Biosynthesis of Methyl-Branched Poly(β-hydroxyalkanoate)s by *Pseudomonas oleovorans*

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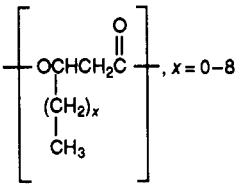
ABSTRACT: *Pseudomonas oleovorans* was grown on 6-methylnonanoic acid (6-MNA), 7-methylnonanoic acid (7-MNA), 8-methylnonanoic acid (8-MNA), 9-methyldecanoic acid (9-MDA), 7-methyldecanoic acid (7-MDA), and 2,6-dimethylhept-5-enoic acid (2,6-DMHA). Poly(β-hydroxyalkanoate)s (PHAs) were obtained when this bacterium was grown on pure 9-MDA, 8-MNA, 7-MNA, and 6-MNA, but not on 7-MDA and 2,6-DMHA. The mixtures of *n*-nonanoic acid (NA) and either 7-MDA or 2,6-DMHA gave almost pure nonanoic acid homopolymers. For the other branched substrates, ^{1}H and ^{13}C NMR studies showed that the polymers obtained contained the type of methyl branching expected from the starting alkanoic acid. The melting points of most polymers were similar to that of nonanoic acid except for that of the polymer from 6-MNA, which was considerably higher. DSC measurements also showed that this polymer crystallized much faster than the others. ^{13}C NMR spectra of the polymer containing 6-methyl-3-hydroxynonanoate units showed that equal amounts of the two chiral isomers were present. From the GPC measurements the number-average molecular weights (M_n) of the polyesters produced were in the range of 70 000–384 000 with polydispersity indices (M_w/M_n) in the range of 1.37–2.55. Wide-angle X-ray diffraction measurement revealed that the crystalline structure of the polymers obtained from 6-MNA was different from that of the polymer obtained from *n*-nonanoic acid.

Introduction

Poly(β-hydroxyalkanoate)s (PHAs) of the general structure shown below are accumulated by many diverse microorganisms:[1–4]



The ability of bacteria to accumulate massive amounts of PHAs is a natural mechanism used to provide the cell with an organic reserve acid in a reduced form that is osmotically inert. For this purpose, the PHAs are accumulated as a result of nutrient imbalance when the environment still contains an excess of a suitable carbon source.[3,4] The polymer is formed as intracellular inclusion bodies, which are subsequently consumed by enzymatic depolymerization reactions under energy or carbon starvation conditions. In addition to its biodegradability, the lower members of the PHA series with $x = 0$ and 1 are also biocompatible and of interest for biomedical applications.[5]

The microorganism *Pseudomonas oleovorans* is known to form PHAs with long alkyl chains, $x = 2$ or higher, from alkanes,[6,7] alkanoic acids,[8,9] and alkanols.[7,10] Recently, octanoic acids with the methyl groups at the 5-, 6-, and 7-positions of the side chain ($x = 5$) were also used as substrates to grow *P. oleovorans*, and polyesters containing methyl-branched 3-hydroxyalkanoate units were obtained.[11] In this manner, a PHA containing 7-methyl-3-hydroxyoctanoate units was produced by this microorganism when it was grown on pure 7-methyloctanoic acid. No PHAs were obtained when 5- or 6-methyloctanoic acids were used as the only substrates, but copolymers containing these methyl-branched units were produced when *P. oleovorans* was grown with either 5- or 6-methyloctanoate and a second good polymer-producing substrate. In the present study, we evaluated longer chain methyl-branched alkanoic acids, including the methylnonanoic, -decanoic, and -heptanoic acids as substrates both alone and with cosubstrates.

Experimental Section

Synthesis of Substrates. 8-Methylnonanoic Acid (8-MNA). A procedure similar to that reported in the literature[11,12] was used to synthesize methyl-branched nonanoic and decanoic acids. A typical example for the synthesis of 8-MNA is as follows: 43.44 g (0.240 mol) of 5-bromovaleric acid was dissolved in 320 mL of anhydrous tetrahydrofuran (THF),[13] the solution was cooled to −20 °C under a dry argon atmosphere, and then 85 mL (0.255 mol) of a 3 M methylmagnesium chloride solution in THF was added dropwise while keeping the temperature below −15 °C. After 15 min of stirring, 24 mL (0.240 mol) of a 0.1 M Li_2CuCl_4[14] in THF was added, followed by the dropwise addition of a solution of the Grignard reagent prepared from 42.28 g (0.280 mol) of 1-bromo-3-methylbutane and 6.8 g (0.280 mol) of magnesium in 300 mL of anhydrous THF. The temperature was kept between −25 and −20 °C. The mixture was allowed to warm to room temperature and stirring was continued overnight, after which the solution was poured into 1 L of an ice cold, 20% H_2SO_4 aqueous solution. The aqueous phase which separated was saturated with NaCl and extracted with ether. The combined organic phases were extracted with a solution of 50 g of KOH in 100 mL of water. After these extracts were acidified with 1 L of 20% H_2SO_4 solution, an oily layer separated. This layer was dried over Na_2SO_4 and distilled under vacuum. Yield 31.4 g (76.1%); bp 133–137 °C/11 Torr; NMR (200 MHz, $CDCl_3$, TMS) δ = 0.85 (d, 6 H, CH_3), 1.05–1.45 (m, 9 H, H-4, H-5, H-6, H-7, H-8), 1.45–1.70 (m, 2 H, H-3), 2.33 (t, 2 H, H-2), 11.7 (s, 1 H, COOH).

7-Methylnonanoic Acid (7-MNA). This substrate was also prepared as described above from 46.8 g (0.240 mol) of 6-bro-

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mohexanoic acid and 38.4 g (0.280 mol) of 2-bromobutane to yield 27.0 g (65.4%) of distilled product: bp 125–129 °C/11 Torr; NMR (200 MHz, $CDCl_3$, TMS) δ = 0.85 (m, 6 H, CH_3), 1.05–1.45 (m, 9 H, H-4, H-5, H-6, H-7, H-8), 1.45–1.70 (m, 2 H, H-3), 2.35 (t, 2 H, H-2), 11.7 (s, 1 H, COOH).

6-Methylnonanoic Acid (6-MNA). This substrate was prepared as described above from 43.44 g (0.240 mol) of 5-bromovaleric acid and 42.30 g (0.280 mol) of 2-bromopentane to yield 23.5 g (56.9%) of distilled product: bp 127–131 °C/11 Torr; NMR (200 MHz, $CDCl_3$, TMS) δ = 0.87 (m, 6 H, CH_3), 1.05–1.50 (m, 9 H, H-4, H-5, H-6, H-7, H-8), 1.55–1.77 (m, 2 H, H-3), 2.35 (t, 2 H, H-2), 11.7 (s, 1 H, COOH).

9-Methyldecanoic Acid (9-MDA). This substrate was synthesized as described above from 46.8 g (0.240 mol) of 6-bromohexanoic acid and 42.30 g (0.280 mol) of 1-bromo-3-methylbutane to yield 22.2 g (47.2%) of distilled product: bp 143–146 °C/11 Torr; NMR (200 MHz, $CDCl_3$, TMS) δ = 0.90 (d, 6 H, CH_3), 1.05–1.45 (m, 11 H, H-4, H-5, H-6, H-7, H-8, H-9), 1.55–1.70 (m, 2 H, H-3), 2.35 (t, 2 H, H-2), 11.7 (1 H, COOH).

7-Methyldecanoic Acid (7-MDA). This substrate was also prepared as described above from 46.8 g (0.240 mol) of 6-bromohexanoic acid and 42.3 g (0.280 mol) of 2-bromopentane to yield 22.5 g (47.8%) of the distilled product; bp 136–139 °C/11 Torr, NMR (200 MHz, $CDCl_3$, TMS) δ = 0.85 (m, 6 H, CH_3), 1.05–1.45 (m, 11 H, H-4, H-5, H-6, H-7, H-8, H-9), 1.55–1.70 (m, 2 H, H-3), 2.35 (t, 2 H, H-2), 11.7 (s, 1 H, COOH).

2,6-Dimethylhept-5-enoic Acid (2,6-DMHA). This substrate was synthesized by a procedure adapted from the literature.[15] A mixture of 30 g of 2,6-dimethylhept-5-enal, 10 g of 10% NaOH aqueous solution, 150 mL of water, and 35 g of Ag_2O was reacted at 30–35 °C by stirring continuously. The reaction mixture was filtered after 1 h, and the aqueous layer was acidified with concentrated HCl. After cooling with ice, an oily layer was extracted with ether, dried with sodium sulfate, and distilled under vacuum: yield 26.5 g (84%); bp 98–101 °C/11 Torr; NMR (200 MHz, $CDCl_3$, TMS) δ = 1.18 (d, 3 H, CH_3 on C-2), 1.60 (s, 3 H, CH_3 on C-5), 1.69 (s, 3 H, CH_3 on C-5), 1.95–2.20 (m, 2 H, H-2), 2.45–2.6 (m, 1 H, H-3), 5.1 (t, 1 H, H-4 vinylic proton), 11.7 (s, 1 H, COOH).

Fermentation. Stock cultures of *P. oleovorans* (ATCC 29347) were used throughout the experiments. The strains were maintained at 4 °C on nutrient agar plates using the modified mineral E* medium[11] described below with a 20 mM *n*-nonanoic acid as the carbon source. The culture was grown in a 1-L solution of mineral medium containing $(NH_4)_2HPO_4$ (1.1 g), K_2HPO_4 (5.8 g), KH_2PO_4 (3.7 g), 10 mL of 0.1 Mg SO_4, and 1.0 mL of a microelement solution (this microelement solution contained $FeSO_4 \cdot 7H_2O$ (2.78 g), $MnCl_2 \cdot 4H_2O$ (1.98 g), $CoSO_4 \cdot 7H_2O$ (2.81 g), $CaCl_2 \cdot 2H_2O$ (1.67 g), $CuCl_2 \cdot 2H_2O$ (0.17 g), and $ZnSO_4 \cdot 7H_2O$ (0.29 g) in 1 L of 1 N HCl). The alkanoic acids were added at a total concentration of 20 mM, and the pH was adjusted to 7.00. For small-scale fermentations, the cells were grown under aerobic conditions in either 250-mL, 1-L, or 1.5-L cultures, which were agitated at 250 rpm at 30 °C in a rotary shaker (Labline Orbit Environ shaker). Individual carbon sources (20 mM concentration) or mixtures of sources (10 mM of each) of either 1- or 1.5-L volume were inoculated with either 10.4 or 60 mL of an inoculum obtained from the cultures described above with 20 mM *n*-nonanoic acid as the carbon source, respectively.

Polymer Yield Determinations. Samples of 5 mL were withdrawn periodically from a 250-mL culture, and the optical density was determined at 660 nm on a 1-cm layer thickness. At the maximum optical density the polymer yield in the cells was also at a maximum, and the cells were harvested at this point. A typical plot of optical density *vs* fermentation time for the growth of *P. oleovorans* on 8-MNA is shown in Figure 1.

Harvesting of Cells. The cells grown in 1-L cultures were harvested by centrifugation (Sorvall RC2-B, 5 °C, 4000 rpm) at the maximum polymer yield as estimated by the OD of the cells. The cells were lyophilized on a Freeze Dry System (Labconco). The dry cell weights were determined gravimetrically.

Isolation and Fractionation of Polymer. The polymer was extracted from lyophilized cells in a Soxhlet extractor with 500 mL of chloroform. After the solvent was evaporated to 4–5 mL, the solution was filtered through glass wool and the polymer was precipitated into 300 mL of vigorously stirred methanol. After two precipitation cycles, the polymer was dried under vacuum for 2 days. The results for the harvesting time, total biomass, and polymer yield are listed in Table 1. The polymer that was isolated in this manner was collected, washed with methanol several times, and dried. This product is designated as fraction I. The second fraction, fraction II, was obtained by evaporating the solvent obtained from the precipitation, washing the solid obtained with methanol several times, and drying. Both the first and second fractions of polymer were dried under vacuum for 2 days at room temperature, and total yield of the polymer obtained from the dry biomass was determined gravimetrically. The results are listed in Table 2.

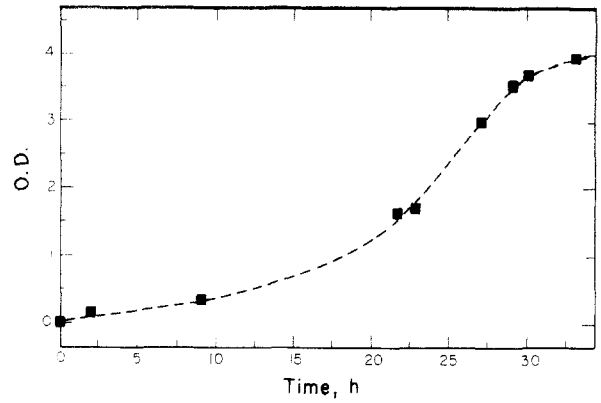


Figure 1. Growth of *P. oleovorans* on 8-MNA.

Table 1. Growth of *P. oleovorans* on Methyl-Branched Alkanoic Acids

substrate[a]	harvesting conditions: OD	harvesting conditions: hours	cell yield, g/L	yield of polymer, g/L	polymer in dry cells, wt %
6-MNA	1.98	27	0.570	0.103	18.2
6-MNA + sodium butyrate	2.70	50	0.840	0.110	13.2
6-MNA + NA	3.70	27	1.550	0.447	28.8
7-MNA	2.91	33	1.380	0.187	13.6
7-MDA	0.56	41	no measurable polymer		
7-MDA + NA	3.60	30	1.500	0.330	22.0
8-MNA	3.96	33	1.760	0.250	14.2
9-MDA	4.96	23	3.050	0.630	20.7
2,6-DMHA	0.58	28	no measurable polymer		
2,6-DMHA + NA	2.76	27	1.600	0.200	12.1
DA	4.92	19	2.540	0.730	28.7
NA	4.50	16	2.320	0.530	22.9

[a] For abbreviations, see text.

Table 2. Molecular Weights of Polymer Fractions

substrate	fractions	%	M_w	M_n	M_w/M_n
6-MNA			861 000	338 000	2.55
6-MNA + sodium bytrate			864 000	384 000	2.26
7-MNA			137 000	100 000	1.37
8-MNA	I	88	163 000	102 000	1.60
	II	12	115 000	70 000	1.64
9-MDA	I	87	258 000	161 000	1.61
	II	13	186 000	118 000	1.58
2,6-DMHA + NA	I	83	329 000	175 000	1.88
	II	17	256 000	140 000	1.83
NA	I	82	269 000	181 000	1.50
	II	12	192 000	120 000	1.58
DA			201 000	140 000	1.41

Molecular Weight Measurements. The molecular weights of the polymer were determined by gel permeation chromatography (GPC) with a Waters Model 6000 A solvent delivery system, a Mode 401 refractive index detector, and a Mode 730 data module with two Ultrastyragel linear columns in series. Chloroform was used as the eluent at a flow rate of 1.0 mL/min. Sample concentrations of 3–5 mg/mL and injection volumes of 150 μL were used. A calibration curve was generated with four polystyrene standards (600 000, 120 000, 22 000 and 2950 g/mol) of low polydispersity, which were purchased from Polysciences.

Methanolysis and GC Analysis. For determination of the polymer composition, 2 mg of the polymer was degraded by methanolysis by heating for 140 min at 100 °C with a solution of 1 mL of 15% sulfuric acid dissolved in methanol containing 1.0 mL of chloroform.[8] The solution was washed with 1.0 mL of water, and the chloroform layer was analyzed by gas chromatography (GC) using a Perkin-Elmer 8500 GC and a Durobond Carbowax megabore capillary column (15 m × 0.54 mm; carrier gas He, 17 mL/min; temperature program: 80 °C for 4 min, then the temperature was increased at 8 °C/min to 160 °C and held for 11 min).

NMR Spectra. ^1H and ^{13}C NMR spectra obtained on chloroform-*d* solutions were recorded at 17 °C on either a Varian XL 200 NMR spectrometer at 200 MHz (^1H) or a Varian XL 300 NMR spectrometer at 70.4 MHz (^{13}C).

Thermal Analysis. The glass transition temperature (T_g), melting temperature (T_m), and heat of fusion (ΔH_m) were measured on a DSC V4.0B DuPont 2000. The weight of each sample was typically 7–11 mg. The polymer samples were heated at a rate of 20 °C/min from −100 to +200 °C, quickly cooled, and then scanned a second time using the same heating rate and temperature range as for 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.

Wide-Angle X-ray Diffraction. WAXD measurements were made under reduced pressure by using a Statton camera and a Siemens K 710 H generator operating at 40 kV and 30 mA. The X-ray beam was pinhole collimated. Nickel-filtered Cu Kα radiation (λ = 1.541 Å) was used. The PHAs obtained from 6-MNA were cast as films (0.2–0.3 mm thick) from chloroform solutions and dried 3 days under vacuum at room temperature. The distance (D) of the film from the camera was calculated from the equations $\tan 2\theta = r/D$ (I) and $\lambda = 2d \sin \theta$ (II), where θ is the Bragg angle, by using $CaCO_3$ standard, taking λ = 1.542 Å, d = 3.036 Å, and radius r = 30 mm for the sharp circle in the diffractogram. For moderate angles, measurements from 10 to 30°, the sample to film distances used were 53.23 mm. The sample and film exposure time was 6–8 h.

Results

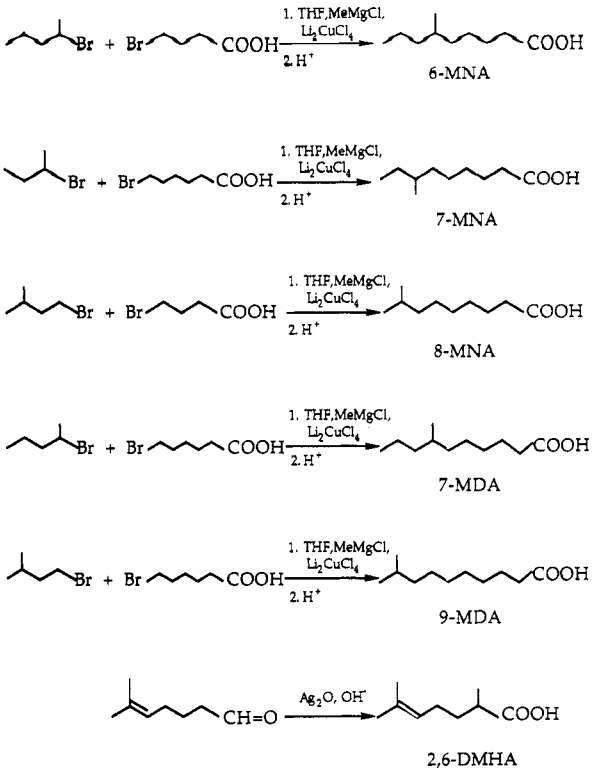
Production of Polyesters. *P. oleovorans* was grown separately on three different methylnoanoic acids (MNA) and two different methyldecanoic acids (MDA), including 6-MNA, 7-MNA, 8-MNA, 7-MDA, and 9-MDA, and on 2,6-DMHA. These substrates were prepared in high yields by the reactions summarized in Scheme I as described in the Experimental Section.

A typical growth plot is given in Figure 1, which shows the rate of growth of *P. oleovorans* with 8-MNA. To obtain a maximum polymer yield, the cells were harvested from the fermentation mixture when the maximum optical density was reached. Data for cell yields and polymer yield for each substrate are collected in Table I.

The growth of *P. oleovorans* on 6-MNA, 7-MNA, 8-MNA, and 9-MDA gave PHAs containing the expected methyl-branched units, but the 7-MDA and 2,6-DMHA substrates did not yield measurable amounts of polymers. Furthermore, cofeeding either 7-MDA or 2,6-DMHA with *n*-nonanoic acid (NA) produced only the PHA expected from NA alone. When compared with the use of NA and *n*-decanoic acid (DA) as single substrates, the polymer yields from 8-MNA, 7-MNA, 6-MNA, and 9-MDA were lower, as previously observed with other methyl-branched substrates, and the polymer yields decreased as the methyl branch was moved closer to the carboxylic acid group.

Table 2 contains the results of molecular weight measurements after separation of the crude polyester products into methanol-insoluble and methanol-soluble fractions as described in the Experimental Section. In all cases, fraction I was obtained in a much larger amount, generally more than 80%, and the number-average molecular weight, M_n, of this fraction was also higher. M_n values ranged between 102 000 and 181 000 for fraction I and between 70 000 and 140 000 for fraction II.

Scheme 1



In earlier investigations in this laboratory on the production of PHAs by *P. oleovorans* when grown on mixed substrates, it was observed that this bacterium can produce either copolymers or polymer mixtures, depending on the combinations used. This possibility was considered in the present study for the PHA product obtained by cometabolism with 6-MNA and NA, and an attempt was made to analyze the type of polymer produced by fractional precipitation. However, it was found that the solubilities of the PHAs obtained from 6-MNA alone and from NA alone were essentially identical in all solvent/nonsolvent combinations evaluated for that purpose, so the question remains unresolved of whether a true copolymer or a physical mixture of two PHAs was produced from cofeeding on 6-MNA and NA.

Compositions of Polyesters. The ^1H NMR spectra of the methyl-branched PHAs were similar to those of the original acids except that the hydrogen on the 3-position shifted to 5.20 ppm in the repeating units from 1.55–1.70 ppm in the alkanoic acids. The ^1H NMR spectra of the polymers obtained by cofeeding of either 7-MDA or 2,6-DMHA with NA had the same integration values for the methyl protons in each case, indicating that the PHAs obtained were essentially identical to the polymer obtained from NA alone.

The ^{13}C NMR spectra of the PHAs obtained from the methyl-branched substrates also confirmed the presence of methyl-branched units. A typical ^{13}C NMR spectrum, that of poly(3-hydroxy-6-methylnonanoate), is shown in Figure 2. The methyl group at the C-6 position in the alkyl substituent in this polymer has a doublet at 19.3 ppm, which can be assigned to the *R* and *S* configurations that are apparently present in equal amounts.

Methanolysis–GC analysis of the polymers obtained either from 6-MNA alone or by prefeeding with sodium butyrate showed the same compositions. From the peak area measurements, these polymers had 63% of 6-MNA units and 37% unbranched units as shown in Table 3. The

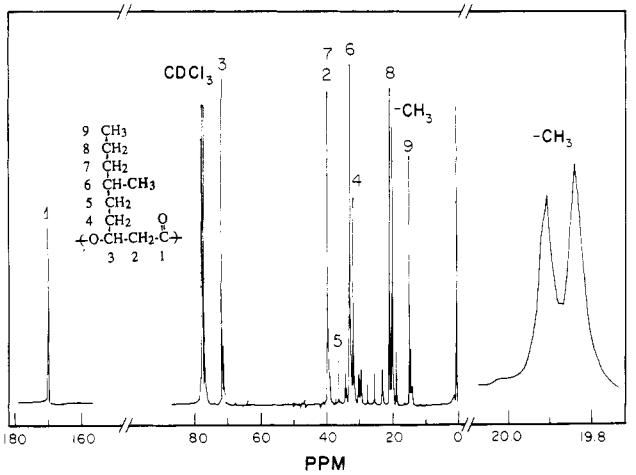


Figure 2. ^{13}C NMR spectrum of the polymer obtained from 6-MNA.

Table 3. Composition of the Polymers Obtained by Growth with 6-MNA Alone and with a Mixture of 6-MNA and Sodium Butyrate[a]

	polymer comp, % of GC peak area	
units in polymers[b]	from 6-MNA alone	from 6-MNA + sodium butyrate
methyl-3-hydroxyvalerate	1.3	0.7
methyl-3-hydroxyhexanoate	6.7	12.0
methyl-3-hydroxyheptanoate	6.3	5.0
methyl-3-hydroxyoctanoate	0.9	1.6
methyl-3-hydroxynonanoate	11.0	8.1
methyl-3-hydroxy-6-methyl-nonanoate	63.0	64.0
methyl-3-hydroxyundecanoate	2.2	1.9
other	9.3	7.0

[a] Repeating unit compositions as determined by GC analysis of the products of methanolysis. [b] From peaks in GC assigned to the methyl esters of these 3-hydroxyalkanoate units obtained by methanolysis.

Table 4. Thermal Analysis of Polymers Produced from 6-MNA, 7-MNA, 8-MNA, 9-MDA, and NA As Determined by DSC

	DSC thermograms					
	1st heating cycle			2nd heating cycle		
substrate	T_g, °C	T_m, °C	ΔH J/g	T_g, °C	T_m, °C	ΔH J/g
6-MNA		65	32.2		58	20.8
6-MNA + sodium butyrate		67	25.2		60	20.6
7-MNA	−45	54	5.3	−44		
	−24			−24		
8-MNA	−14	57	15.4	−19		
				−43		
9-MDA	−16	56	24.8	−21		
NA	−29	58	10.4	−33		

presence of such a large amount of unbranched units in both of the samples in Table 3 is very surprising and difficult to rationalize. The compositions of the polymers obtained from the other substrates in Table 2 were not determined.

Properties of Polyesters. The temperatures observed in the first heating cycle DSC thermograms for the glass transition (T_g onset) and melting transition (T_m) and the enthalpy of fusion (ΔH_m) are compiled in Table 4. The polymers produced with 6-MNA had higher melting points and higher crystallinities than the other methyl-branched polyesters, which had properties that were essentially the same as those obtained from either NA or DA alone.

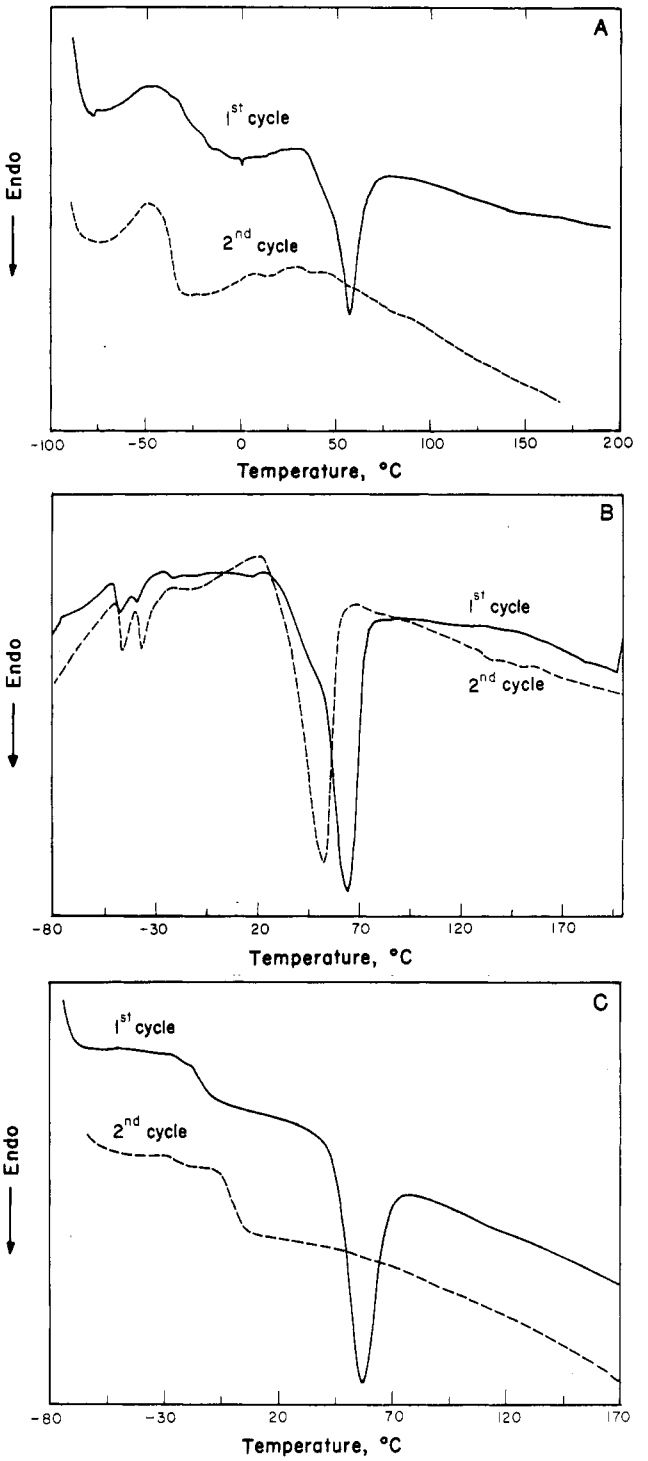


Figure 3. DSC thermograms for the polymers from (A) NA, (B) 6-MNA, and (C) 8-MNA.

Additionally, the second heating cycle thermograms of the polymers obtained for 6-MNA showed that the recrystallization rates were very fast, while polymers from 9-MDA, 8-MNA, 7-MNA, and NA remained amorphous during the second heating, indicating that their crystallization rates were very slow (see Figure 3).

The cells were harvested at maximum optical densities to obtain the highest yields of polyester. Some studies were also carried out to determine the properties of the PHAs which were harvested after that time. The data in Table 5 show the results obtained in that study. Delaying harvesting caused lower polymer yields even though cell yields remained constant. Quite surprisingly, however, the T_m values of the polymer from 6-MNA increased to

Table 5. Polymers Obtained after Longer Harvesting Times

6-MNA	harvesting conditions: OD	harvesting conditions: hours	polymer in dry cells, wt %	thermal properties by DSC[a]: T_g, °C	T_m, °C	ΔH, J/g
6-MNA	0.98	72	5.5		75[b]	22.0
6-MNA + sodium butyrate	1.89	70	3.5		81	22.9
6-MNA + NA	2.16	49	2.9		48	1.5
					70	0.7
7-MDA + NA	2.70	32	4.0	-33	44	1.4
8-MNA	3.00	50	6.7	-14	57	15.0
9-MDA	3.12	46	10.6	-19	56	12.0

[a] Taken from the thermograms for the first heating cycle. [b] The T_m in the second heating cycle thermogam was 65 °C.

75–81 °C from 67 °C while the T_m values of polymers from 8-MNA or 9-MDA stayed at the original value, 57 °C, as shown in Table I.

The wide-angle X-ray diffractograms were recorded on films cast from chloroform solution (see Experimental Section) for the PHA obtained from either 6-MNA alone or by prefeeding with sodium butyrate. The diffraction patterns of these samples contained four sharp lines and one broad halo (d_5) corresponding to $d_1 = 20$, $d_2 = 11$, $d_3 = 9.2$, $d_4 = 7.2$, and $d_5 = 4.7$ Å. These values are different from those obtained for the PHA obtained from NA, which were $d_1 = 19$, $d_2 = 5.0$, $d_3 = 4.6$, and $d_4 = 4.1$ Å. The latter crystalline reflections correspond to the data reported by Marchessault and co-workers for the same polymer.[17]

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