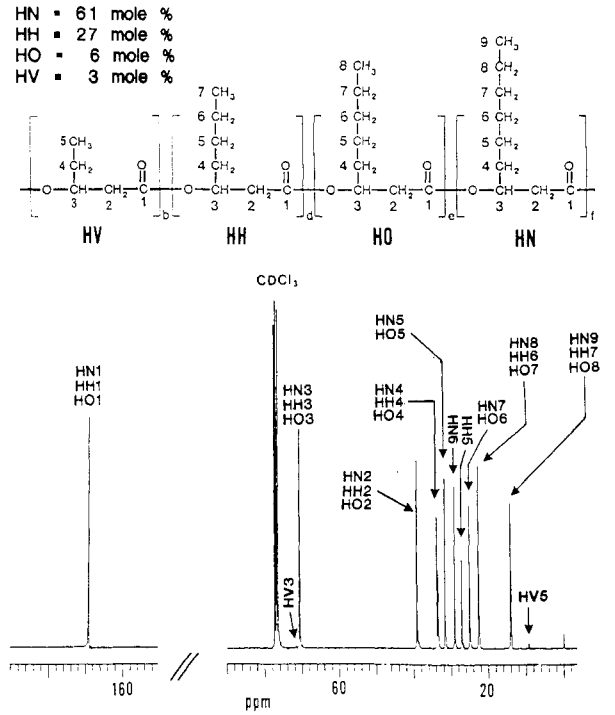

Figure 6. 75.4-MHz ^{13}C NMR spectrum, recorded at 20 °C in chloroform-*d*, of the PHA biosynthesized by method A (see Experimental Section) with nonanoate as the carbon source.

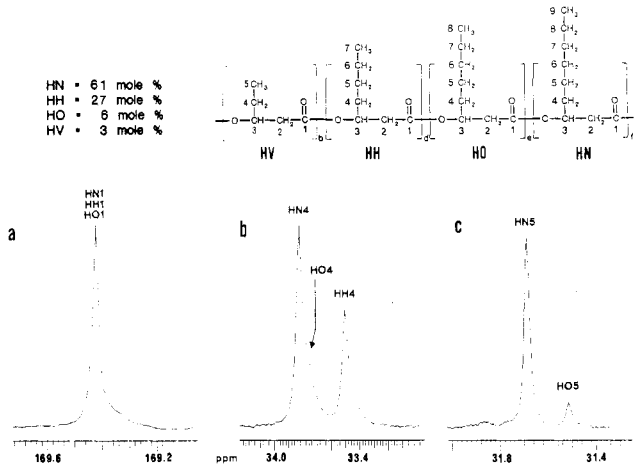

Figure 7. Expanded regions from the ^{13}C NMR spectrum shown in Figure 6 of (a) the carbonyl carbon region, (b) the side-chain methylene carbons closest to the main chain, and (c) the side-chain methylene carbons HN5 and HO5.

in these spectra. Therefore, the minor peaks were assigned as being due to the appropriate carbons of the corresponding repeating units above (see Figures 2–7).

The same repeating units which were found to be present in these PHA samples in quantities greater than 2 mol % by GC analysis (see Table II) were observed as well in the ^{13}C NMR spectra of these samples.

The spectrum in Figure 2 for the PHA produced when caproate was the carbon source has six major peaks for the carbons of HC along with minor peaks showing the presence of HB, HO, and HD in the sample (see Table II). Expansion of the carbonyl carbon region (see Figure 3a) shows that HC1, HO1, and HD1 are not resolved while the chemical shift of the small upfield peak corresponds closely (±0.10 ppm) with HB1. The expansion of the methine carbon region (see Figure 3b) shows clearly HC3 and HB3 as well as the unresolved signals of HO3 and HD3. The spectrum in Figure 4 for the PHA produced when octanoate was the carbon source shows eight major peaks for the carbons of HO, along with minor peaks showing the presence of HC and HD in the sample. The expansion of the methine carbon region (Figure 5a) shows a peak corresponding to HC3 along with a single peak for the unresolved signals of HO3 and HD3. Expansion of the main-chain methylene carbon region (Figure 5b) shows a single peak for the unresolved signals of HO2, HC2, and HD2. The expansion of the methyl carbon region (Figure 5c) shows the signals for HO8 and HC6 partially resolved and a signal corresponding to HD10 which appears as a shoulder on the HO8 peak. The spectrum in Figure 6 for the PHA produced when nonanoate was the carbon source shows nine major peaks for the carbons of HN, along with smaller peaks showing the presence of HV, HH, and HO. Expansion of the carbonyl carbon region (Figure 7a) shows that the signals from HN1, HH1, HO1, and HV1 are not resolved. Expansion of the region for the pendant methylene groups closest to the main chain (Figure 7b) shows the signals for HN4 and HH4, with HO4 as a shoulder on the HN4 peak. In Figure 7c the expansion of the signals from HN7 and HO6 are shown, providing clear evidence from ^{13}C NMR for the metabolism of nonanoate (the nine-carbon *n*-alkanoate growth substrate) to the eight-carbon repeating unit HO.[30]

The chemical shifts for the carbons of HC, HH, HO, HN, or HD did not change (within ±0.05 ppm), whether that monomer unit composed most of the PHA sample (for example, HO for the PHA produced from the carbon source octanoate) or was a minor component (for example, HO for the PHA produced from the carbon source caproate). If, in fact, the PHA's synthesized here approximate a statistically random comonomer sequence distribution,[31] then the above result would represent an insensitivity of the ^{13}C NMR (at 75.4 MHz) chemical shifts of these repeating units to the *n*-alkyl pendant group length (when greater than or equal to *n*-propyl) of neighboring repeating units.

The data in Table V show that increases in the length of the *n*-alkyl pendant group (from *n*-propyl to *n*-heptyl) has a negligible effect on the chemical shifts (within ±0.05 ppm) of the main-chain carbon atoms 1–3, with the exception of HC3. Another interesting feature which is apparent in Table V is the extremely low value for the chemical shift of HV5 relative to the methyl carbons of the other repeating units. In addition, their is an increase in the chemcial shift values for the methyl carbons from HC (*n*-propyl pendant group) to HN (*n*-hexyl pendant group), where the values for HN and HD are equivalent (within experimental error). The above may very well be due to the relative probability of conformations which allow close proximity of the methyl carbon in the pendant group to the main-chain carbonyl group, where a higher probability would decrease the chemical shift. The close proximity which may occur between HV5 and the carbonyl group on a neighboring repeating unit is clearly seen by the 2_1 helical conformation which exists in the solid states of P(HB-*co*-HV).[11,32,33]

Thermal Analysis. The DSC thermograms for the first heating scan on solution-precipitated PHA samples (see PHA isolation, Experimental Section) are shown in Figures 8–10. The biosynthesis of the samples studied was carried out by method A for the carbon sources octanoate and nonanoate and method B for the carbon sources caproate, heptanoate, and decanoate (see Experimental Section for a description of these methods). The values obtained from these thermograms for the glass transition temperature (T_g onset), the peak melting temperature (T_m), and the enthalpy of fusion (ΔH_m) are compiled in Table VI.

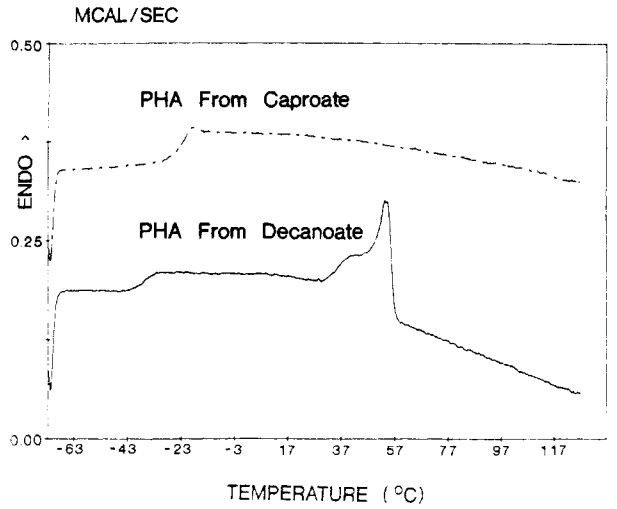

Figure 8. DSC thermograms during the first heating scan of the PHA samples biosynthesized with caproate (- - -) and decanoate (—) as carbon sources.

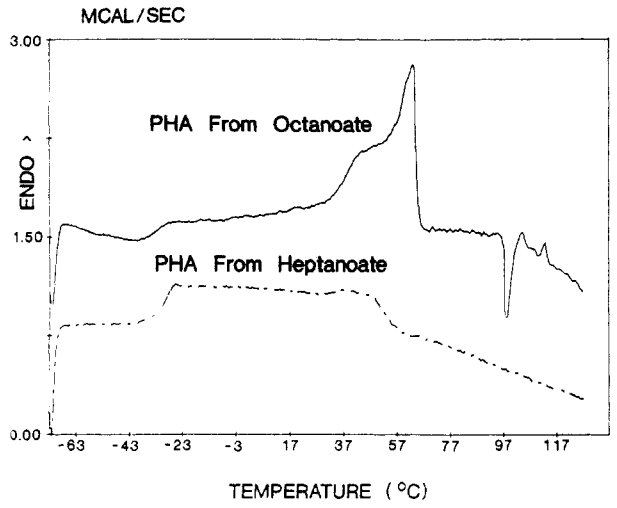

Figure 9. DSC thermograms during the first heating scan of the PHA samples biosynthesized with octanoate (—) and heptanoate (- - -) as the carbon sources.

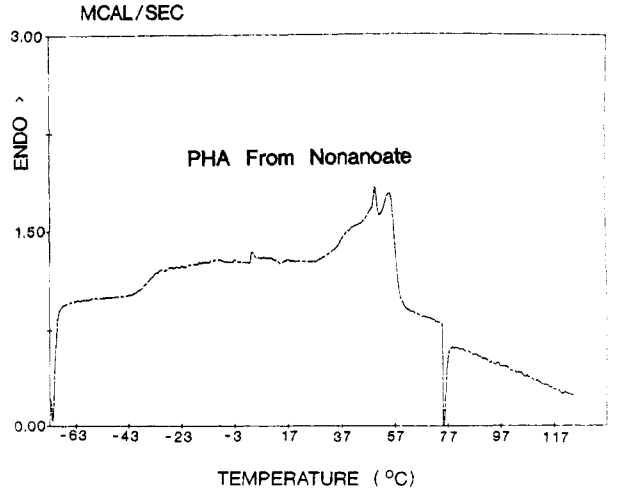

Figure 10. DSC thermogram during the first heating scan of the PHA sample biosynthesized with nonanoate as the carbon source.

In Figure 8 it can be observed that the PHA produced when caproate was the carbon source did not show a melting endotherm, whereas the PHA produced when decanoate was the carbon source clearly shows this transition. In the latter case, the predominance (total of 95 mol %) of the relatively longer *n*-alkyl pendant groups, specifically *n*-heptyl and *n*-pentyl (see Table II), is therefore favorable for sample crystallinity.

Table VI
Thermal Analysis Measurements Using Differential Scanning Calorimetry[a] of PHA Samples[b] from *P. oleovorans*

carbon source	T_g onset, °C	T_m, °C	ΔH_m, cal/g
caproate[d]	−25		
heptanoate[d]	−33	45	1.3
octanoate[c]	−36	61	8.3
nonanoate[c]	−39	54	5.4
decanoate[d]	−40	54	4.9

[a] The data reported in this table were for the first heating scan. [b] The samples were analyzed after two precipitations and removal of the solvent in vacuo at ambient temperature (see Experimental Section). [c] The biosynthesis was carried out by using method A (see Experimental Section). [d] The biosynthesis was carried out by using method B (see Experimental Section).

In Figure 9 is shown the DSC thermograms for the PHA samples produced when heptanoate and octanoate were the carbon sources. In the latter case we see again that the predominance of the relatively longer *n*-alkyl pendant groups (see Table II) *n*-pentyl (85%) and *n*-heptyl (6%) is favorable for sample crystallinity. The PHA produced with heptanoate as the carbon source, which contains 86 mol % of the shorter *n*-butyl pendant groups, shows a comparably much smaller endotherm suggesting a lower degree of sample crystallinity.

The DSC thermogram for the PHA sample produced with nonanoate as the carbon source in Figure 10 clearly shows a melting endotherm. This polymer which contains mainly the *n*-hexyl pendant group (61 mol %, see Table II) appears crystalline, consistent with the DSC results presented above for the PHA samples which contain predominantly *n*-pentyl or longer *n*-alkyl side chains.

Observation of Table VI shows a decrease in the value of T_g from the PHA produced using the carbon source caproate to that produced from decanoate, corresponding to an increase in the average length of the predominant side-chain group. The largest ΔH_m value and melting point observed was for the polymer produced with octanoate as the carbon source (see Table VI). This may be due to a higher content of a single monomer type (85 mol % HO) in this sample relative to 61 mol % HN and 47 mol % HD in the PHA samples produced from the carbon sources nonanoate and decanoate, respectively (see Table II). A higher content of a single monomer type in the PHA samples containing predominantly *n*-pentyl, *n*-hexyl, or *n*-heptyl side chains may, therefore, result in less disruption of the crystalline unit cells present in these samples. It is of interest to note, though, that the PHA sample produced with heptanoate as the carbon source had the highest content of a single monomer type (86 mol % HH) but showed a small melting endotherm (see Figure 9). The weak melting endotherms observed by DSC for the PHA samples which contained predominantly *n*-propyl and *n*-butyl side chains will be discussed further below. The values for T_m in Table VI agree extremely well with the T_m values observed for poly(*n*-alkyl acrylates) (PA), polyvinyl esters (PVE), and poly(*n*-alkylacrylamides) (PAA), which contain long (greater than dodecyl) *n*-alkyl pendant groups.[34]

X-ray Diffraction Analysis. The X-ray diffractograms were recorded for unoriented films cast from acetone solution (see Experimental Section) of PHA samples biosynthesized in the present study. From these diffractograms *d* spacings were calculated, and their values are compiled in Table VII. The X-ray diffractograms for

Table VII
Interplanar *d* Spacings (Å) for PHA Samples Biosynthesized by *P. oleovorans*[a,b]

carbon source	diffraction maxima (reflections), Å								
	d_1 (±0.2)	d_2 (±0.04)	d_3 (±0.04)	d_4 (±0.04)	d_5 (±0.03)	d_6 (±0.03)	d_7 (±0.03)	d_8 (±0.03)	d_9 (±0.03)
caproate[c]	11.2 (s)[d]		4.52 (s)[d]						
heptanoate[c]	12.6 (s)[d]		4.53 (s)[d]						
octanoate	18.4 (vs)	5.01 (m)	4.54 (s)	4.09 (m)	2.91 (vw)	2.68 (vw)	2.49 (vw)	2.23 (vw)	2.05 (vw)
nonanoate	19.4 (vs)	5.00 (w)	4.59 (m)	4.14 (m)		2.69 (vw)	2.47 (vw)	2.23 (vw)	2.04 (vw)
decanoate	19.9 (vs)		4.73 (m)[d]	4.12 (m)		2.63 (vw)	2.44 (vw)	2.20 (vw)	2.03 (vw)

[a] Intensity of the reflections are given in parentheses: s, strong; m, medium; w, weak; v, very. [b] The biosynthesis was carried out by using method B (see Experimental Section). [c] The sample was only observed in position 3 (see Experimental Section). [d] The reflection was very disperse.

unoriented and partially oriented films (see Experimental Section) of the PHA produced from nonanoate as the carbon source (biosynthesized using method B, see Experimental Section) are shown in parts a and b of Figure 11, respectively.

From observation of Table VII, we see that the d_1 long spacings have larger values as the length of the predominant *n*-alkyl pendant group of the PHA sample increases. Many of the other interplanar spacings for the PHA samples produced from the carbon sources octanoate, nonanoate, and decanoate have almost identical values: d_4 from 4.09 to 4.14; d_6 from 2.63 to 2.68; d_7 from 2.44 to 2.49; d_8 from 2.20 to 2.23; d_9 from 2.03 to 2.05. These interplanar spacings are then independent of the most abundant side-chain length and the mole percent of other repeating units present in the sample (Table II). Of the above small spacing reflections only d_4 is of medium intensity while d_6, d_7, d_8, and d_9 were very weak (see Table VII).

The PHA samples which have relatively shorter side chains, produced from caproate and heptanoate as the carbon sources, show only very disperse reflections (see Table VII), indicating low sample crystallinity. This is in agreement with the DSC results above and the observation that films of these samples cast from acetone solution were rather sticky and weak after they had stood at room temperature for more than 3 weeks.[35]

The long *n*-alkyl side-chain polymers and copolymers of PA, poly(*n*-alkyl methacrylates) (PMA), and PV ethers and esters are believed to crystallize in a layered packing order.[34] Their X-ray diffraction patterns show many common features to those described above for long side-chain PHA's (where the majority of the *n*-alkyl pendant groups are equal to or greater than *n*-pentyl). The patterns for long *n*-alkyl side chain PA, PMA, and PV ethers and esters look identical for the small spacing reflections, irrespective of the side-chain length (assuming its longer than approximately *n*-octyl). They contain only three small spacing reflections: a sharp and strong reflection at 4.15–4.19 Å and very weak reflections at 2.40–2.43 and 2.08–2.10 Å. For long side chain PHA's the values and intensities of the *d* spacings d_4, d_7, and d_9 are in very good agreement with the above results (see Table VII). In addition, practically all classes of long side chain polymers[34] show a long spacing of maximum intensity which corresponds to d_1 in this study. The values of the long spacings are determined by the distance between neighboring main chains and increase with a rise in the length of the *n*-alkyl pendant groups.[34] This was observed, as well, for d_1 in the present study.

From observation of Figures 11, parts a and b, it is clear that partial orientation of a sample by stretching the film (see Experimental) results in meridional and equatorial reflections which correspond to *d* spacings at 4.14 Å (d_4) and 19.4 Å (d_1), the very strong intensity long spacing reflection, respectively. This was seen, as well, for the

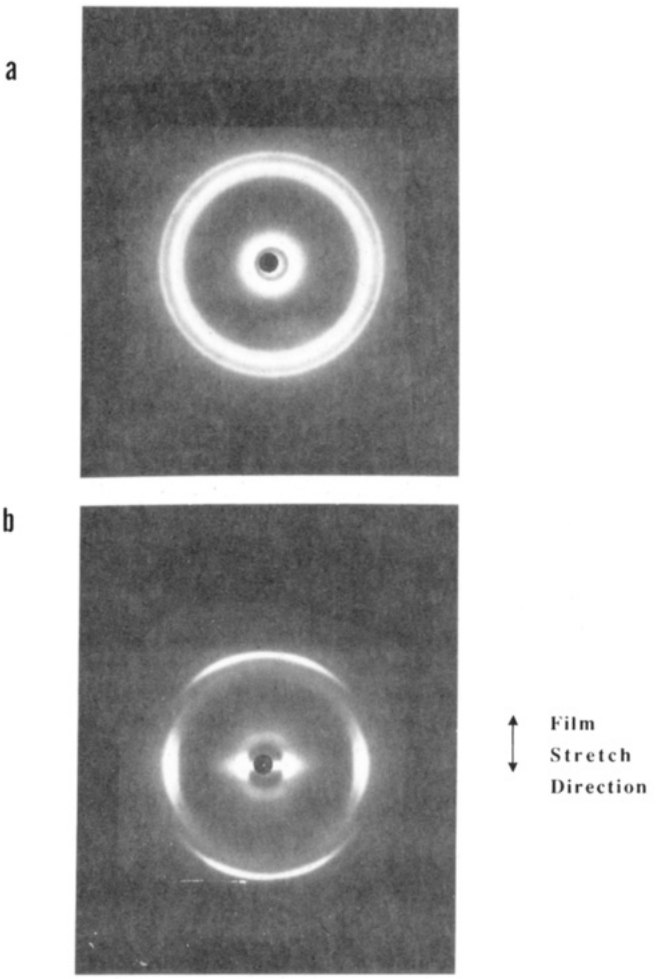

Figure 11. X-ray diffractograms for unoriented (a) and partially oriented (b) films of the PHA produced with nonanoate as the carbon source.

X-ray pattern of a partially oriented long side chain PMA, which showed a meridional reflection at 4.19 Å and a long spacing equatorial reflection.[34] The reflection observed at 4.19 Å, corresponding to d_4 in the present study, is believed to have its origin from the interplanar spacing for crystalline long-chain hydrocarbons,[36] while the origin of the long spacing reflection corresponding to d_1 in the present study was discussed above. The observed meridional and equatorial reflections for the partially oriented PHA sample described above are therefore consistent with the general scheme for a layered packing order, which is believed to exist in PA, PMA, PV ethers, PV esters, and other long side chain polymers, where the *n*-alkyl pendant groups often exists at right angles to the main chain.

The DSC and X-ray diffraction results, therefore, suggest that the long side chain PHA samples biosynthesized

in this work crystallize with participation of both the main and side chains in a similar manner as described for other classes of polymers bearing long side chains.[34] The low sample crystallinity observed for the PHA samples which contained predominantly *n*-propyl and *n*-butyl side chains may be explained by the inability of these samples to crystallize via close proximity of their main chains (possibly due to small quantities of longer side chain repeating units present in these samples), while having side chains which are too short to allow a layered packing order. Work is currently in progress to better understand the exact nature of side- and main-chain order in long side chain PHA's.[33]

Suggested Mechanism. The DP for PHA's synthesized by *P. oleovorans* when grown on caproate and heptanoate was approximately twice as large as the DP for PHA's which were produced when using octanoate, nonanoate, and decanoate as carbon sources (reported above, see Table IV). This result was not anticipated due to the higher production of PHA on the longer chain length carbon sources. An explanation for the above may be found if one considers that an overall relatively higher efficiency of enzymatic conversion from a carbon source to polymer does not mean that the final enzymatic step of polymerization shows a similarly high specificity for the predominant monomer structure. It is quite possible that the degree of polymerization achieved is highly dependent on the binding constant (K_m) of the polymerase enzyme to the chain end. The value of K_m may be a function of both the substrate–enzyme fit as well as the hydrophobicity of the terminal repeating unit of the growing chain which is bound to the enzyme. A pendant group with a longer chain length would, of course, introduce greater hydrophobicity to the enzymatic active site of polymerization which could very well play a role if the polymerase enzyme is located, as is generally believed to be the case,[37] in the protein membrane at the interface between the hydrophylic cytoplasm and the relatively hydrophobic granule. Therefore, when the pendant group is propyl or butyl, the binding constant K_m described above may be smaller, leading to less events of chain transfer relative to propagation. Isolation of the polymerase enzyme in *P. oleovorans* both bound and free from the PHA granule followed by direct measurement of K_m values with various β-hydroxythiocoenzyme A ester substrates in a cell-free system would be useful in evaluating the above hypothesis.

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Registry No. Sodium caproate, 10051-44-2; sodium heptanoate, 10051-45-3; sodium octanoate, 1984-06-1; sodium nonanoate, 14047-60-0; sodium decanoate, 1002-62-6.

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(8) The synthesis of P(HB-*co*-HV) copolymers with up to 93 mol % HV content has been carried out in our laboratory by using the photosynthetic microorganism *Rhodospirillum rubrum*. A manuscript on this work is currently in preparation.
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(23) The studies carried out in ref 17 used culture conditions which are described as method A in this work (see Experimental Section). Growth on octanoate or nonanoate by method A gave values for cell yield and polymer content not exceeding 1.2 g/L and 28%, respectively.
(24) For details on the procedure and chromatographic conditions used here for the GC and GC-MS analyses of PHA samples, see the Experimental Section in this paper and in ref 17, above. See also ref 16, above, for additional information on the methanolysis procedure.
(25) It was recently shown in our laboratories for the photosynthetic bacterium *Rhodospirillum rubrum*, using *n*-alkanoate and β-hydroxyalkanoate carbon sources of variable chain length, that the incorporation of HB and HV into the PHA's produced is favored but smaller quantities of longer *n*-alkyl pendant groups (up to butyl) could also be incorporated into the PHA's. Copolyesters of HB and HV produced were found to have a random comonomer sequence distribution. Manuscripts on this work are in preparation.
(26) It would be anticipated that PHA's which contained longer pendant group chain lengths, for the same value of DP, would have larger hydrodynamic radii.
(27) P(HB-*co*-HV) copolymers synthesized in our laboratory (see ref 25) were studied by ^{13}C NMR using the conditions identical

with those described in the Experimental Section. Chemical shift values obtained in the above manner on a P(HB-*co*-HV) sample containing 16 mol % HV are reported in Table V.
(28) See Doi et al. and Bluhm et al. in ref 9.
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(30) A detailed discussion concerning the metabolism of a carbon source to the various repeating units which were identified in the corresponding PHA is found in ref 16 and 17, above.
(31) A random comonomer sequence distribution for PHA copolyesters is not unprecedented; see ref 25 and 28.
(32) Studies by ^{1}H NMR on P([*R*]-HB) indicate a preference, in chloroform, for the gauche conformation related to the 2_1 helix of P([*R*]-HB) and P([*R*]-HV) observed in the solid state; see: Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1986**, *19*, 1274.
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(35) See the Experimental Section, X-ray Diffraction, for additional observations on the physical behavior of PHA films cast from acetone solution.
(36) Chapman, D. *The Structure of Lipids by Spectroscopic and X-ray Techniques*; Methuen, 1965. Vereshagin, A. G. *Uspek. Khim.* **1971**, *40*, 1995.
(37) For an idealized scheme of a proposed mechanism for PHA biosynthesis, see: Dawes, E. A.; Senior, P. J. Page 234 of ref 4.

Photoresponsive Polymers. Photostimulated Aggregation–Disaggregation Changes and Photocontrol of Solubility in Azo-Modified Poly(glutamic acid)

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ABSTRACT: The photochromic properties of poly(L-glutamic acid) containing 21, 52, and 85 mol % azobenzene units in the side chains were investigated. In suitable conditions, depending on the azo content, the trans ⇌ cis photoisomerization of the azobenzene units was accompanied by aggregation–disaggregation processes and precipitation–dissolution of the polymers. The phenomena were fully reversible and could be photoregulated by irradiating at the appropriate wavelength. The polymer solubility was studied as a function of the cis/trans isomeric composition of the azobenzene side chains. It was found to change as a sharp phase transition, the midpoint occurring at about a 50/50 cis/trans isomeric ratio. Findings are consistent with a molecular mechanism in which aggregation and precipitation processes occur through hydrophobic interactions and ordered stacking between the azobenzene moieties. Such interactions can be favored or inhibited depending on the trans ⇌ cis photoisomerization of the azo units, thus causing the photoresponse effects.

Introduction

Polymers containing azobenzene moieties have recently attracted considerable interest, mainly because of their potential use as photoresponsive systems.[1-3] In particular conditions, in fact, the trans ⇌ cis photoisomerization of the azo groups can be accompanied by reversible variations of the macromolecular conformation and the physical properties of the polymers. Interesting light-induced conformational changes were found to occur in azo polypeptides,[4-10] which exist in ordered conformations, such as α-helix and β-structure, and more closely approximate biological systems.

In preliminary papers, we have also reported the occurrence of photostimulated aggregation–disaggregation processes[11] and reversible variations of solubility induced by light.[12] The former phenomenon was observed in aged solutions of azo-modified poly(L-glutamic acid) containing about 20 mol % azobenzene units in the side chains. The latter was exhibited by an analogous azo polypeptide, having a much higher content of azobenzene groups. In order to have a complete and detailed picture, these photoresponse effects have been investigated in samples of azo-modified poly(L-glutamic acid) having various contents of azobenzene units in the side chains, in various solvent conditions, and at different wavelengths of the incident light.

The results obtained show that aggregation–disaggregation or precipitation–dissolution processes can be actually photoregulated by irradiating at the appropriate wavelength. the findings also provide a clear and well-fitting explanation of the molecular events that are responsible for the photoresponse effects described.

Experimental Section

Materials. Trifluoroethanol (TFE) and hexafluoro-2-propanol (HFP) were of spectroscopic grade. Trimethyl phosphate (TMP) was purified by fractioned distillation; its absorbance at 200 nm in a 1-cm cell was 0.8.[13]

***N*-Acetyl-α-(phenylazo)-L-glutamanilide (I).** *N*-Acetyl-γ-benzyl-L-glutamic acid (2.79 g, 10 mmol) was dissolved in chloroform (100 mL) and the solution cooled to 0 °C. *p*-Aminoazobenzene (1.97 g, 10 mmol) and dicyclohexylcarbodiimide (2.26 g, 11 mmol) were added in that order, and the mixture was stirred for 2 h at 0 °C and overnight at room temperature. The precipitated dicyclohexylurea was removed and the solution was washed with 5% $NaHCO_3$, 5% acetic acid, and water. After evaporation of the solvent, the product was crystallized from ethyl acetate/petroleum ether to give 3.2 g (7 mmol) of *N*-acetyl-γ-benzyl-*p*-(phenylazo)-L-glutamanilide.

Benzyl groups were removed by treating the above product (0.32 g, 0.70 mmol) with 0.1 N NaOH (8 mL) in dioxane (30 mL) for 1 h at room temperature. The solvent was evaporated, then water was added, and aqueous solution washed with ether and acedified to pH 4. Finally the oily product was extracted with ethyl acetate and crystallized by adding petroleum ether. Absorption spectra:

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