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## Preparation, characterization, and modification of poly-[beta]-hydroxyalkanoates from *Pseudomonas oleovorans*/

Young Baek Kim  
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PREPARATION, CHARACTERIZATION, AND MODIFICATION OF  
POLY- $\beta$ -HYDROXYALKANOATES FROM *PSEUDOMONAS OLEOVORANS*

A Dissertation Presented

by

YOUNG BAEK KIM

Submitted to the Graduate School of the University  
of Massachusetts in partial fulfillment of the  
requirements for the degree of

DOCTOR OF PHILOSOPHY

May 1991

Polymer Science and Engineering

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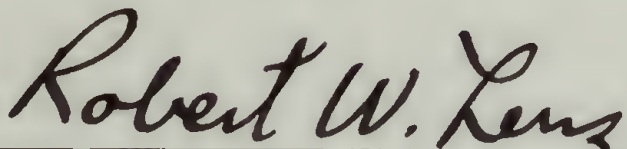
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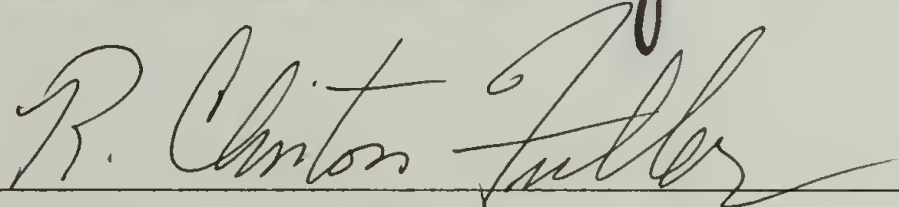
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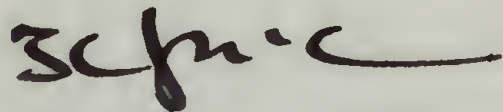
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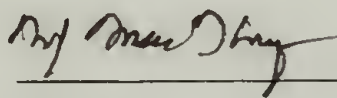
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## ABSTRACT

# PREPARATION, CHARACTERIZATION, AND MODIFICATION OF POLY- $\beta$ -HYDROXYALKANOATES FROM *PSEUDOMONAS OLEOVORANS*

MAY 1991

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Polymer production by *P. oleovorans* grown with various carbon substrates was investigated. Under the experimental conditions employed in this study, no limitation of nutrient was necessary to induce for this microorganism to produce polymers, but limiting nitrogen improved the polymer yield. The main repeating unit in polymers produced from n-alkanoic acids longer than heptanoic acid was either 3-hydroxyoctanoate or 3-hydroxynonanoate.

Carbon sources examined for growth and PHA production were classified according to growth results. Both physical and chemical properties of the carbon substrate affected cell growth and PHA production. When a substituent was present, the better growth and PHA production were, the longer the separation between the carboxylic acid and the substituent was.

Polymers containing various unusual groups such as olefin, nitrile, ester, bromine, alcohol, cyclohexane, and phenyl group were obtained by growing *P. oleovorans* with



either single carbon substrate or mixtures of two carbon substrates.

Ninety nine percent of repeating units in a PHA prepared from cells grown with solely with 10-undecenoic acid, UND:, contained olefin group. PHAs produced from mixtures of two carbon substrates both of which support cell growth and PHA production were generally random copolymers with the exception of the polymer produced from mixtures of 5-phenylvaleric acid and either n-nonanoic acid, NA, or n-octanoic acid, OA.

Many carbon substrates that support cell growth without PHA production were incorporated into the polymer when these substrates were co-fed to *P. oleovorans* with NA or OA.

Polymers containing repeating units with bromine group could be prepared only when  $\omega$ -bromoalkanoic acids were fed in the presence of NA, but a polymer containing nitrile group were obtained when 11-cyanoundecanoic acid was fed in the presence of either NA or OA.

PHAs containing unsaturated units were crosslinked by heating in the air or by heating with peroxides under nitrogen atmosphere to get rubber elastic products. Epoxidation of these polymers was also partially investigated.

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## CHAPTER I

### GENERAL INTRODUCTION

For decades, the plastics industry has worked on formulating materials that are durable, long lasting, and resistant to environmental factors. Synthetic polymers fulfill most of such demands and therefore most of these synthetic materials are not biodegradable.

The amount of plastic production has grown to nearly 60 billion pounds per year in U.S., and almost a third of plastics production is used for relatively short-term, disposable purposes. Packing waste is about one third by weight of consumer waste which contains paper (48 %), glass (27 %), and plastics (11 %) [Thayer, 1990] and the plastic component of municipal solid waste is more than 7 % by weight and 18 % by volume [Sadun et al., 1990].

The tremendous production and use of plastic materials around the world has created problems of massive waste disposal, both planned and random. For example, the effects of plastic wastes on marine environments have been investigated, and it was estimated that one million marine animals are killed by nondegradable plastic debris discarded into marine environments [Bean, 1987; Leaversuch, 1987; Pruter, 1987; Wilber, 1987].

In respond to the attention focused on plastic wastes, industry began to develop plastics that disintegrate readily, and several of these new materials are on the market for

six-pack connectors, trash bags, grocery bags, and backing for disposable diapers. These products are claimed to be "biodegradable" by the manufacturers, and several different mechanisms are employed in these products to render them "biodegradable".

One approach to "biodegradation" is to incorporate photosensitive groups into the polymers so that sunlight initiates photochemical reactions that lower molecular weight of the polymers. As a result, the plastic article made of these polymers become brittle when they are exposed to sunlight so that fragmentation can occur, and the resulting low molecular weight products can be utilized by living organisms as carbon or energy source.

A second approach is to blend a small amount of a natural, biodegradable polymer, generally starch, with the principal polymer. Microorganisms will readily digest the starch, presumably weakening and breaking down the plastic article, and the manufacturers claim that through the use of additives the remaining plastic fragments can be either degraded photochemically when exposed to light, or can break down oxidatively when buried. If not degraded completely by these processes, an increase in exposed surface area or a reduction in molecular weight is believed to make the fragments more susceptible for microorganisms to attack. However, in studies investigating biodegradability of "biodegradable plastics" prepared by blending with starch



there was no evidence that biodegradation was obtained [Thayer, 1990; Sadun et al., 1990; Gilmore et al., 1990].

In another approach, a starch-grafted polymethyl methacrylate copolymer was developed having grafted side chains with molecular weight of approximately 500,000 [Dennenberg et al., 1978]. Films of this polymer showed excellent susceptibility to fungal growth, and some samples lost more than 40 % of their weight after 22 days of incubation with *Aspergillus niger*.

Polyolefins can be biodegraded if they are shortened to C<sub>12</sub>-C<sub>40</sub> molecules as many organisms metabolize alkanes in this range by conversion of the end carbons to carboxylic groups [Traxler and Flannery, 1968; Foster, 1962b].

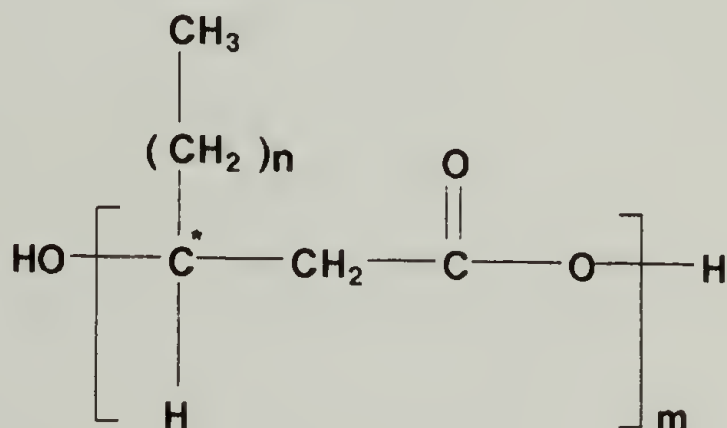
Naturally occurring polymers are supposed to be biodegradable by nature, and studies on biodegradation of natural rubber [Taysum, 1966; Spence and Van Niel, 1936; Lightbody et al., 1954; MacLachlan et al., 1966; Turner, 1967; Heap and Morrel, 1968; Dunn and Hart, 1969] and polysaccharides have been carried out [Siu, 1951; Wrick, 1968 a, b; Selby, 1969]. Besides these polymers biodegradable poly- $\beta$ -hydroxyalkanoates have been discovered in various microorganisms as discussed in next section.

#### A. Occurrence of Poly- $\beta$ -hydroxyalkanoates (PHAs)

PHAs are produced by a wide variety of microorganisms as intracellular energy and carbon storage material. The

microorganisms capable of accumulating PHAs are listed in Table 1 with the carbon sources that support PHA production in each microorganism. Most of the organisms are capable of accumulating PHA in amounts varying from 30 to 80 % of their cellular dry weight.

PHAs are an ideal form of reserve material because it is a highly reduced molecule, and being virtually water insoluble, it exerts a negligible osmotic pressure. PHAs have the following structure:



Poly-β-hydroxyalkanoate

Every β-hydroxyalkanoate unit in a PHA has a chiral center at the β position from the ester group, and all of the units have R configuration. This type of isotacticity is thought to be important in the biodegradation of PHAs, and no PHA produced by microorganisms containing repeating unit with S configuration has been reported.

The first discovered and most abundant PHA in nature is poly-β-hydroxybutyrate, PHB (n=0), which was isolated from *Bacillus megaterium* by Lemoigne in 1925 [Lemoigne, 1925].

PHB had been thought to be the only kind of storage polyester produced by microorganisms until polyesters containing repeating units longer than 3-hydroxybutyrate were isolated from microorganisms in sewage sludge in 1974 [Wallen and Rohwedder, 1974]. The polymer contained 3-hydroxybutyrate (C4) and 3-hydroxyvalerate units (C5) as major repeating units with 3-hydroxyhexanoate (C6) and possibly 3-hydroxyheptanoate (C7) units as minor components. The polymer extracted from marine sediments was found to contain at least 11 short-chain 3-hydroxyalkanoate units, the principal ones being C4 and C5 [Findlay and White, 1983]. Also, PHAs containing C4, C6, and C8 units were discovered in sewage sludge [Odham et al., 1986]. The compositions of various PHAs found in specific microbes and in environmental samples are collected in Table 2.

PHA production by *A. eutrophus* and *P. oleovorans* has been most intensively studied. *A. eutrophus* produces PHB when it is grown with various carbon substrates such as ethanol, glucose, and  $H_2/CO_2$ /air.

3-Hydroxybutyrate/3-hydroxyvalerate copolymers (PHB/PHV) are produced from *A. eutrophus* grown with glucose in the presence of either propionic acid or valeric acid as a cosubstrate. *A. eutrophus* grown with only propionic acid afforded a copolymer containing 57 mol% C4 and 43 mol% C5, and the PHA content of the bacteria was 35 wt% [Doi et al., 1986]. A copolymer containing 90 mol% C5 units was obtained



from *A. eutrophus* grown with only valeric acid [Doi et al., 1987a].

Terpolymers of C4, C5, and 5-hydroxyvalerate were obtained from *A. eutrophus* grown with mixtures of 5-chloropentanoic acid and pentanoic acid [Doi et al., 1987b], and PHAs containing C4 units and 4-hydroxybutyrate units have been prepared from *A. eutrophus* grown with mixtures of glucose and  $\gamma$ -butyrolactone [Doi et al., 1988]. The mole fraction of 4-hydroxybutyrate unit was varying as a function of the concentration of carbon sources and pH of the culture.

The majority of bacteria investigated accumulate PHA in respond to a nutrient limitation. The reported nutrient absence of which in the growth medium induced polymer production for various microorganisms are listed in Table 3.

PHB is formed within the cell's cytoplasm as granule inclusions, which can be observed under the light microscope as refractile bodies [Shively, 1974; Dawes and Senior, 1973; Clayton and Siström, 1978]. The shape of the PHB granules is usually spherical, and the size varies according to organism. The diameter of PHB granules accumulated in *Bacillus megaterium* is in the range 0.2 to 0.7  $\mu\text{m}$ . These granules are enclosed by membranes of between 2.5 and 4.5 nm thickness which have the PHB polymerase tightly bound to them. The granule membranes of some bacteria, e.g., *Rhodospirillum rubrum* and *Azobacter beijerinckii*, also possess PHB depolymerase and so these granules are self-hydrolyzing,

whereas in other organisms the depolymerase is a soluble enzyme.

Ballard and coworkers investigated the changes of number and size of granules in *A. eutrophus* grown under nitrogen-limited conditions at different growth times and they concluded that the number of granules per cell was fixed at the earliest stages of polymer accumulation [Ballard et al., 1987]. That is, the number of granules was not increasing, but the size of granules increased uniformly during the PHA accumulation. Polymer production in *A. eutrophus* ceased when a PHB content of 80 wt% was attained, although PHB synthetase activity remained high. This result suggested that physical constraints operated and the cell was unable to accommodate more polymer within the fixed existing amount of cell wall material, despite the availability of substrate and active synthetase. The average molecular weight of PHB granules was found to be  $5 \times 10^9$  as determined by light scattering [Ellar et al., 1968]. Therefore, there were at least 1,000 polymer chains in a single PHA granule considering that the molecular weight of isolated PHB was between  $10^3$  to  $10^6$  depending on the microorganism. The molecular weight of PHA was characteristic for a microorganism [Anderson and Dawes, 1990].

In the early X-ray diffraction studies of solid PHB it was concluded that PHB granules *in vivo* are crystalline [Alper et al., 1963; Lundgren et al., 1965]. However, in recent studies it was concluded that the PHB granules in



living cells are amorphous [Bernard and Sanders, 1988; Bernard and Sanders, 1989; Kawaguchi and Doi, 1990].

### B. Properties of PHAs

PHB is a brittle polymer with a compact right-handed helix with a twofold screw axis and a fiber repeat of 0.596 nm [Cornibert and Marchessault, 1972; Okamura and Marchessault, 1967]. Investigations on the solution behavior of PHB showed that this helical structure was retained in chloroform solution as studied by viscometry, light scattering, optical rotatory dispersion, and  $^1\text{H}$  NMR spectroscopy [Akita et al., 1976; Cornibert et al., 1970; Doi et al., 1986; Marchessault et al., 1970, 1981; Alper et al., 1963; Lundgren et al., 1966]. The Mark-Howwink-Sakurada parameters of PHB in various solvents at various temperatures are listed in Table 4.

PHB and polypropylene have similar degrees of crystallinity and  $T_g$ 's, although their chemical structures are completely different. Barham and coworkers investigated crystallization behavior in detail [Barham, 1984; Barham et al, 1984]. PHB has much less solvent resistance, but it has better natural resistance to UV weathering. The physical properties of PHB and polypropylene are compared in Table 5 [Blembergen, 1985; Winton, 1985; Howell, 1982; King, 1982].

The toughness and flexibility of PHB are improved by incorporating C5 units into the polymer which reduces  $T_m$  and



$T_g$ . The degree of crystallinity and crystallization rate are reduced with increased fraction of C5 units in the polymer [Bloembergen et al., 1986]. PHB/PHV copolymers are isodimorphic with a melting temperature minimum at a C5 content of approximately 30 mole%. For compositions of higher fraction of C5 units than 30 mole%, C4 units crystallizes in the PHV lattice and for compositions of lower fraction of C5 units, C5 units crystallizes in the PHB lattice. The  $T_m$  and heat of fusion ( $\Delta H_m$ ) are plotted against the mol% of C5 units in the PHB/PHV copolymers in Figure 1 and 2 [Bluhm et al., 1986].

*P. oleovorans* produces PHAs containing side chains longer than ethyl group when it is grown with n-alkanes, n-alkenes, alkanoic acids, or alkenoic acids [De Smet et al., 1983; Brandl et al., 1989; Gross et al., 1989; Fritzsche et al., 1990 a,b,c]. PHAs produced from *P. oleovorans* are described in further detail in the appropriate sections in this thesis.

### C. Biosynthesis of PHA

As PHA is a storage material, it is appropriate to consider its metabolism as a cycle of synthesis and degradation. In most of the organisms so far investigated, PHB is synthesized from acetyl coenzyme A (acetyl-CoA) by a sequence of three reactions catalyzed by 3-ketothiolase (acetyl-CoA acetyltransferase), acetoacetyl-CoA reductase

(hydroxybutyryl-CoA dehydrogenase), and poly- $\beta$ -hydroxybutyrate synthetase. The PHB production in *Azobacter beijerinckii* was studied in most detail. This microorganism accumulates PHB under oxygen limited conditions. PHB synthesis and degradation pathway in this organism is shown in Figure 3. Two molecules of acetyl-CoA are condensed by the action of  $\beta$ -ketothiolase to release CoASH and form acetoacetyl-SCoA, which is then reduced to D(-)-3-hydroxybutyryl-SCoA, a reaction catalyzed by 3-hydroxybutyryl-SCoA dehydrogenase (acetoacetyl-SCoA reductase), an enzyme which utilizes NADPH at about fivefold the rate of NADH. 3-Hydroxybutyryl-SCoA is then the substrate for the granule membrane-bound PHB synthetase (polymerase) and simultaneously liberates CoASH. 3-Ketothiolase has been purified from various PHB-synthesizing bacteria [Dawes and Senior, 1973; Haywood et al., 1988a, Oeding and Schlegel, 1973; Nishimura et al., 1978; Suzuki et al., 1987; Tomita et al., 1983]. The presence of stereoselective acetoacetyl-SCoA reductase and PHB synthetase is responsible for the production isotactic [R] PHB in microorganisms [Fukui et al., 1987; Haywood et al., 1989; Haywood et al., 1988b,c].

A regulatory enzyme in PHB metabolism is acetyl-CoA acyltransferase, which is inhibited by high concentrations of free coenzyme A. Under balanced growth conditions, CoASH levels are high and the synthesis of PHB is inhibited. In nutrient limitation by carbon excess, the build up of NADH

inhibits citrate synthetase (TCA cycle), and acetyl-CoA levels rise to the point where inhibition by CoASH is overcome. The condensation reaction to acetoacetyl-CoA is possible, and PHB is synthesized.

Polymer degradation is controlled by the enzyme 3-hydroxybutyrate dehydrogenase through the oxidation of monomeric 3-hydroxybutyrate, which is formed by enzymatic hydrolysis.

These main regulatory elements of the cycle are supplemented by a second level of control: intermediates of the tricarboxylic acid cycle cause feed back inhibition of the enzymes. Thus synthesis and breakdown of PHB is linked to the metabolic state of the cell and to the carbon flux through intermediary metabolism.

A proposed PHB synthesis and degradation pathway in *P. oleovorans* is presented in Figure 4 [Lageveen et al., 1988].

#### D. Degradation of PHAs

Two mechanisms are responsible for *in vivo* degradation of PHA. Under sterile or aseptic conditions, PHA is degraded by a hydrolytic mechanisms, especially at high pH [Holland et al., 1987; Miller and Williams, 1987]. This type of degradation is important for medical applications, such as for use of PHA in drug release carriers or surgical sutures.



Extracellular enzymes capable of depolymerizing PHB and oligomers derived from the polymer have been found in a few organisms such as *Alcaligenes faecalis* [Taino et al., 1982], *Pseudomonas lemoignei* [Delafield et al., 1965; Lusty and Doudoroff, 1966; Nakayana et al., 1985], *Penicillium simplicissimum* [McLellan and Halling, 1988], or *Eupenicillium* sp. [McLellan and Halling, 1988], but research has concentrated on the depolymerase excreted by *Alcaligenes faecalis*. In addition to PHB polymerase, *A. faecalis* possesses a quite distinct extracellular oligomer hydrolase [Shirakura et al., 1983].

PHBs containing [S]-3-hydroxybutyrate units were not biodegradable as demonstrated in a biodegradability study of [R,S] stereoblock copolymer and [R,S] stereoatactic copolymer [Lenz et al., 1989].

Doi and colleagues compared the rates of degradations of various polyester films in buffer solutions, soil, and activated sludge. The thickness of the films was 0.07 mm. The copolymers containing 4-hydroxybutyrate and 3-hydroxybutyrate degraded more rapidly than either PHB or PHB/PHV copolymers under all conditions tested. The presence of C5 units in the PHB/PHV copolymers had no significant effect on biodegradation rate compared to the degradation rate of the PHB [Kunioka et al., 1989; Doi et al., 1989].

## E. Cloning and Expression of Genes

The genes involved in the biosynthesis of PHB by *A. eutrophus* have been cloned, and expressed in *E. coli*. [Peoples et al., 1987; Ploux et al., 1988]. The recombinant strains of *E. coli* were capable of accumulating a substantial amount of PHB as intracellular granules. Peoples and Sinskey have sequenced that structural genes for the three biosynthetic enzymes of *A. eutrophus* and shown that transcription occurs in the sequence polymerase, thiolase, reductase [Peoples and Sinskey, 1989a,b].

Recently, Huisman et al. identified two polymerases and a depolymerase in *P. oleovorans* [Huisman et al., 1991]. They cloned some of the genes which encode PHA polymerases from *P. oleovorans*, and expressed in *E. coli*. The recombinant strain of *E. coli*. cloned with the gene encoding one polymerase, and the strain cloned with the gene encoding another polymerase and depolymerase gave PHAs of identical composition.

Table 1. The accumulation of PHAs in various microorganisms  
[Brandl, 1990].

Geneus	Group <sup>a</sup>	PHA wt%	PHA producing substrate
<i>Aconetobacter</i>	10	<1	glucose
<i>Alcaligenes</i>	7	96	fructose
<i>Aphanothece</i>	CB	<1	NS
<i>Aquaspirillum</i>	6	ND	NS
<i>Azospirillum</i>	6	57	$\beta$ -hydroxybutyrate
<i>Axobacter</i>	7	73	glucose
<i>Bacillus</i>	15	25	glucose
<i>Beggiatoa</i>	2	57	acetate
<i>Beijerinckia</i>	7	38	glucose
<i>Caulobacter</i>	4	36	glucose/glutamate/yeast extract
<i>Chloroflexus</i>	1	<1	yeast extract/glycylglycine
<i>Chlorogloea</i>	CB	10	acetate, CO <sub>2</sub>
<i>Chromatium</i>	1	20	acetate
<i>Chromobacterium</i>	8	37	glucose/peptone
<i>Clostridium</i>	15	13	tryptour/peptone/glucose
<i>Derxia</i>	7	26	glucose
<i>Ectothiorhodospira</i>	1	ND	NS
<i>Escherichia<sup>b</sup></i>	8	ND	tryptone/glucose/yeast extract
<i>Gamnosphaeria</i>	CB	ND	ND
<i>Haemophilus<sup>b</sup></i>	8	ND	brain-heart-infusion
<i>Halobacterium</i>	13	38	glucose
<i>Hyphomicrobium</i>	4	ND	methanol, glucose
<i>Lamprocystis</i>	1	ND	NS
<i>Lampropedia</i>	10	ND	NS
<i>Leptothrix</i>	3	67	pyrubate
<i>Methylobacterium</i>	7	47	methanol
<i>Methylocystis</i>	ND	70	methane
<i>Methylosinus</i>	7	25	methane
<i>Micrococcus</i>	14	28	pentone/trypton
<i>Microcoieus</i>	CB	<1	NS
<i>Microcystis</i>	CB	ND	ND

Continued, next page



Table 1-Continued

<i>Moraxella</i>	10	ND	NS
<i>Mycoplana</i>	17	ND	methanol
<i>Nitrobacta</i>	12	ND	NS
<i>Nitrococcus</i>	12	ND	NS
<i>Nocardia</i>	17	14	butane
<i>Oceanospirillum</i>	6	ND	NS
<i>Paracoccus</i>	10	ND	NS
<i>Photobacterium</i>	8	ND	NS
<i>Pseudomonas</i>	7	67	methanol
<i>Rhizobium</i>	7	57	mannitol
<i>Rhodobacter</i>	1	60	acetate
<i>Rhodospirillum</i>	1	47	acetate
<i>Sphaerotilus</i>	3	45	glucose/peptone
<i>Spirillum</i>	6	40	lactate
<i>Spirulina</i>	CB	6	CO <sub>2</sub>
<i>Streptomyces</i>	17	4	glucose
<i>Syntrophomonas</i>	9	30	crotonate
<i>Thiobacillus</i>	12	ND	glucose
<i>Thiocapsee</i>	1	ND	NS
<i>Thiocystis</i>	1	ND	NS
<i>Thiodictyon</i>	1	ND	NS
<i>Thiopedia</i>	1	ND	NS
<i>Thiosphaera</i>	1	ND	acetone, CO <sub>2</sub>
<i>Vivrio</i>	8	ND	NS
<i>Xanthobacter</i>	7	ND	NS
<i>Zoogloea</i>	7	ND	yeast extract/casamino acids

a, after Bergey's manual

Group	Bacterium	Group	Bacterium
1	Phototrophic bacteria	14	Gram-positive cocci
2	Gliding bacteria	15	Endospore-forming rods and cocci
3	Sheathed bacteria	17	Actinomycetes
13	Archaeobacteria		

b, PHB found in cell membranes; ND(S), not detected(selected)

Table 2. Compositions of various PHAs found in specific microbes and in environmental samples [Brandl et al., 1990].

Source	C4	C5	C6	C7	C8	C9	C10	C11	C12
<i>R. rubrum</i>	x	x	x	x					
<i>Rb. sphaeroids</i>	x	x							
<i>P. oleovorans</i>	x	x	x	x	x	x	x	x	x
<i>P. cepacia</i>	x	x							
<i>A. eutrophus</i>	x	x							
<i>B. megaterium</i>	x	x							
<i>Aphanotheco</i>	x	x							
<i>Microvoleus</i>	x	x							
Marine sediments	x	x	x	x	x				
swage	x		x		x				
swage sludge	x	x	x	x					

Cx,  $\beta$ -hydroxyalkanoate monomer unit with a chain length of x carbon atoms

Table 3. List of limiting compounds leading to PHA formation  
[Brandl, 1990].

Limiting Compound	Organism
Ammonium	<i>Alcaligenes eutrophus</i>
	<i>Alcaligenes latus</i>
	<i>Pseudomonas oleovorans</i>
	<i>Pseudomonas cepacia</i>
	<i>Rhodobacter sphaeroides</i>
	<i>Speudomonas</i> sp. K.
	<i>Methylocystus oarvus</i>
	<i>Thiosphaera pantotropha</i>
	<i>Rhizobium</i> ORS 571
	<i>Spirillum</i> sp.
Carbon	<i>Hyphomicrobium</i> sp.
	<i>Azospirillum brasiliense</i>
Iron	<i>Pseudomonas</i> sp. K.
Magnesium	<i>Pseudomonas</i> sp. K.
	<i>Azotobacter vinelandii</i>
Oxygen	<i>Azobacter beijerinckii</i>
	<i>Rhizobium</i> ORS571
	<i>Rhodospirillum rubrum</i>
Phosphate	<i>Rhodobacter sphaeroids</i>
	<i>Caulobacter crescentus</i>
	<i>Pseudomonas oleovorans</i>
Potassium	<i>Bacillus thuringiensis</i>
	<i>Pseudomonas</i> sp. K.
Sulfate	<i>Pseudomonas oleovorans</i>
	<i>Rhodospirillum rubrum</i>
	<i>Rhodobacter sphaeroids</i>



Table 4. The Mark-Houwink-Sakurada parameters of PHB in various solvents at various temperatures.

Solvent	Temp °C	$K \cdot 10^4$	a	$\{\eta\} = K \cdot (M_w)^{a_{dl}} / g$	Reference
Chloroform	30	0.77	0.82		Marchessault et al., 1970
	30	1.90	0.74		Mola et al., 1975
	30	1.18	0.78		Akita et al., 1976
	30	1.66	0.76		Hirosye et al., 1979
Trifluoroethanol	30	2.51	0.74		Corbinet et al., 1970
	30	1.25	0.80		Akita et al., 1976
	25	2.22	0.76		Miyaki et al., 1977
	25	1.75	0.78		Hirosye et al., 1979
1,2-Dichloroethane	30	1.68	0.74		Hirosye et al., 1979

Table 5. Chemical and physical properties of polypropylene (PP) and poly-β-hydroxybutyrate (PHB) [Bloembergen, 1985; Winton, 1985; Howell, 1982; King, 1982].

Parameter	PP	PHB
Melting point, °C	171-186	171-182
Glass transition temperature °C	-15	5-10
Crystallinity, %	65-70	65-80
Density, g/cm <sup>3</sup>	0.905-0.94	1.23-1.25
Weight average molecular weight, x 10 <sup>-5</sup>	2.2-7	1-8
Flexural modulus, GPa	1.7	3.5-4
Tensile strength, MPa	39	40
Extension to break, %	400	6-8
UV resistance	poor	good
Solvent resistance	good	poor
Oxygen permeability, cm <sup>3</sup> /m <sup>2</sup> /atm/d	1700	45
Biodegradability	-	+
Approx. U.S. annual production, million ton	1.8	0

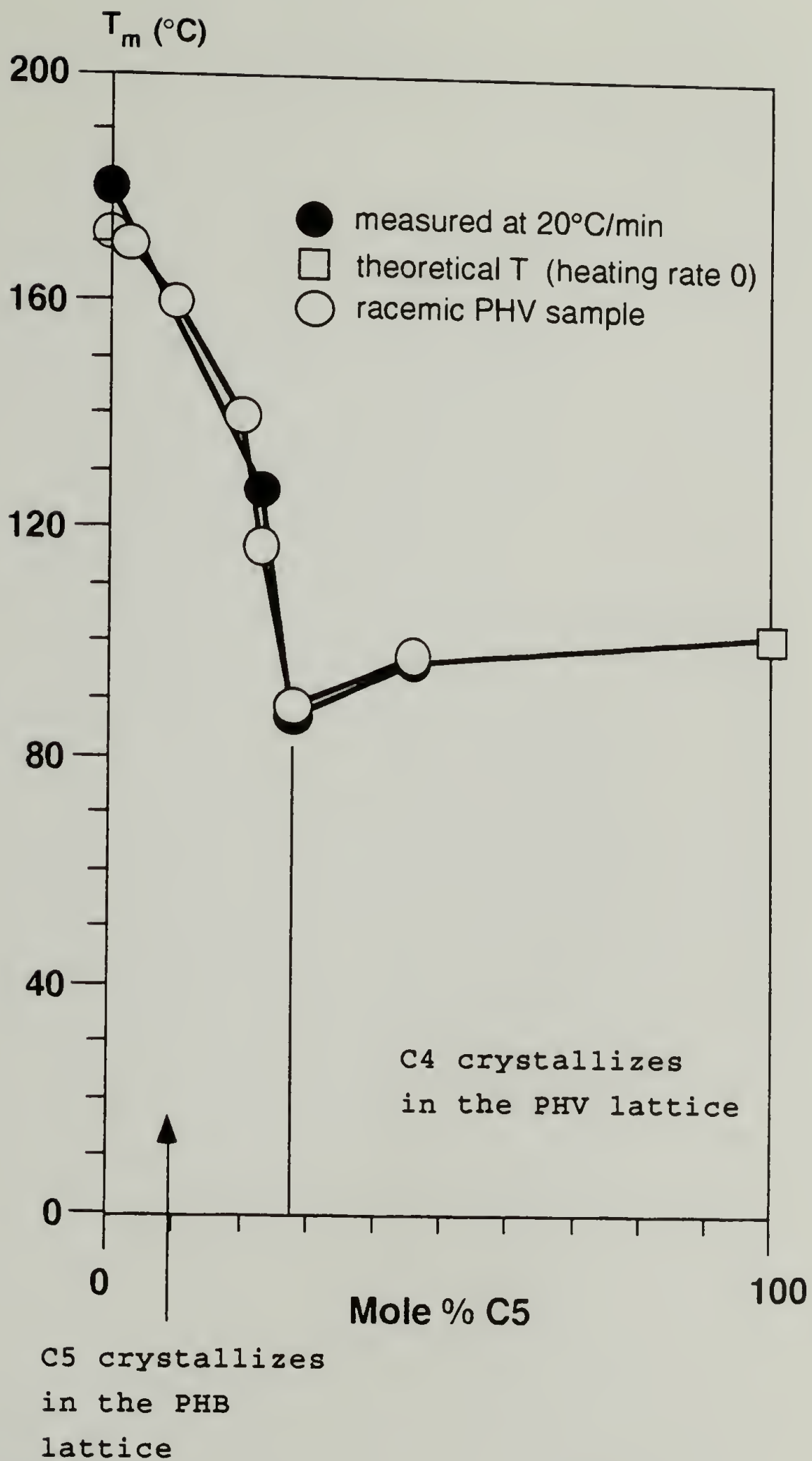


Figure 1. Melting point ( $T_m$ ) vs. composition curve for PHB/PHV copolymer.



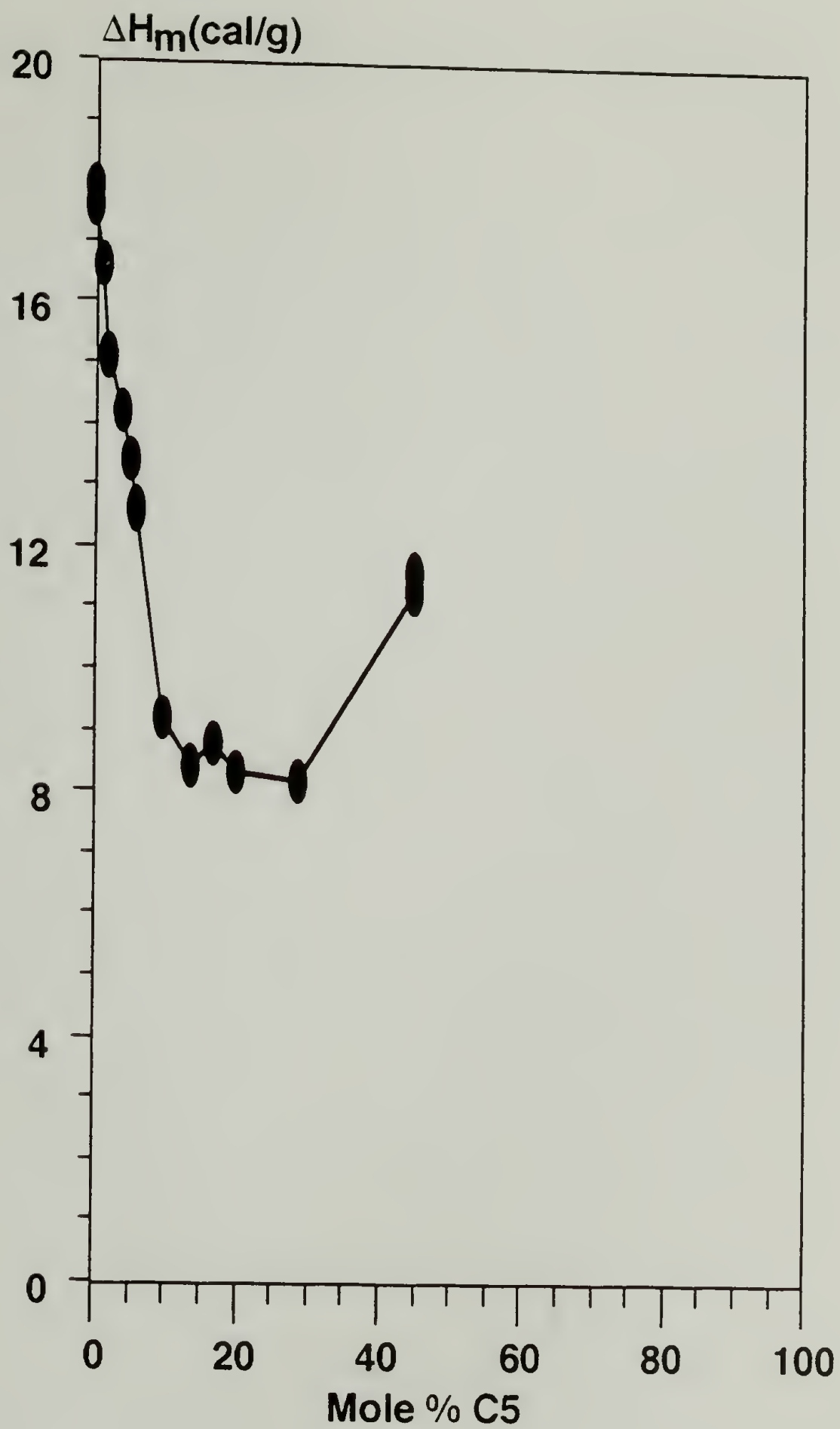


Figure 2. Heat of fusion vs. composition curve for PHB/PHV copolymers.

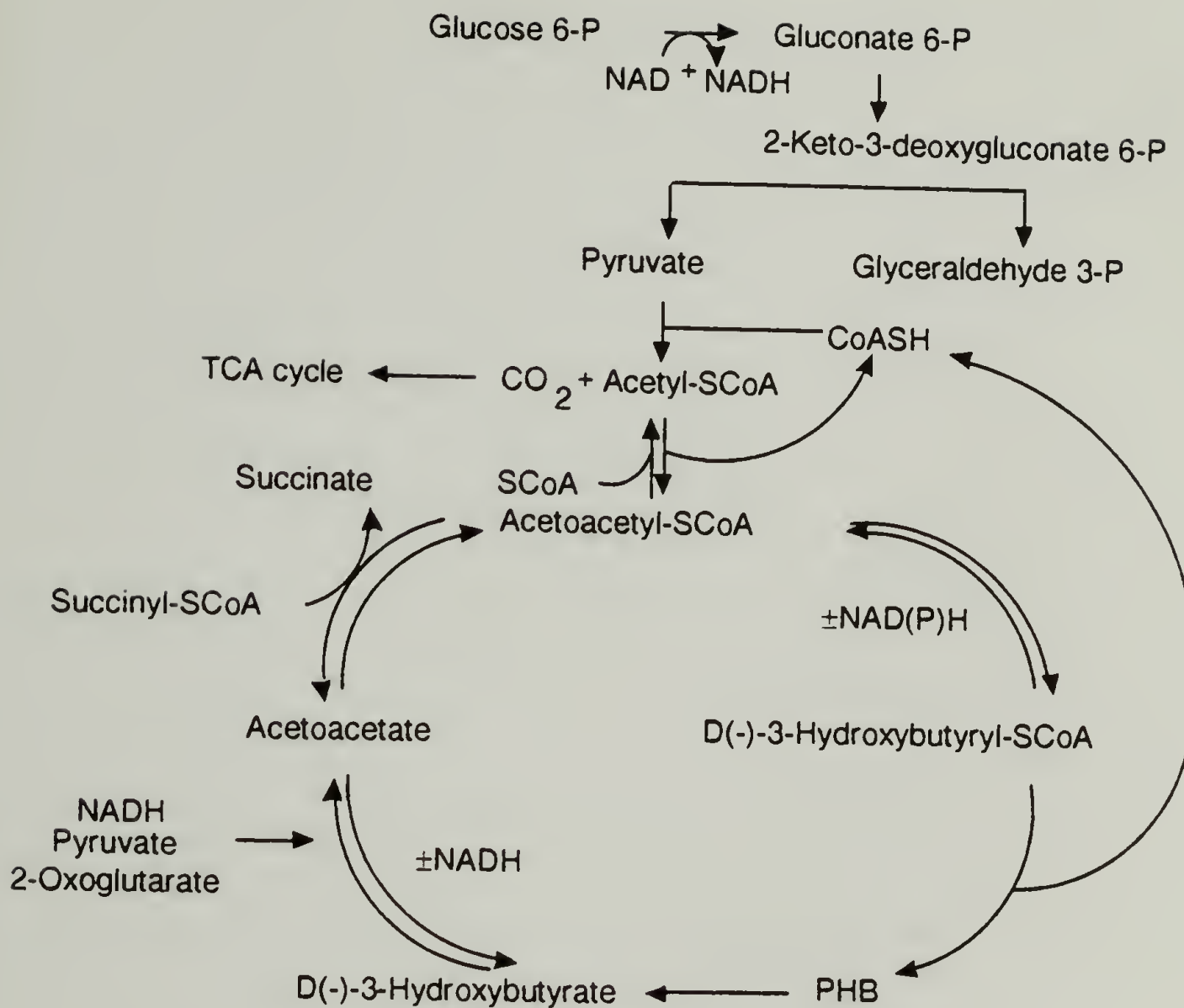


Figure 3. PHB synthesis and degradation pathway in *Azobacter beijerinckii*.





CHAPTER II  
EXPERIMENTAL SECTION

A. Materials

The following chemicals were obtained from the indicated sources.

Acetic acid (F)	Hexanoic acid (A)
Acetonitrile (A, F)	Heptanoic acid (A)
Benzene (F)	Octanoic acid (A)
Chloroform (A)	Decanoic acid (A)
4-Cyclohexylbutyric acid (A)	Undecanoic acid (A)
Chloroform-d <sub>1</sub> (A)	Dodecanoic acid (A)
6-Aminohexanoic acid (A)	Tridecanoic acid (A)
8-Aminooctanoic acid (A)	Tetradecanoic acid (A)
11-Aminoundecanoic acid (A)	Hexadecanoic acid (A)
6-Bromohexanoic acid (A)	Octadecanoic acid (A)
8-Bromooctanoic acid (A)	10-Undecanoic acid (A)
11-Bromoundecanoic acid (A)	Trimethylchlorosilane (A)
11-Cyanoundecanoic acid (A)	Methanol (F)
Sebacic acid (A)	Ethyl ether (F)
Oleic acid (A)	Ethanol (A)
Benzyl alcohol (F)	Sulfuric acid (F)
BH <sub>3</sub> Solution (10 <sup>-3</sup> M) (A)	Hydrochloric acid (F)
Acetone d <sub>6</sub> (A)	Iodomethane (A)
Hexane (F)	Iodine (F)
Heptane (F)	Triethylamine (E)

Cycloheptanone (A)

NaHCO<sub>3</sub> (F)

Na<sub>2</sub>CO<sub>3</sub> (F)

(NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (F)

K<sub>2</sub>HPO<sub>4</sub> (F)

KH<sub>2</sub>PO<sub>4</sub> (F)

MgSO<sub>4</sub> (F)

Phenyloctane (A)

Diazald\* (A)

FeSO<sub>4</sub>•7H<sub>2</sub>O (F)

CaCl<sub>2</sub>•2H<sub>2</sub>O (F)

CuCl<sub>2</sub>•2H<sub>2</sub>O (F)

ZnSO<sub>4</sub>•7H<sub>2</sub>O (F)

NaOH (F)

Deionized Water \*\*

A : Aldrich Chemical Co.

F : Fisher Scientific Co.

E : EM Science Co.

\* : CH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>SO<sub>2</sub>N(CH<sub>3</sub>)NO

\*\* : Filtered through a Branstead NANOpure filtering system

## B. Biosynthesis of Poly- $\beta$ -hydroxyalkanoates

### 1. Preparation of Growth Medium

The growth medium was prepared according to the formula shown in Table 6. The pH of the medium was adjusted to 7.0 using 10 N NaOH solution and concentrated HCl solution. Then the medium was sterilized by autoclaving at 120 °C for 50 minutes. The initial concentration of carboxylic acids was 10 mM. Therefore, a growth medium containing two carboxylic acids (A and B) in a ratio of a:b was prepared by dissolving  $\frac{10 \cdot a}{(a+b)}$  millimole of A and  $\frac{10 \cdot b}{(a+b)}$  millimole of B per one liter of medium. The carbon sources which are volatile (alkanes) or can be hydrolysed (esters of alkanolic acids) were added to the medium after autoclaving and cooling down to room temperature. The volume of the growth medium was usually decreased after sterilization due to the evaporation of water. If the loss of volume was more than 1 liter out of 12 liters or 30 milliliters out of 250 milliliters, that medium was discarded.

### 2. Preparation of Inoculum

a. Stock cultures of *P. oleovorans* (ATCC 29347) Solid medium for storing *P. oleovorans* was prepared by adding 1.5 % of agar to the liquid medium prepared as described above



containing octanoic acid, OA, as the sole carbon source. Cells were grown aerobically in liquid culture with nonanoic acid, NA, as the sole carbon source for 24 hours. A sample was transferred to agar plates and grown for another 24 hours. The plates were then stored at 4 °C. Cells were used as long as the result of preparation of inoculum was reproduced as described below. Usually, the cells were useful for two months.

b. Preparation of Inoculum Inoculum was prepared by growing *P. oleovorans* aerobically as 250 mL batch cultures. To a 250 mL of medium containing NA as the carbon source, a small amount of cells was transferred from the agar plate. The culture transfer was carried out carefully to keep the amount of cells transferred constant. The culture was incubated in a shaker at a temperature of 30 °C. After 12 to 14 hours of growth, the optical density of the culture was between 1.2 and 1.4 A.U. The shape of cells was homogeneous and was approximately 10 µm long. If the optical density or the cell length deviated from these values, that inoculum was discarded.

### 3. Final Growth Experiment

a. Feeding a Carbon Source Once Final growth experiments for PHA production were carried out under aerobic conditions as 12 l batch cultures using a New Brunswick

temperature controlled fermenter (30 °C, 100 r.p.m., 2 liters of air per minute). The amount (milliliters) of the inoculum added to the 12 L medium was determined by dividing 150 by the optical density of the inoculum to keep the number of cells used for inoculation the same. After inoculation the growth was monitored by measuring the optical density at 660 nm. A Varian Cary 2300 UV-Vis-NIR spectrophotometer or a Bausch & Lomb Spectronic 20 was used for the measurement. The sample was diluted to keep the measured optical density around 1.0 A.U. As will be discussed in Section III. B, the highest PHA yield was obtained when the growth reached the stationary phase. However, as the procedure for harvesting a 12 L culture took three hours, harvesting was started late in the deceleration phase. To reduce the growth rate and PHA degradation the temperature was lowered and aeration was stopped upon harvesting. Cells were collected by centrifugation which was carried out using a Sorvall RC2B at 12,000 g for 10 minutes (4 °C). The cell pellets were resuspended with a minimum amount of 10 mM Tris-HCl buffer (pH 7.5) and then re-centrifuged. The cells were washed with excess of distilled water, frozen at dry ice-acetone temperature, and dried using a freeze drier.

b. Sequential Feeding of Carbon Substrates A carbon source was fed twice in sequence to examine the effect of feeding frequency on the repeating unit composition of the PHA, and possibly obtain block copolymers. The total mole

equivalent of the used carbon sources was fixed to be 120 mmole for 12 L cultures, except when NA was fed twice. In the growth experiment feeding NA twice, 120 mmole of NA was fed each time. In the experiment of feeding two carbon sources (A, B) in a ratio of a:b, a 12 L growth medium was prepared as described above using  $\frac{120 \cdot b}{a+b}$  mmole of A and was inoculated following the normal procedure. The second addition of carbon source was prepared by dissolving  $\frac{120 \cdot a}{a+b}$  mmole of B in 500 mL of deionized water. The pH was adjusted to 7.0 using concentrated HCl or 10 N NaOH solution and sterilized at 120 °C for 20 minutes. The second carbon source was added to the culture when the growth with the first carbon source reached the deceleration phase. Cells were harvested when the second growth reached the stationary phase.

#### 4. Isolation of PHA from Dry Cells

The polymers were isolated from lyophilized cells by extraction with hot chloroform in a Soxhlet extractor. When the amount of cells were about 10 grams, the extraction was carried out for 6 hours. The temperature of the heating bath was 100 °C. The solvent was evaporated and the weight of crude extract was measured. The crude polymer product was dissolved in chloroform at a ratio of the volume of



chloroform (milliliters) to weight of the crude extract (grams) of 5:1. The solution was usually passed through a glass funnel containing a cotton plug into rapidly stirred methanol. The ratio of methanol to chloroform was 10:1 (v/v). After 30 minutes of stirring, the liquid layer was decanted, and the precipitated polymer was dried in vacuum (1 mmHg) at a temperature above 35 °C for 16 hours. The product was weighed and the precipitation was repeated twice more to obtain the final product. If the solution of the polymer was not clear which was usually caused by contamination of cell materials, the solution was filtered through a filter paper. Commonly, about 40 % of the weight of the crude product was lost after first precipitation. About 0.1 to 0.2 g of PHA was lost by further precipitation regardless of the amount of the polymer. Therefore, weight loss from the second precipitation was most likely due to polymer sticking to the glassware. In the experiment determining the changes of variables with respect to time, PHAs were isolated by stirring dry cells with excess amount of chloroform at room temperature as will be described in following section. The whole process from 1 to 5 are schematically shown in Figure 5.

### C. Determination of Variables With Respect to Growth Time

#### 1. Biomass Yield, PHA Yield, Repeating Unit Composition, and Molecular Weight vs. Growth Time

The changes of biomass yield, PHA yield, repeating unit composition of the polymer with respect to growth time were determined by harvesting a 12 L culture partially at different growth times. Usually 2 liters of the culture were harvested during the exponential growth and 1 liter of the culture was harvested after exponential phase. The harvested cultures were treated following the standard procedures as described above. The weights of dry cells were usually less than 1 g. Extraction of PHA was carried out by stirring the cells with an excess amount of chloroform (30 mL for 1 g of dry cell) at room temperature for 12 hours. The cells were removed by filtering through a filter paper. The PHA was precipitated twice as described before. When the polymer slurry did not precipitate well, the mixture was centrifuged or allowed to stand at room temperature for further agglomeration of the polymer. The gas chromatographic analysis of the methanolized sample of the cells after extraction showed that the amount of PHA remaining in the cells was less than 1 %.

## 2. The Fraction of Carbon Sources Remaining in the Culture vs. Growth Time

The fraction of carbon sources remaining in the culture during the growth was determined by gas chromatography as follows. Sixty milliliters of the culture was taken at different growth times. Cells were removed by centrifugation and exactly 25 mL of the aqueous layer was taken. To this solution exactly 2 mL of 20 mM aqueous sodium octanoate solution was added as the internal standard. The solution was acidified with 2 mL of concentrated HCl. Litmus paper was used to confirm the acidic condition. The precipitated carboxylic acids in the mixture were extracted into two fractions of 4 mL of chloroform. Then the chloroform solution of remaining carbon sources and OA was dried over anhydrous magnesium sulfate. After concentrating the solution to approximately half of the original volume, one milliliter of the dry chloroform solution and 1 mL of 3 % (v/v) H<sub>2</sub>SO<sub>4</sub> solution in methanol were mixed into a test tube with a screw cap. The cap was hand tightened and the tube was placed in an oil bath which was pre-heated to 100 °C. After four hours, the tube was cooled to room temperature and the reaction mixture was washed with 2 mL of distilled water. The organic layer was taken and dried over anhydrous magnesium sulfate. One to three µL of the solution was injected into a Perkin-Elmer 8500 gas chromatography equipped with a Durabond Carbowax M15 megabore capillary column (15 m,



0.54 mm i.d., J&W Scientific) and a flame ionization detector. The injector was used in splitless mode. The conditions for the gas chromatographic analysis were as follows.

Initial Temperature	: 80 °C
Initial Time	: 4 minutes
Ramp	: 20 °C/minute
Final Temperature	: 180 °C
Final Time	: 2 minutes
Carrier Flow Rate (He)	: 22 mL/minute
Injector Temperature	: 250 °C
Detector Temperature	: 270 °C

The fractions of remaining carbon substrates were calculated from the peak areas of each compound using the peak of methyloctanoate as the internal standard. The equation for calculating the fraction of carbon sources remaining in the medium is,

$$\begin{array}{l} \text{Fraction of carbon} \\ \text{source remaining} \\ \text{in the culture} \end{array} = \frac{A_{is} \cdot A_s}{A_{iss} \cdot A_{ss}}$$

$A_{is}$  : Peak area of internal standard (methyloctanoate)

$A_s$  : Peak area of the carbon source to be determined

$A_{iss}$ : Peak area of the internal standard in the standard run (the sample at time = 0)

$A_{ss}$  : Peak area of the carbon source to be determined in the standard run

## D. Analysis of PHAs

### 1. Chemical Analysis of PHAs

$\beta$ -Hydroxyalkanoate units in the polymers were identified by gas chromatographic analysis of the acid-catalysed methanolysis products of the polymers using various types of detectors. Gas chromatographies equipped with a Flame Ionization Detector (FID), a Mass Selective Detector (MSD), and an Atomic Emission Detector (AED) were used for qualitative and quantitative analysis. GC/FID was used for comparison of retention times and quantitative calculation of each repeating unit. GC/MSD was used to examine the presence of characteristic ion fragments from methyl- $\beta$ -hydroxyalkanoates. GC/AED was used to selectively detect repeating units containing bromine or nitrogen.

a. Acid-Catalysed Methanolysis of PHAs Three to five milligrams of the polymer were weighed into a test tube with a screw cap. One milliliter of 3 % (v/v) sulfuric acid solution in methanol and 1 mL of chloroform was added to the tube. The screw cap was hand tightened and then the tube was allowed to stand in an oil bath at 100 °C for 4 hours. The reaction mixture was cooled to room temperature and 2 mL of distilled water was added, and the mixture was shaken vigorously. The organic layer was taken and dried over

anhydrous  $\text{MgSO}_4$ . One to three microliters of the chloroform solution was injected into the gas chromatography.

b. GC-FID Analysis A Perkin-Elmer 8500 GC equipped with a Durabond Carbowax M15 megabore capillary column (15 m, 0.54 mm i.d., J&W Scientific) and an FID was used. The injector was used in splitless mode. <sup>flame ionization detector</sup> The conditions for gas chromatographic analysis were as follows.

Initial Temperature	: 80 °C
Initial Time	: 4 minutes
Ramp	: 10 °C/minute
Final Temperature	: 180 °C
Final Time	: 10 minutes
Carrier Flow Rate (He)	: 22 mL/minute
Injector Temperature	: 250 °C
Detector Temperature	: 270 °C

The retention time of each peak was compared with that of each standard material. The gas chromatogram of a mixture of standard materials (methyl- $\beta$ -hydroxybutyrate to methyl- $\beta$ -hydroxydodecanoate) obtained under these conditions is shown in Figure 6. In early studies, the amount of each repeating unit was determined quantitatively using a calibration curve established with these standard materials. The calibration curves used are collected in Table 7.



c. GC-MSD Analysis A Hewlett-Packard 5970 Mass

Selective Detector attached to a Hewlett-Packard 5890A gas chromatography equipped with a Carbowax capillary column (25 m, 0.32 mm i.d.) was used. The MSD was equipped with a quadropole detector and an ion impact ionizer. The fragmentation pattern for the production of characteristic ion fragments from methyl- $\beta$ -hydroxyalkanoates is shown in Figure 7. The mass spectra of standard materials, when available, were used to determine the repeating units of the PHAs. Otherwise, the presence of ion fragments of  $m/z$  of 103 and 74 was investigated to determine if that unit was a  $\beta$ -hydroxyalkanoate or not. The mass spectra of standard materials and repeating units of some polymers are presented in the Appendix and in corresponding sections.

d. GC-AED Analysis A Hewlett-Packard 5921A AED attached to a Hewlett-Packard 5890A gas chromatography was used. Emission of lights of 478.6 nm and 174.2 nm was monitored for the detection of bromine and nitrogen, respectively. The HP 5921A detector is described in literature [Baum, 1989].

e. Elemental Analysis Elemental analyses were carried out by the Microanalysis Laboratory at the University of Massachusetts at Amherst.

## 2. Physical Analysis of PHAs

a. Molecular Weight Measurement Molecular weights of polymers were determined by gel permeation chromatography (GPC). The instruments used include a Waters Model 6000A solvent delivery system, a Waters Model 401 refractive index detector, and a PC compatible for data acquisition and analysis. The A/D converter was a Data Translation 2811 board and the software used for acquiring signal was Acquire published by LabNotebook. The output signals collected as ASCII files were imported into Lotus 1-2-3 electronic spread sheet and the molecular weight was calculated by a macro program presented in Table 8. The calibration curve for the molecular weight was obtained using polystyrenes of narrow molecular weight distributions.

b. NMR spectroscopy  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded using Varian XL-200 or XL-300 NMR spectrometer at 17 °C. Approximately 15 mg and 50 mg of a polymer was dissolved per 1 mL of chloroform- $d$  or acetone- $d_6$  for  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra, respectively. The delay time between sampling pulses for both  $^{13}\text{C}$  and  $^1\text{H}$  NMR measurements was 3.0 seconds. The  $^{13}\text{C}$  NMR spectra taken were proton-decoupled with a 16,500 Hz spectral width, 30 K data points, and a  $56^\circ$  pulse (10  $\mu\text{s}$ ). Typically 10,000 transients were accumulated.

c. Thermal Analysis The glass transition ( $T_g$ ), melting transition ( $T_m$ ) and the heat of fusion ( $\Delta H_m$ ) were measured by using a Perkin-Elmer Model DSC-4 or a Dupont DSC 2000. The weight of polymer used for DSC measurements was 7 to 10 mg. The temperature range for the DSC measurement was -100 to 100 °C. For the low temperature region, mercury was used for temperature calibration. Polymer samples were heated at a rate of 10 or 20 °C per minute.

d. X-ray Diffraction X-ray diffraction measurements were made using a stretched sample of a small piece of PHA 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 K $\alpha$  radiation ( $\lambda = 0.1542$  nm) was used. The PHA films were prepared by melt moulding. A mold (2.5 cm x 5 cm x 0.1 cm) was prepared with glass plates. Chunks of polymers were placed in the well and melt in a vacuum oven at a temperature between 80 and 100 °C. The polymer was kept at that temperature for several hours until the polymer melt flowed to fill the mold and then cooled to room temperature under vacuum. The film was allowed to stand at room temperature at least for 24 hours. The polymer became hard enough to be peeled out of the glass plate after 24 hours. A small piece of the film was cut and manually stretched until it broke. The distances between the polymer sample and film were 74.7 mm and 194.5 mm for moderate angle ( $10^\circ < 2\theta < 30^\circ$ ) and small



angle ( $2\theta < 10^\circ$ ) measurements, respectively. The samples were exposed for 6 hours (74.7 mm position) and 14 hours (194.5 mm position). The arrangement for the X-ray scattering measurement and calculation of d spacings are shown in Figure 8.

e. Others Infrared spectra were recorded on a Perkin Elmer 1600 series FTIR in the form of a coated film on a NaCl cell. Typically 100 transients were accumulated.

### C. Organic Reactions

#### 1. Synthesis of 7-Octenoic Acid

The reaction pathway for the synthesis of 7-octenoic acid is shown in Figure 9. To a 250 mL round bottom flask containing 50 mL of dry DMF (treated with barium oxide and distilled under vacuum) and 43 mL of freshly distilled trimethylamine, 20 mL of 98 % trimethylsilylchloride (0.16 mole) was added. To this mixture 16.3 mL of 97 % cycloheptanone (0.14 mole) was added for approximately 30 minutes under nitrogen atmosphere at room temperature. When the addition was completed, the mixture was heated to 120 °C and allowed to stand for 48 hours at that temperature. The reaction mixture was cooled in an ice bath and extracted with 400 mL of pentane. The pentane solution was washed three times with 300 mL of ice-cold saturated  $\text{NaHCO}_3$  solution. Then

the solution was washed with 50 mL of ice-cold 1.5 M HCl solution and then again with 100 mL of ice-cold NaHCO<sub>3</sub> solution. The pentane solution was dried over anhydrous MgSO<sub>4</sub>. After evaporation of the pentane under reduced pressure, trimethylsilyl ether of 1-cyclohexenol, B in Figure 9, was isolated by vacuum distillation. The fraction boiling between 85 and 90 °C under 45 to 45 mmHg was collected. The product forms a very foamy head so a large flask was used for distillation. The product mixture was analyzed using thin layer chromatography using plates precoated with silica gel. The rate of flow (R<sub>f</sub>) values of B and cycloheptanone were 0.91 and 0.5 when a 2:1 mixture of pentane and ether was used as the eluent. The yield of the product was approximately 87 % after distillation.

Simons reaction (B to C in Figure 9) was carried out as follows. A mixture of 6.73 g of zinc powder, 1.03 g of CuCl, and 16 mL of ether was refluxed for 30 minutes. To this Zn/Cu complex, 9 grams of B and 4 mL of CH<sub>2</sub>I<sub>2</sub> were added. The reaction mixture was refluxed for 24 hours. Approximately 30 mL of ether was added and the mixture was filtered through a filter paper with celite layer. The solution was washed with 25 mL of ice-cold 10 % HCl solution. The layer was separated as fast as possible and the aqueous layer was extracted with two portions of 20 mL of ether to recover product in the aqueous layer. The combined ether extract were washed successively and rapidly with 20 mL of cold 10 % HCl. The product, C in Figure 9, was isolated by vacuum

distillation. The fraction boiling between 97 and 99 °C under 16 mmHg was collected. The TLC analysis showed that the R<sub>f</sub> value of the product was approximately 0.7 as analyzed using the same conditions as described above. The yield of C from B was approximately 50 %. To 5 g of C, 32 g of lead tetraacetate and 23 mL of dry acetic acid (distilled over P<sub>2</sub>O<sub>5</sub>) were added. The mixture was stirred at room temperature for eight hours. The brown slurry was filtered through a filter paper with celite layer. The product was extracted using three portions of 300 mL of hexane. After evaporation of the hexane, 7-octenoic acid was isolated by column chromatography using silica gel (yield 75 %). A 3:1 mixture of hexane and ether was used as the eluent. The overall yield of 7-octenoic acid was approximately 33 %. NMR spectrum of the product showed the presence of olefin units and the elemental analysis was corresponding to the theoretical formula (calc'd for C<sub>8</sub>H<sub>14</sub>O<sub>2</sub>: H 9.92 C 67.57 O 22.5; found: H 10.2 C 66.8 O 23).

## 2. Synthesis of Methyl-β-hydroxyalkanoates

### a. Preparation of β-Hydroxyalkanoates

β-Hydroxyalkanoates used in this study were prepared by Dieter Boeckh or Herb Ulmer following a procedure described in other literatures [Mulzer et al., 1980, 1981; Moersch and Burkett, 1971; Adam et al., 1972]. The reaction pathway is shown in Figure 10. The general procedure is as follows



[Gross et al., 1989]. Dry THF and 14.0 mL (0.100 mole) of anhydrous diisopropylamine (distilled from  $\text{CaH}_2$ ) was transferred to a three neck flask. This solution was cooled to 0 °C and 40 mL (0.100 mole) of 2.5 M n-butyllithium in hexane was added dropwise with stirring over 0.5 hour to the three neck flask at 0 °C. Then 2.9 mL (0.050 mole) of glacial acetic acid in 10 mL of dry THF was added to 0 °C. The three neck flask was then heated with stirring to 45-50 °C for 2 hours, producing a milky white suspension. After this, 0.050 mole of an n-alkyl aldehyde in 15 mL of dry THF was added dropwise over 1 hour followed by an additional 2.5 hour reaction period with stirring at 40 °C. To this mixture, 5 mL of distilled water 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 6N 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 %.

#### b. Preparation of $\beta$ -Hydroxyalkanoic Acid Methyl Esters

Diazomethane was used for methyl esterification of  $\beta$ -hydroxyalkanoic acids. Diazomethane was prepared from Diazald® (p-tolylsulfonylmethylnitrosoamide) supplied by Aldrich and using a clear seal glassware kit as shown in

Figure 11. To the 250 mL round bottom flask 3 g KOH solution in water, 50 mL of ethyl alcohol, and 20 mL of ether were added. The separatory funnel was charged with diazald solution (10 g in 50 mL ether). The temperature of the oil bath was set between 50 and 70 °C. Upon distillation of the ether, the contents in the separatory funnel was slowly added. The rate of dropping was controlled to be the same as the rate of distillation. When the dropping was complete, 30 mL of ether was added and distillation was continued until the color of the distillate became colorless.

To 0.050 mole of a  $\beta$ -hydroxyalkanoic acid dissolved in 100 mL of ether at 0 °C, the the diazomethane solution was added dropwise until no additional gas was evolved and the color of the methyl ester solution stayed slightly yellow. The solvent was removed and the crude methyl- $\beta$ -hydroxyalkanoates were purified by short path fractional distillation under reduced pressure. The overall yields were greater than 50 %. Methyl- $\beta$ -hydroxybutyrate was purchase from Aldrich Chemical Co.

c. Esters of Carboxylic Acids Methyl esters and benzyl esters of carboxylic acids were prepared by acid-catalysed esterification.

Benzyl-10-undecenoate was prepared by refluxing a mixture of 100 g of 10-undecenoic acid (UND:), 110 g of benzyl alcohol, 2 ml of sulfuric acid, and 200 mL of chloroform for 6 hours. The fraction boiling at



185 °C/10 mmHg was collected. The overall yield was higher than 70 %.

Monobenzylester of sebacic acid was prepared by refluxing a mixture of 180 g of sebacic acid, 214 g of benzyl alcohol, 4 mL of sulfuric acid and 500 mL of chloroform. The product was recrystallized from methanol to afford 40 g of product (calc'd for  $C_{17}H_{24}O_4$ : C 69.84 H 8.27 O 21.89; found: C 69.88 H 8.23 O 21.89).

d. Preparation of 6-Methoxy-6'-hydroxybiphenyl To a 500 mL flask equipped with mechanical stirrer, 51.6 g of biphenol, 26 g of NaOH, and 220 mL of distilled water were charged. To this mixture, 26.5 mL of DMS was added for 15 minutes. The reaction was exothermic and the flask was cooled with a water bath. The solid product were formed and the color of the mixture turned green. The product was filtered. The dimethyl ether was removed by dissolving the product in 200 mL of 10 % NaOH aqueous solution and filtering. The filtrate was acidified at 70 °C and filtered and dried overnight to afford 21.6 g of product (yield 39.4 %).

e. Others Trimethylsilyl ether of undecanol was prepared by stirring a mixture of 150 mL of undecanol, 111 mL of trimethylsilylchloride, 120 mL of triethylamine and 200 mL of THF at room temperature for 16 hours. The reaction mixture was washed three times with 200 mL of water. The



organic layer was taken and dried over anhydrous  $\text{MgSO}_4$ . The product was isolated by fractional vacuum distillation. Three fractions were taken with boiling points of 30 °C, 30 to 113 °C, and 113 °C under 10 mmHg. The final fraction was the desired product. The overall yield was approximately 50 %.

### 3. Reactions on Polymers

a. Epoxidation of PHA-UND: Epoxidation of the PHA-UND: was carried out using metachloroperbenzoic acid (MCPBA). To 60 mL of purified chloroform (distilled over sodium), 0.5 g of PHA-UND:, and 0.63 g of MCPBA was dissolved under nitrogen atmosphere. The solution was stirred at room temperature for 6 hours. The remaining peracid was broken by washing twice with 30 mL of 10 % aqueous solution of sodium sulfate. The organic layer was separated and dried over anhydrous  $\text{MgSO}_4$ . Approximately 0.35 g of product was obtained after evaporating the solvent. Approximately 67 % of olefinic units disappeared after the reaction. The product crosslinked after a few days of exposure to air at room temperature.

#### b. Crosslinking of PHAs Containing Unsaturated Units

PHAs containing unsaturated units were crosslinked by heating them at 100 °C in the air or with benzoylperoxide under vacuum at 100 °C. The degree of crosslinking was not

determined. These PHAs went through crosslinking even at room temperature when they were exposed to air. The gas chromatographic analysis results of the crosslinked product showed that there was no noticeable compositional change caused by the crosslinking, suggesting that the degree of crosslinking was very low. These polymers crosslinked in a low degree had the the same glass transition and melting transition temperatures as the uncrosslinked polymers.

#### c. Hydrogenation of the PHA Containing Benzylesters

Reduction of the benzylesters in the PHA prepared from a mixture of NA and sebacic acid monobenzyl ester was attempted by Pd/C catalysed hydrogenation. One hundred milliliters of chloroform, 0.4 g of the PHA, and 50 mg of activated Pd/C (10 wt%) were mixed and stirred for 6 hours under hydrogen using an apparatus shown in Figure 12. However, no reduction occurred by this reaction and no further investigation was carried out.

#### d. Hydroboration of the PHAs Containing Unsaturated

Units Hydroboration of PHAs containing unsaturated units was carried out using  $10^{-3}$  M solution of  $\text{BH}_3$  in THF. The molecular weights of various PHA-NON•UND:s were calculated from the composition of the PHAs. The mole ratio of the polymer to  $\text{BH}_3$  was 1:2. The amounts of PHAs and  $\text{BH}_3$  solution ( $10^{-3}$  M) used for boration were as follows.

Carbon Source NON/UND:	PHA, g	BH <sub>3</sub> Solution (mL)
1/1	0.1589	1
2/1	0.1050	0.44
1/1 sequential	0.1311	1.2
1:2	0.1411	1.0

To the chloroform solution of each PHA (5 mL for 1 g) stirred vigorously under nitrogen atmosphere, BH<sub>3</sub> solution was added using a syringe. Gelation occurred immediately. After stirring for 30 minutes, an excess amount of chloroform was added to the flasks and stirred for 4 hours to extract soluble materials. The product was filtered through filter paper. The chloroform was evaporated and the remaining materials were weighed. The crosslinked polymer was methanolized and analyzed using gas chromatographic method as described above.

e. Reaction of the PHA Containing Brominated Units With 6-methoxy-6'-hydroxybiphenyl (MHB) To a solution of 0.25 g of PHA-NON•11BRUA in 5 mL of THF, 0.15 g of MHB ( $8 \times 10^{-4}$  mole) and 30 mg of NaOH were added. The mixture was stirred at room temperature (18 °C) for 6 hours. Excess amount of water was added and polymeric materials, if any, were extracted with chloroform. However, no polymeric material was obtained.



Table 6. Composition of growth medium for preparation of PHA from *P. oleovorans*.

(a) E\* medium, 10.6 g/L

Component	Amount (g)
$(\text{NH}_4)_2\text{HPO}_4$	1.1
$\text{K}_2\text{HPO}_4$ (dibasic)	5.8
$\text{KH}_2\text{PO}_4$ (monobasic)	3.7

(b) Microelement solution  
(1 mL/L)

Component	Amount g per 1L 1N HCl soln.
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	2.78
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	1.67
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.17
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.29
$\text{MnCl} \cdot 4\text{H}_2\text{O}$	1.98
$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	2.81

(c) 10 mL of 100 mM  $\text{MgSO}_4$  solution per 1 L culture.

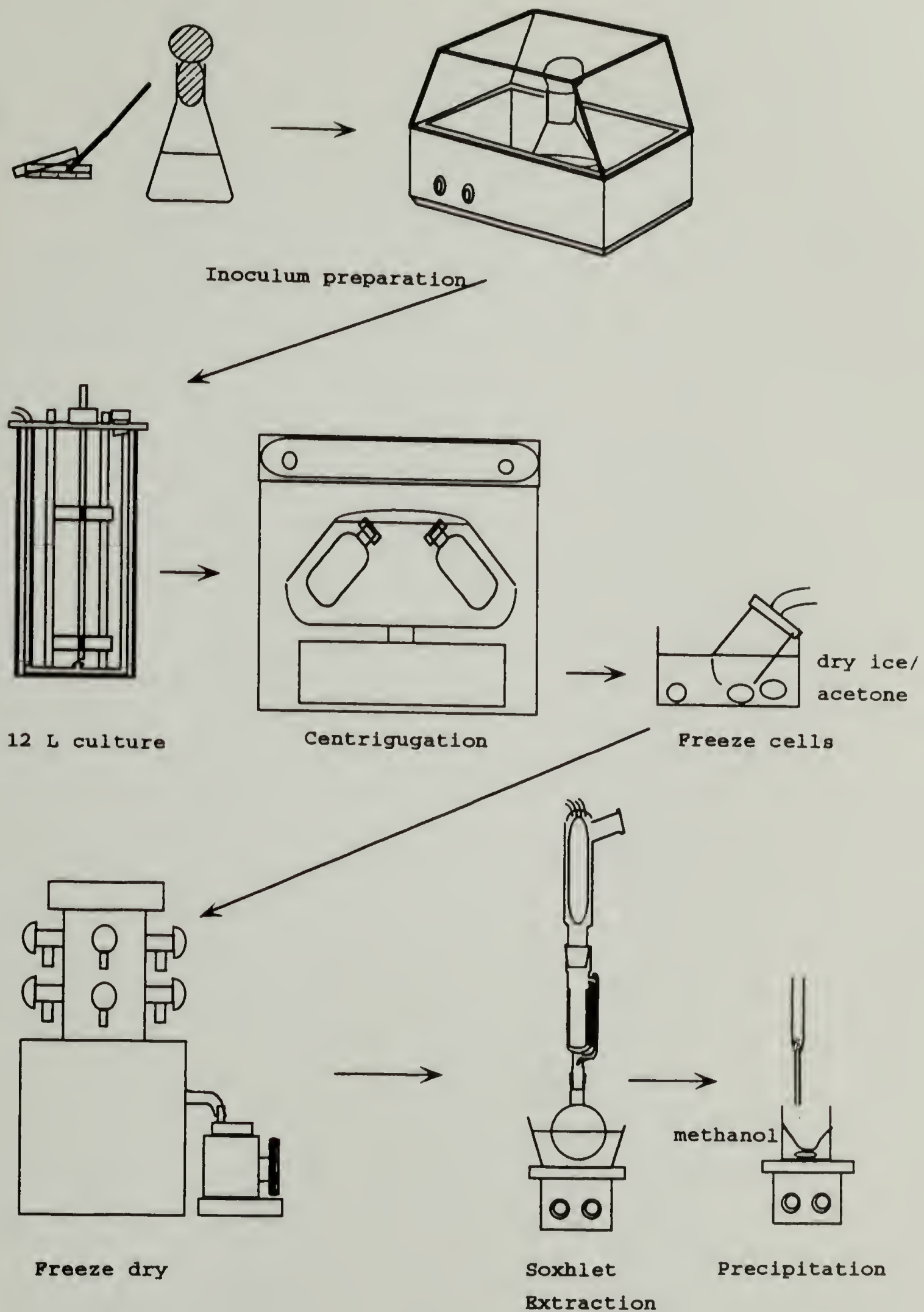


Figure 5. Experimental procedure of PHA preparation.

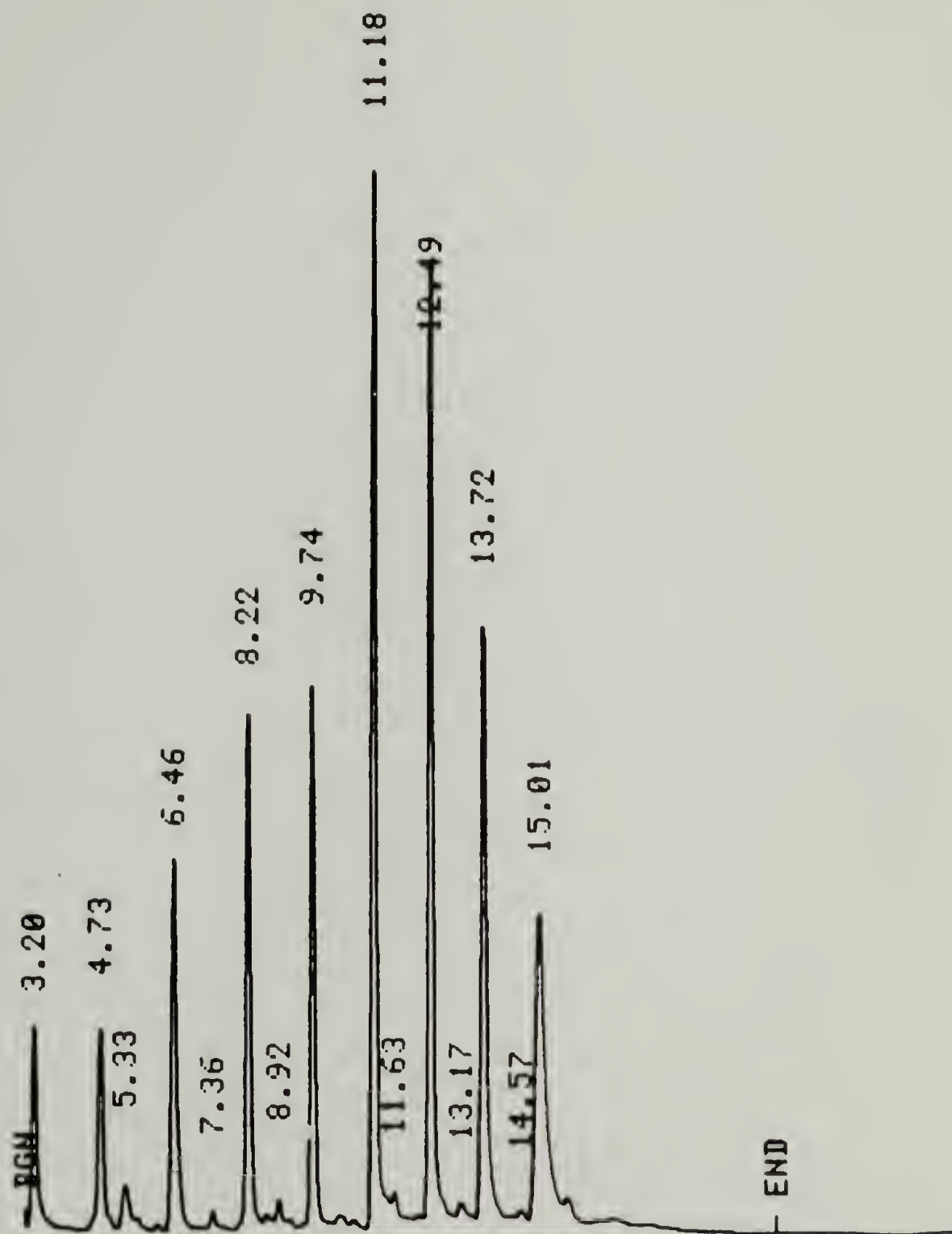


Figure 6. Gas chromatogram of a mixture of standard methyl-3-hydroxyalkanoates from C4 to C12.



Table 7. Calibration table for methyl-3-hydroxyalkanoates.

(a) methyl-3-hydroxybutyrate

weight of sample	slope	intercept	correlation
4.855 to 33.985	0.30166	0.02547	1
48.55 to 339.85	0.3183	-0.6478	0.9999
486 to 3399	0.3731	22.4340	0.9928

(b) methyl-3-hydroxyvalerate

weight of sample	slope	intercept	correlation
5.045 to 35.315	0.3019	2.8250	0.9976
50.45 to 353.15	0.3286	1.1936	1
505 to 3532	0.4207	-23.9662	0.9958

(c) methyl-3-hydroxyhexanoate

weight of sample	slope	intercept	correlation
5.110 to 35.770	0.5230	-0.1121	0.9992
51.10 to 357.70	0.5082	-0.0635	0.9998
511 to 3577	0.6599	-44.2594	0.9954

(d) methyl-3-hydroxyheptanoate

weight of sample	slope	intercept	correlation
5.520 to 38.640	0.5279	-0.5266	0.9961
55.20 to 386.40	0.4768	0.0923	0.9998
552.0 to 3864	0.6576	-70.6365	0.9943

Continued, next page

Table 7-Continued

## (e) methyl-3-hydroxyoctanoate

weight of sample	slope	intercept	correlation
5.765 to 40.355	0.5428	-0.8394	0.9997
57.65 to 403.55	0.4467	0.1353	0.9993
577 to 4036	0.7308	-127.8361	0.9948

## (f) methyl-3-hydroxynonanoate

weight of sample	slope	intercept	correlation
5.345 to 37.415	0.1600	-0.4759	0.9960
53.45 to 374.20	0.4282	-8.7989	0.9989
534.5 to 2672.5	0.4878	69.0691	0.9923

## (g) methyl-3-hydroxydecanoate

weight of sample	slope	intercept	correlation
5.34 to 37.38	0.0335	0.0157	0.9867
53.40 to 373.80	0.4296	-14.0293	0.9983
534.0 to 2670	0.5252	8.5920	0.9924

## (h) methyl-3-hydroxyundecanoate

weight of sample	slope	intercept	correlation
5.355 to 37.485	0.005042	0.0446	0.9897
53.55 to 374.85	0.2781	-13.6784	0.9863
535.5 to 2677.5	0.3592	-23.3804	0.9922

## (i) methyl-3-hydroxydodecanoate

weight of sample	slope	intercept	correlation
5.565 to 38.955	0.2650	-2.1250	1
166.95 to 389.55	0.1924	-27.566	0.9865
558.5 to 2792.5	0.2727	-47.7107	0.9872

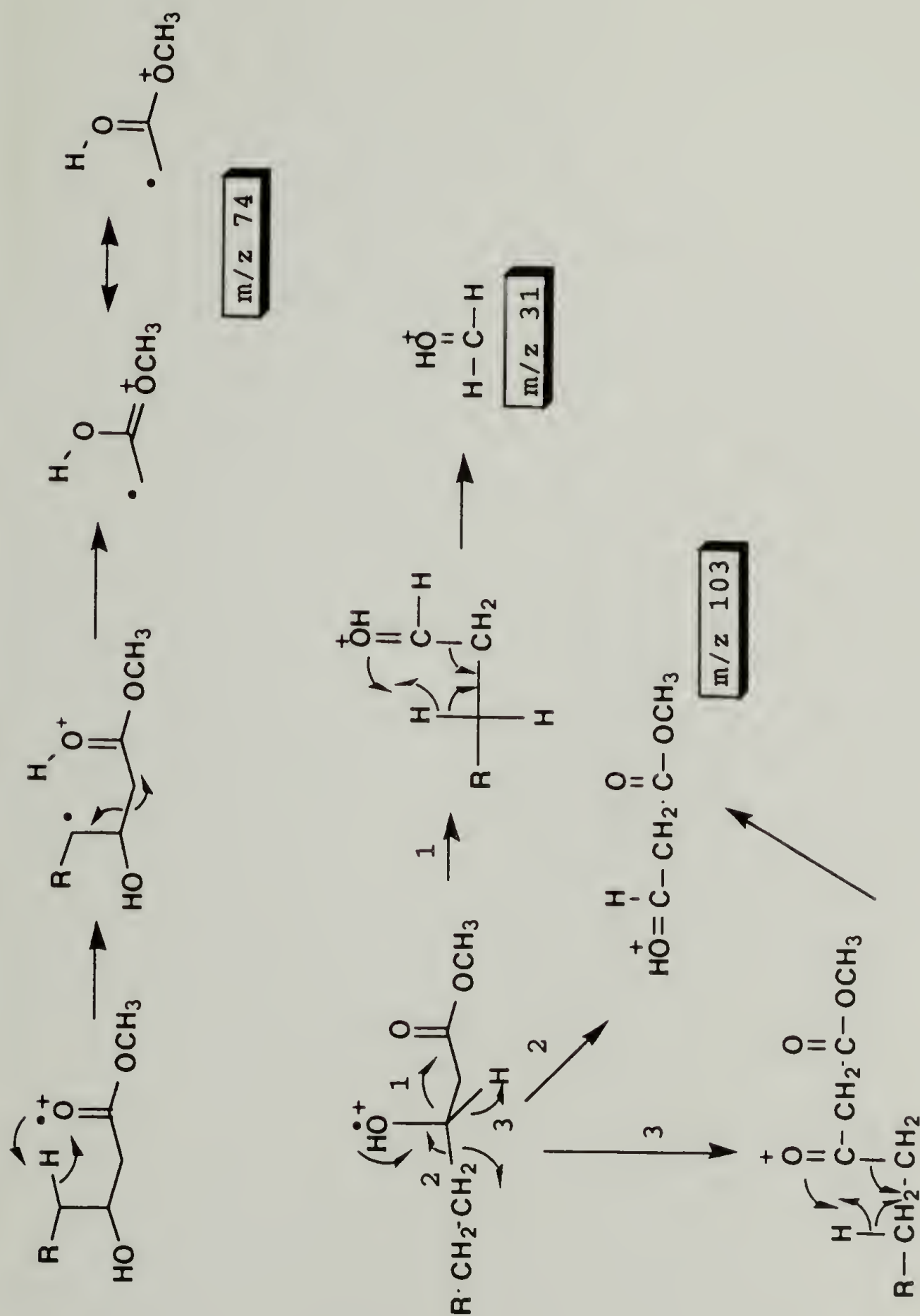
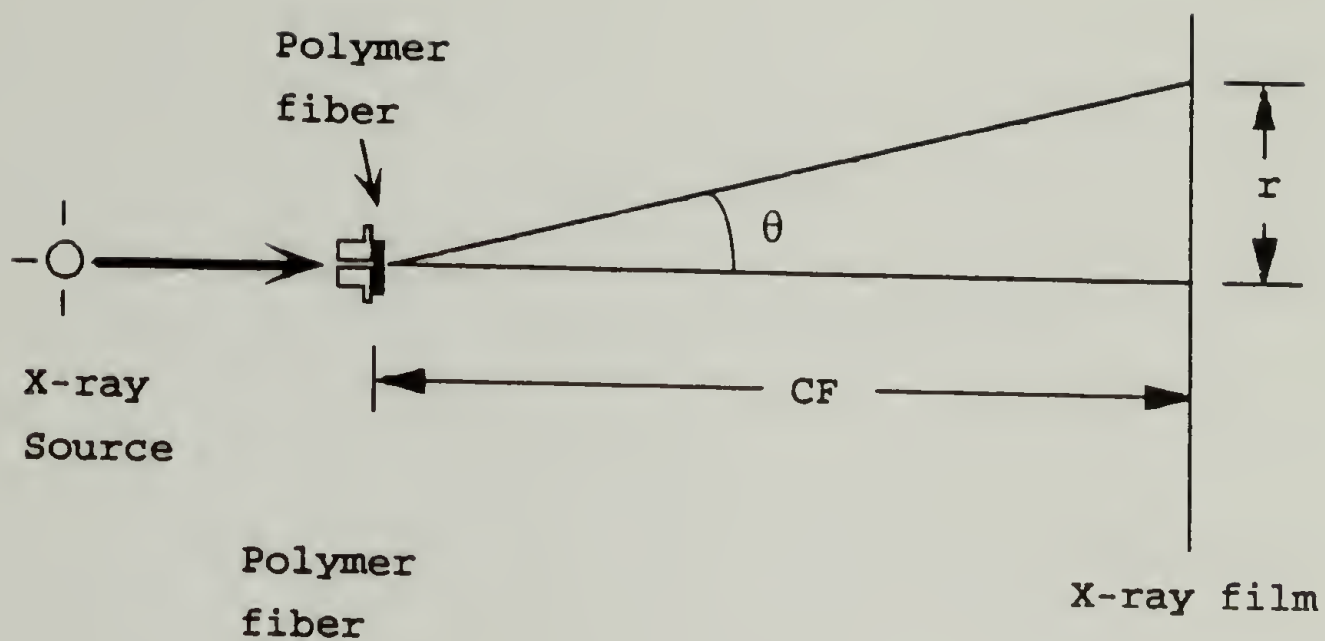


Figure 7. Pathways for production of major ion fragments from methyl-β-hydroxyalkanoates.



Table 8. Macro-commands for molecular weight calculation using lotus or vpp electronic spreadsheet.

A	<pre>{home}GPC calculation program~{down} You have to know your standard curve~{down} and your data file name.~ {getnumber Slope of your std. curve,slope} /rea1.a5~ {getnumber Intercept of your std. curve,intercept} {goto}a1~Slope:~{down}Intercept:~ {down}Now your data file (d:\file.ext)~{goto}a10~/in{?}~ Look at the chromatogram and decide which part to expand.~/grg{esc}{down}When ready hit Alt+b~</pre>
B	<pre>/grg{esc}{esc}{goto}a15~/gtxx.{end}{down}~{esc}{esc}{goto}b15~/gofa1{esc}{esc}a.{end}{down}~p {esc}{esc}{esc}/gosxml{?}~u{?}~{esc}{esc}{esc}vosxml{?}~u{?}~{esc}{esc}v {esc}{esc}{esc}{esc} /rea3.a10~{goto}a3~It you chose the start and end time of your peak,~{down}Great! But it you did not hit Alt+b again~{down}If finished hit Alt+c~ You will be asked to enter range to copy.~{down}Enter a(start+14).b(end+14)then enter~</pre>
C	<pre>/rec1.g2000~/c{?}~c1~ {goto}c1~/c~Start~ {goto}c1~{end}{down}/c~end~ {goto}d1~/c~y1~ {goto}d1~{end}{down}/c~y2~ {goto}e1~ 10^(bs1*c1+bs2)~/c~{down}.{left}{end}{down}{right}~ {goto}l1~(d1-c1*kS3-k\$4)/e1~/c~{down}.{left}{end}{down}{right}~ {goto}g1~+e1*f1~/c~{down}.{left}{end}{down}{right}~ {goto}g1~+g1*e1~/c~{down}.{left}{end}{down}{right}~ {goto}a4~Mn~{down}Mw~{down}PDI~{goto}b4~ {goto}i1~@sum({left}.{end}{down})/@sum({left}{left}.{end}{down})~ {goto}i2~@sum({left}{left}{up}.{end}{down})/@sum({left}{left}{left}{up}.{end}{down})~ {goto}i3~+i1/i2~ /ci1.i3~b4~ {goto}a4~Mw~{down}Mn~{down}PDI~</pre>



$$\tan 2\theta = \frac{r}{CF}$$

$$\theta = \frac{1}{2} \operatorname{atan} \frac{r}{CF}$$

$$d = \frac{1}{2 \sin(\frac{1}{2} \operatorname{atan} \frac{r}{CF})}$$

$$\text{CuK}\alpha = 1.542 \text{ \AA}$$

Figure 8. Arrangement of X-ray source, a sample and a camera for X-ray diffraction study.

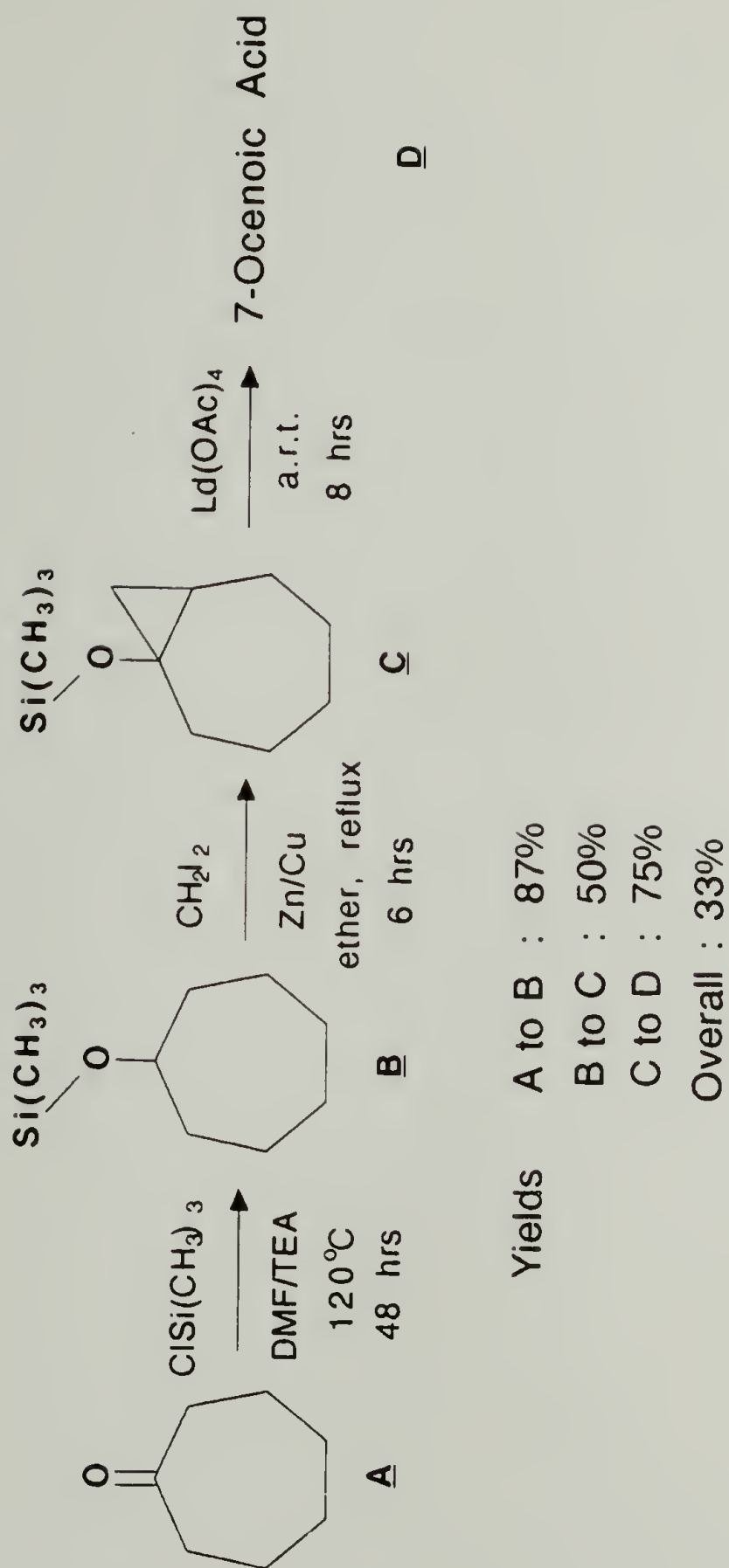


Figure 9. The reaction scheme for preparation of 7-octenoic acid.



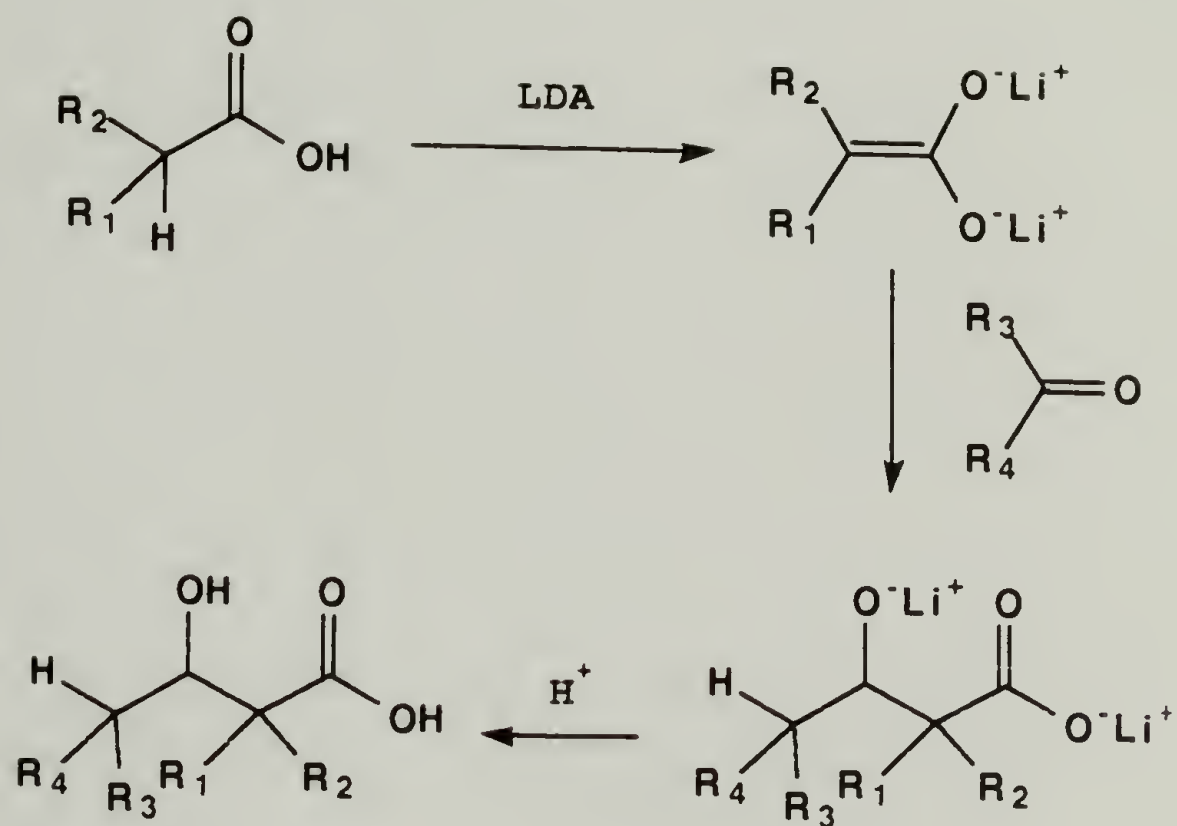


Figure 10. Reaction scheme for the preparation of 3-hydroxyalkanoic acids.

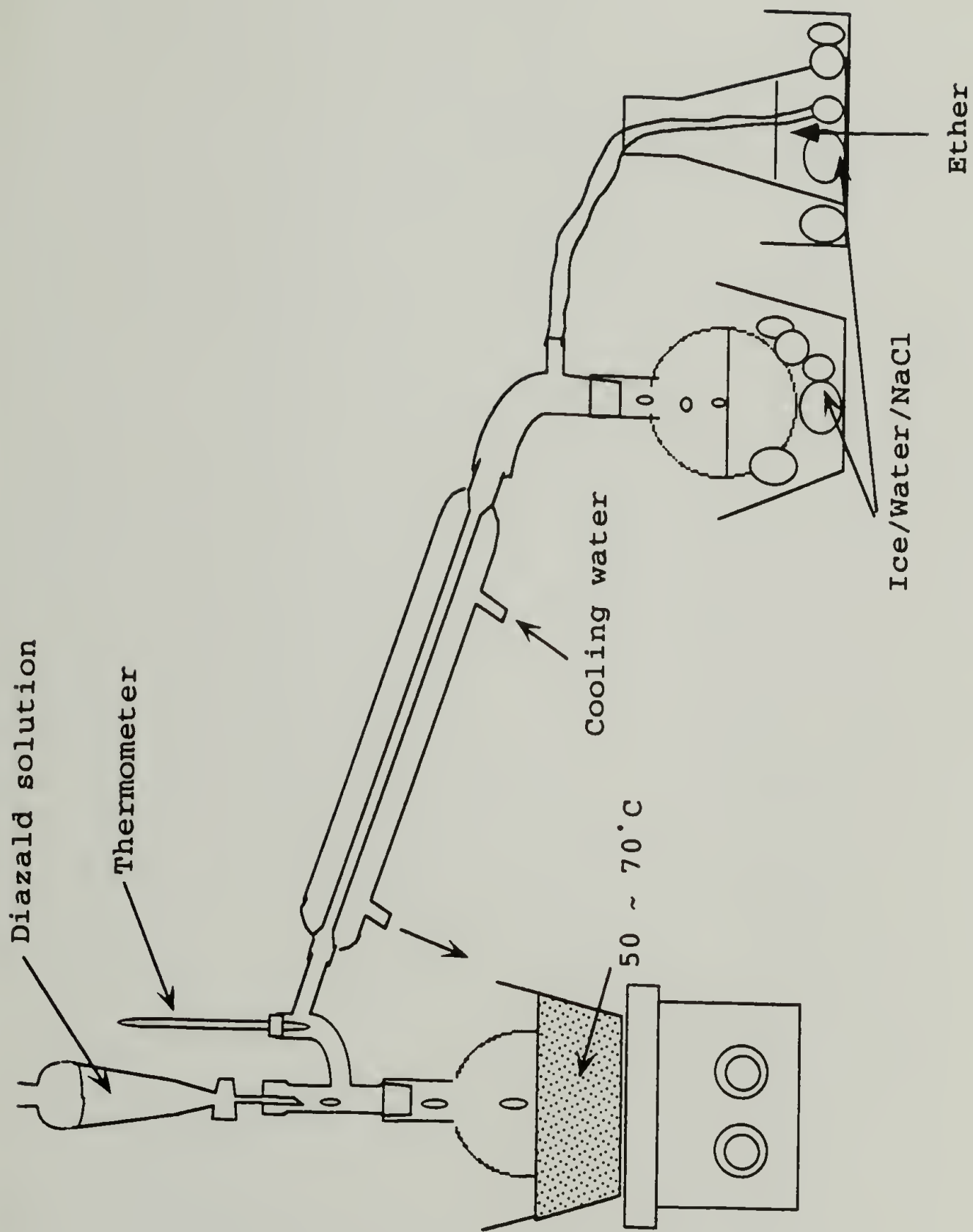
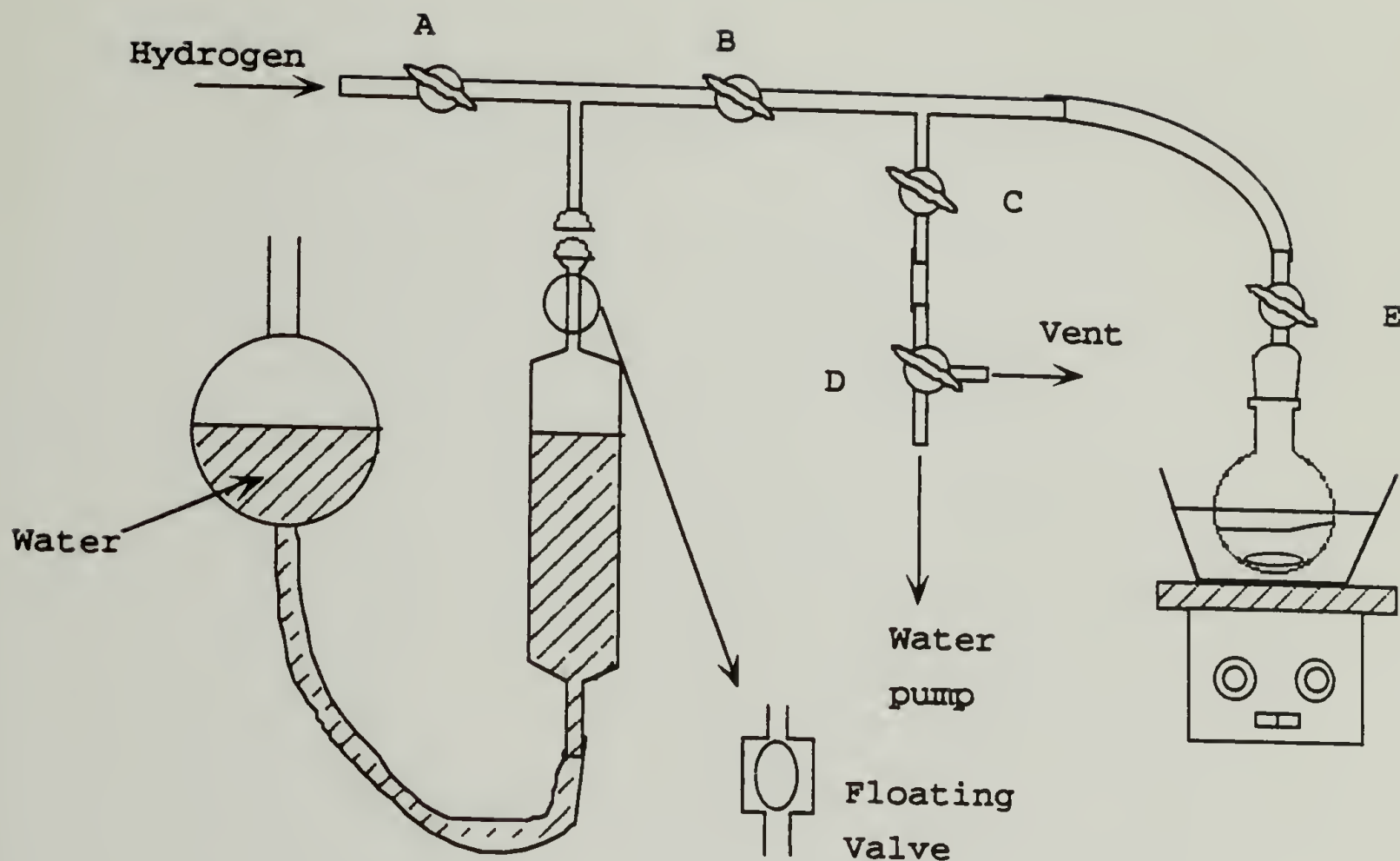


Figure 11. The apparatus for the preparation of diazomethane from Diazald®.



#### Procedure

1. Close A, open B, C, E and D to water pump.
2. Close D and open A.
3. Repeat 1 and 2 three times.
4. Close D, open A and charge hydrogen to a proper level.

Figure 12. The apparatus for Pd/C catalyzed hydrogenation.



## CHAPTER III

### RESULTS AND DISCUSSION

#### A. Objectives

The objectives of this work were preparation and characterization of novel poly- $\beta$ -hydroxyalkanoates (PHAs) from *P. oleovorans*. The most desired polymers were those containing functional groups.

The behavior of *P. oleovorans* was partially investigated by growing this microorganism with various carbon sources under fixed growth conditions, and new potential carbon sources were discovered through this investigation.

Carbon substrates were used as either a sole carbon source or a cosubstrate in the presence of another carbon substrate for PHA production, and the composition and physical properties of the obtained polymer, if any, were determined. The effect of the chemical and physical properties of a carbon substrate on the repeating unit composition and polymer properties was also studied.

Some preliminary investigation on the modification of functional group was carried out.

## B. Examination of Experimental Conditions

### 1. Introduction

In earlier studies on PHA synthesis by *P. oleovorans* carried out in our laboratory, the effect of nutrients on the growth of *P. oleovorans* and PHA production were not well understood, therefore, a logical first step to accomplish the objectives of this thesis work was to understand the behavior of *P. oleovorans* when it was grown with a growth medium formulated as shown in Table 6 and a carbon substrate. To this end, the growth of *P. oleovorans* with a growth medium containing nonanoic acid, NA, as the sole carbon source was investigated carefully to determine how the growth changed with respect to time and which nutrient was responsible for change of growth phase from one to another. Also investigated was how the biomass yield, PHA content, repeating unit composition, and molecular weight distribution of the PHA changed with respect to the growth phase to determine the optimal harvesting moment.

### 2. Growth Conditions

The growth of *P. oleovorans* with alkanolic acids under the conditions described in Chapter II consisted of a lag phase, an exponential phase, a linear phase, a deceleration phase, and a stationary phase. These five phases are

indicated in a growth curve obtained by growing *P. oleovorans* with NA in Figure 13. After a very short lag phase, cells grew exponentially for approximately 6 hours. The exponential phase was followed by a linear growth which lasted for approximately 10 hours. Then the growth rate was decelerated (deceleration phase) and after a few hours the growth stopped (stationary phase).

The length of the lag phase when a fresh medium is inoculated depends on both the change in nutrient composition (if any) experienced by the cells and the age and size of the inoculum [Bailey et al., 1986]. The lag phase in Figure 13 is very short because the initial composition of the growth medium used for inoculum preparation was identical to the composition of the growth medium used for the final growth experiment. Also, the cells in the inoculum were young when the inoculum was used.

Cells can grow exponentially only under balanced growth conditions, i.e., none of the ingredients in the medium are limiting the growth during the exponential phase. In batch culture growth, the amounts of nutrients remaining in the medium are decreasing as cells consume them, and the growth becomes limited as the amounts of certain nutrients remaining in the medium become insufficient to support exponential growth.

The first transition of growth phases in Figure 13 is from exponential phase to linear phase. The linear growth occurs when the rate of increase in cell number is constant.



This would happen when a nutrient is supplied to the cells at a fixed but insufficient rate while there is no other nutrient limiting the growth. The only nutrient supplied to cells at a fixed rate in this study was oxygen. Oxygen was supplied to cells by forcing compressed air into the culture at a rate of 2 liters per minute. Therefore, oxygen was the most probable factor responsible for the linear growth. A growth study recently carried out in our laboratory showed that the amount of oxygen taken by the cells increased rapidly as cells grew, and quickly became insufficient to support an exponential growth [Wolf et al., 1990a]. The aeration rate was much higher than 2 liters per minute and the agitation rate was much higher than 100 r.p.m. Therefore, the linear growth observed in this study was induced by the shortage of oxygen.

The effect of oxygen concentration on PHA production and growth was partially examined in this study. The growth curve shown in Figure 14 was obtained from an experiment in which the aeration rate was kept extremely low for the first 60 hours. The aeration rate was maintained at about two bubbles a second, approximately 100 ml per minute, then returned to normal, 2 liters per minute. The optical density increased for the first 24 hours to approximately 0.6 A.U., after which there was only a slight increase until the aeration rate was resumed to normal. The cell morphology was homogeneous and did not change while the aeration rate was low. The length of cells was approximately 10  $\mu\text{m}$ . However,

the cell morphology became heterogeneous when normal aeration was resumed. The length of cells then varied from 2  $\mu\text{m}$  to 10  $\mu\text{m}$ . The PHA content in the biomass harvested after 24 hours and after 60 hours were 30 % and 20 % (w/w), respectively.

During growth experiments using various carbon sources, a heterogeneous cell morphology was commonly observed when cultures were in their late stationary phase. At this time, a significant number of PHA granules disappeared and the disappearance was correlated with consumption of PHA molecules by *P. oleovorans*. The heterogeneous cell morphology observed in this study after resumption of normal aeration rate might also be related to PHA degradation. However, the distribution of cell length was continuous, but there were only two types of cells, long or short, present when PHA degradation took place in the old culture grown under normal conditions. The shapes of cells in these two cultures as observed by optical microscopy are drawn in Figure 15.

A lower PHA content under a higher oxygen concentration was also obtained in a study in which octanoic acid, OA, was used as the carbon source [Wolf et al., 1990b]. In that study *P. oleovorans* was grown in two types of flasks, normal Erlenmeyer flasks and dented Erlenmeyer flasks, containing the same growth medium. The dented flasks were used to improve the efficiency of aeration which would increase the oxygen concentration in the medium. The biomass obtained



from the culture grown in normal flasks contained higher content of PHA than the biomass obtained from the culture grown in dented flasks. However, PHA yields were almost the same regardless of the type of flasks. This study showed that more cells containing smaller amount of PHA were obtained from the culture grown under higher oxygen concentration.

Considering what has been discussed above, it can be concluded that oxygen affects PHA production by either (1) increasing cell growth, or (2) decreasing cell growth, or (3) increasing consumption of PHA during stationary phase.

During the deceleration phase, the rate at which the number of cells increases continually decreases. This change will occur when the available amount of a nutrient is not sufficient to support a full growth and decreasing. Therefore, the nutrient responsible for the growth phase transition from a linear phase to a deceleration phase should be one of those provided initially when the medium was prepared. The effects of the initial concentrations of various nutrients on the biomass yield were investigated by growing *P. oleovorans* with 20 mM OA [Wolf et al., 1990b]. The results are shown in Figure 16, and it shows that the growth was limited when the initial concentration of nitrogen was lower than 10 mM under the growth conditions used in that study. However, this result can not be applied to the present study because the concentration of NA in this study was 10 mM, and that amount should be depleted earlier than



when the initial concentration of OA was 20 mM. Also, the physical parameters such as aeration rate and agitation rate were different.

The effect of the initial NA concentration on the specific growth rate was examined from growth curves of *P. oleovorans* with 5 mM and 10 mM NA media concentrations. The plots for the exponential growth phases are expanded and shown in Figure 17. The duration of exponential growth was the same regardless of the concentration of NA. The specific growth rates, the slopes of the curve of  $\log(\text{O.D.})$  vs. growth time, were also very close to each other, 0.58 and 0.53  $\text{h}^{-1}$  for growth with 5 mM and 10 mM NA, respectively. These values are close to those found in other studies in which *P. oleovorans* was grown with OA [Wolf et al., 1990a; Lageveen et al., 1988]. These results showed that the exponential growth rate was not affected by the different initial concentration of NA. However, the time taken for growth to reach the stationary phase was much shorter when the initial concentration of NA was 5 mM as shown in Figure 18. The optical density of the culture at the stationary growth phase was higher for the growth with 10 mM NA. The fact that the growth stopped earlier when the initial concentration of NA was 5 mM shows that the growth was limited by the shortage of NA.

The limitation of growth by the carbon source in the culture grown with 10 mM NA was confirmed by measuring the amount of NA remaining in the medium with respect to the

growth time. The results presented in Figure 19 shows that almost half of NA had been consumed during the exponential growth and that the deceleration phase started after approximately 17 hours as the concentration of carbon source became low and the growth stopped after approximately 25 hours when there was no carbon source remaining in the medium. The concentration of the carbon source was, therefore, responsible for deceleration and initiation of the stationary phases under the growth conditions used in this study.

PHA production by *P. oleovorans* during the exponential growth was examined by determining the PHA content in the biomass harvested at different times during that phase with results shown in Figure 20. The biomass yield and PHA contents increased during the exponential phase which indicates that *P. oleovorans* produced PHA during the exponential phase. This result shows that no limitation of any nutrient is required for induction of PHA production by *P. oleovorans*.

### 3. Polymer Composition Change with Respect to Growth Time

The fractions of C7 unit and C9 units in the PHAs isolated from the biomass harvested earlier than 10 hours are presented in Figure 21. The compositions were calculated from the peak areas in the gas chromatograms. C5 and C11 units were not included in the calculation so as to enhance



the variation. Even though the variation was very small, it can be seen that the amount of C7 units decreased while the amounts of C9 units increased with respect to growth time. A possible explanation for this result may be that NA was used more for energy production than for PHA production in the young culture which means NA was used more for acetate production, resulting in the increase of the amount of 3-hydroxyheptanoate which would result in increased amount of C7 units in the polymer. The decreasing amount of C7 units in the PHAs produced may suggest that the pathway of utilizing NA was changing even during the early growth phase. The concentration of oxygen might be responsible for this change.

The overall change of repeating unit composition of the PHAs obtained at different growth times is shown in Figure 22. The mole fractions of C7 in the PHAs isolated at 5.5 and 6.2 hours were slightly higher than those at other times. The difference cannot be noticed in Figure 22. As a whole, the change of repeating unit composition was small enough to be able to conclude that the repeating unit composition of the PHA did not change with respect to time.

The molecular weight of the PHA was not dependent on the harvesting time. In Figure 23, the gel permeation chromatograms of crude extracts from biomasses harvested at different times are presented. Crude extracts were used for molecular weight determination to avoid possible molecular weight change caused by purification. However, the molecular



weights of the purified PHAs were the same as those of crude extracts.

As will be discussed later in this section, the PHA content in the biomass decreased significantly after 21 hours as *P. oleovorans* digested PHA molecules for growth. That is, the PHA isolated from biomass harvested after 21 hours contained PHA granules that were undergoing degradation. Therefore, the identical repeating unit compositions of PHAs obtained before and after 21 hours means the repeating unit composition was not affected by PHA degradation.

The constant repeating unit composition of the PHA obtained even after degradation suggests that these PHAs were random copolymers. The molecular weight of the PHA was not affected by degradation, either. This result could occur if *P. oleovorans* degraded and consumed entire PHA molecules one after another in such a way that the number of PHA molecules being degraded at any given time remained low. As a result either the amount of low molecular weight PHA would be too small to be reflected in the measured molecular weight or no low molecular weight fraction remained.

There is another very interesting feature observed commonly in old cultures of *P. oleovorans* grown with various carbon sources. As shown in Figure 15, the cells were either long or short so that there were cells which contained either the maximum number of PHA granules or contained no visible PHA granule. This result showed that the cells which started to utilize PHA did so by digesting them completely while

other cells kept their PHA without digesting. This result suggested that, once started, the depolymerization was so fast that cells containing active depolymerase consumed the PHA very fast. However, this result does not explain how depolymerization was inhibited in other cells.

There is a possibility that the cells digesting PHA may release small molecules produced from PHA into the medium which can be used as the carbon source. This suggestion is supported by the presence of acetate in the growth medium when *P. oleovorans* was grown with OA [Brandl et al., 1988]. It was observed that when there is an excess amount of carbon source PHA granules were not degraded [Wolf et al., 1990a]. Therefore, it is likely that acetate produced by digesting PHA was released into the medium for the growth of other cells, and as long as the amount of acetate was sufficient, the other cells would not start to digest PHA molecules.

In Figure 24, the fate of the carbon source is presented schematically based on the PHA synthesis cycle presented in Chapter I. According to Figure 24 and the PHA synthesis cycle, it may be possible that the 3-hydroxyalkanoate produced by PHA degradation was incorporated into new polymer. If this happens in a significant amount, the repeating unit composition of PHA in the late stationary phase should change. As discussed above, the repeating unit composition of PHA obtained after PHA degradation was identical to the repeating unit composition of PHA obtained earlier. The results obtained by others from a molecular



biological study supported this conclusion Huisman et al., 1990]. In that study, a gene containing the codes for polymerase and depolymerase and a gene containing the codes only for polymerase were cloned separately into two different strains of *E. coli*, which were then grown with OA. The repeating unit compositions of PHAs obtained were identical. This result also shows that depolymerization does not affect the repeating unit composition.

#### 4. Optimal Harvesting Time

The biomass yield, PHA yield, and PHA contents per unit biomass were determined with respect to growth time. The results are presented in Figure 25. The maximum PHA content and maximum PHA yield were obtained at slightly different growth times, which were approximately 21 hours and 24 hours, respectively. The PHA content decreased significantly during this time from 45 % (w/w) to 38 % (w/w). A similar result was obtained from a study of the preparation of PHA from *P. oleovorans* grown with OA [Wolf et al., 1990a]. The decrease of PHA content with increases of PHA yield and biomass yield showed that growth, PHA production, and PHA degradation were taking place at the same time during this period.

After 34 hours, both the PHA content in the biomass and the PHA yield decreased. The decreases of both PHA content and PHA yield showed that *P. oleovorans* was mainly degrading



the stored polymers. The biomass yield remained constant during this period of time but the amount of non-PHA cellular materials, (biomass yield)-(PHA yield), increased. This result shows that PHA was used not only for energy production but also for production of other cellular materials. These results show that changes of biomass yield, PHA yield, PHA content, and non-PHA cellular material yield were all related to the amount of carbon source remaining in the medium.

Figure 25 and the growth curve of *P. oleovorans* with 10 mM NA show that cells should be harvested at the early stage of stationary growth phase for the highest PHA yield. Since the harvesting procedure for a 12 L culture usually takes 3 hours, harvesting should begin at the latest stage of the deceleration phase as described in Chapter II. Even though the change of PHA yield was not determined, if aeration was stopped the PHA granules were not degraded as observed by optical microscope.

### C. Reproducibility Study of PHA Preparation

#### 1. Introduction

In earlier studies in our laboratory on PHA synthesis by *P. oleovorans*, the experimental results tend to be inconsistent in respect to repeating unit composition, molecular weight, biomass yield, and PHA yield. This poor reproducibility made analysis of the results obtained under

new experimental conditions difficult, therefore, it was desired to attain reproducibility of PHA production. In this section, the reproducibility of PHA production in respect to repeating unit composition, molecular weight, biomass yield, and PHA yield is examined. Also, the effect of feeding frequency on the repeating unit composition of the PHA is examined.

## 2. Reproducibility of PHA Preparation

The biomass yield, PHA yield and molecular weight distribution of PHAs obtained from repeated batch culture fermentations using various carbon sources are listed in Table 9. Preliminary growth experiments carried out following the experimental procedure described in Chapter II showed growth with the carbon sources listed in Table 9 commonly reached the stationary phase approximately after 20 hours. Therefore, the harvesting was started 19 hours after inoculation for all the growth experiments listed in Table 9.

Data in Table 9 indicate that the reproducibility of biomass yield, PHA yield, and molecular weight distribution was excellent for the growth experiments using carbon sources other than 90 % NA. For the growth experiments using 90 % NA, the optical density of the inoculum was not measured when it was used, therefore, it was likely that the number of cells and the age of cells in the inoculum used for each



growth experiment were not constant. Because the age and size of the inoculum affect the length of the lag phase when a fresh medium is inoculated [Bailey et al., 1988], cells might have not been in the same growth phase when they were harvested in experiments using 90 % NA.

As discussed in Section III.B, the yield of non-PHA cellular materials, (biomass yield)-(PHA yield), increased with time. Therefore, the low biomass yield, low PHA yield, and low non-PHA cellular material yield obtained in experiment A indicated that the culture was harvested before full growth was achieved. On the other hand, the low PHA yield, high biomass yield and high non-PHA cellular material yield from experiment C were obtained because the culture was harvested after *P. oleovorans* began to digest the stored PHA granules for survival.

The constant molecular weight of the PHAs prepared using 90 % NA corresponded to the constant molecular weight of PHA if it was harvested regardless of growth phase, as discussed in Section III.B. In addition, data in Table 9 show that the molecular weights of the PHAs were not dependent on the type of carbon source and all of the PHAs prepared in this thesis research had the same molecular weight distribution regardless of the carbon source or the growth conditions used. The number average molecular weight was between 50,000 and 60,000 and the PDI was between 1.8 and 2.2. There were a few exceptions in which slightly higher molecular weights were obtained which was likely caused by the use of a



different carbon source such as 5-phenylvaleric acid, PVA, and 10-undecenoic acid, UND:. The number average molecular weights of PHA obtained from these carbon source were between 60,000 and 70,000. It should be pointed out that these molecular weights were determined by gel permeation chromatography using a standard relationship established for polystyrene standards but because PHAs contain long side chains, the true molecular weight should be different from the molecular weights calculated in this manner. Nevertheless, the molecular weight determined by GPC are useful for comparison of the closely related polymers.

In general, the molecular weights of PHAs produced seem to depend on the microorganisms involved [Anderson and Dawes, 1990]. For example, the number average molecular weights of PHAs obtained from *A. eutrophus* are generally in the range of hundreds of thousand while the number average molecular weights of PHAs obtained from *P. oleovorans* are generally less than 100,000. Also the results of an early study in our laboratory suggested the molecular weight distribution may depend on the carbon source [Brandl et. al., 1988]. Further, according to the results of that study, the molecular weight was higher when the carbon source was a lower alkanolic acid. For example, the molecular weights of the PHA obtained from hexanoic acid (PHA-HEX), and pentanoic acid (PHA-PEN) were twice as high as those of other PHAs. However, it should be considered that these PHAs have shorter side chains than others, and their behavior in solution might be different.

In early experiments in our laboratory very large amounts of PHAs were loaded on the GPC column to improve the size of peaks because the sensitivity of the refractive index detector to the PHA was very low. Overloading a column can result in higher apparent molecular weight because the packing materials can become saturated and excess PHA can elute faster. The significantly higher molecular weights of PHA-NON and PHA-OCT reported in the early studies than those measured by other researchers in our laboratory might have been caused by the overloading.

The repeating unit compositions of the PHAs produced in this study are presented in Table 10. The repeating unit compositions obtained were reproduced to an excellent degree in all growth experiments. The constant repeating unit compositions of the PHAs prepared from 90 % NA corresponded to the result discussed in Section III.B that the repeating unit composition was not dependent on the growth time. The repeating units containing even number of carbons in these PHAs could have resulted either from the impurities in the carbon source or from reaction causing the loss of one carbon atom from the substituent.

According to the results shown in Table 9, the repeating unit compositions and molecular weight were not affected either by growth time or by poor control of inoculum. Therefore, the poor reproducibility with respect to repeating unit composition and molecular weight reported from early experiments in our laboratory were rather unusual.



### 3. PHAs Obtained by Feeding Nonanoic Acid Once and Twice

The repeating unit compositions of PHAs produced from *P. oleovorans* grown by feeding alkanolic acids either once or twice consecutively were reported to be significantly different [Gross et al., 1989]. For example, a PHA-NON obtained from cells grown by feeding NA once was reported to consist of 3 mole% C5, 27 mole% C7, 6 mole% C8, 61 mole% C9, 2 mole% C10, and 1 mole% C11 units while a PHA-NON produced from cells grown by feeding NA twice consisted of 3 mole% C5, 20 mole% C7, 5 mole% C8, 61 mole% C9 units. However, it was not clear if these differences were caused by feeding frequency of NA.

The effect of feeding frequency of the carbon source on the repeating unit composition of the PHA produced was reexamined by feeding NA either once or twice following the procedure used in the early study [Gross et al., 1989]. The repeating unit compositions of PHAs obtained by feeding *P. oleovorans* NA either once or twice consecutively are compared in Figure 26. There was no compositional difference between these two PHAs, and the molecular weights of these PHAs were also identical. Similar results were also obtained from fed-culture growth experiments in which OA was fed up to eight times consecutively [Gagnon, 1990; Peres, 1990]. The repeating unit compositions and molecular weights of PHAs were constant regardless of the feeding frequency.



These results showed that the frequency of feeding a carbon source to *P. oleovorans* affected neither the repeating unit composition nor the molecular weight of the PHA produced. It is likely, therefore, that the different composition of the PHAs prepared by feeding carbon sources either once or twice, which were reported in the early studies, might be caused by poor characterization of PHAs.

#### 4. Compositions of Repeating Units from a Single Carbon Source

During growth on a single carbon source, such as OA, *P. oleovorans* produces a polymer containing units other than C8, especially C6 and C10 units. It has been discovered in the present study that two different carbon sources are used by the bacteria for polymer production, the relative proportion of repeating units derived from each carbon source was not altered.

For this interpretation, it was assumed that when the bacterium was co-fed with NA and OA, the C5, C7, C9 and C11 units were produced from the former and C6, C8 and C10 units were produced from the latter. In experiments in which the co-substrate was UND:, C7:, C9: and C11: units were considered to be produced only from that source. On that basis, a relative amounts of the repeating units from each carbon source was calculated by ignoring the presence of repeating units from the other carbon source. For example,

the relative amount of a given repeating unit from NA was calculated by the following equation:

$$\frac{\text{mole fraction of C7 or C9 or C11 unit}}{\text{mole fraction of C7 + C9 + C11 unit}} \times 100 (\%)$$

The results for the repeating unit compositions which originated from OA, NA, and UND: in cofeeding experiments are shown in Figure 27. It was not possible to accurately determine the C11 unit in the PHAs containing C11: unit by the standard gas chromatographic method because the retention times of the methyl esters of these units were very close to each other and the amount of C11 unit was too small to be separated from the relatively broad peak of methyl-3-hydroxyundecenoate. Therefore, the C11 units in the PHAs prepared from cells grown with mixtures of NA and UND: were not included in the calculation.

Figure 27 shows that the relative proportions of the repeating units produced from a carbon source does not change significantly in the presence of another carbon source. This result would be expected if the PHAs isolated from cells grown with mixtures of two carbon sources were physical mixtures of two different PHAs, even though each of these PHAs were random copolymers, as will be discussed in following sections. However, in terms of preparing PHAs containing new repeating unit compositions, the constant



relative amount of repeating units from two carbon sources was a quite disappointing finding.

An attempt was made to alter the relative repeating unit composition by growing *P. oleovorans* with NA in the presence of acetic acid. Acetic acid was considered to be a carbon substrate that could affect the repeating unit composition because *P. oleovorans* can remove acetate from NA for energy production. Therefore, if acetate is available in the medium, *P. oleovorans* may not take acetate from NA. Also, because acetic acid supports cell growth but does not support PHA production, the repeating unit composition of the PHAs produced under such conditions may be more like a homopolymer in composition.

For this experiment, *P. oleovorans* was grown with a medium containing an equimolar mixture of NA and acetic acid (5 mM each). The growth curve so obtained is shown in Figure 28. Portions of the culture were sampled during two growth phases, the late exponential phase (6.5 hours) and the early stationary phase (13 hours). From 1 liter of culture harvested after 6.5 hours, 0.183 g of biomass containing 16.4 % (w/w) PHA was obtained. This result is additional evidence for PHA production during exponential phase as discussed in Section B. From 1 liter of culture harvested after 13 hours, 0.673 g of biomass containing 21.4 % (w/w) PHA was obtained.

The amount of PHA obtained from the biomass harvested after 13 hours was slightly lower than one-half of the amount



of PHA obtained from an experiment in which *P. oleovorans* was grown with 10 mM NA and harvested in the early stationary phase. However, the amount of non-PHA cellular materials excluding PHA, (biomass yield)-(PHA yield), in the biomass was close to that in the biomass obtained from an experiment using 10 mM NA as the carbon source in which approximately 0.5 g of cell materials was obtained from 1 liter of culture. These results showed that *P. oleovorans* grew on both NA and acetic acid to produce cellular materials other than PHA but PHA was produced from only NA and was not affected by the presence of acetic acid in the feed because the repeating unit compositions of these PHAs were identical, as described below.

The repeating unit composition of the PHA obtained from cells grown with only NA and the PHAs obtained in this study using an equimolar mixture of NA and acetic acid are compared in Figure 29. Figure 29 shows that the presence of acetic acid in the growth medium did not affect the repeating unit composition of the PHA produced regardless of when the biomass was harvested. The number average molecular weights of all of the PHAs were identical, approximately 55,000 with PDIs of approximately 1.9.

Considering the experimental results discussed so far, it is not likely that PHAs with new composition can be produced from a given carbon source by only modifying physical growth conditions, such as concentration of nutrients, harvesting time and so on. That is, to produce

different PHAs, different carbon sources will have to be used. Using mixtures of carbon substrates is also a useful method to prepare PHAs of new composition as will be described in following sections.

#### D. Classification of Carbon Substrates

##### 1. Introduction

According to the PHA synthesis cycle presented in Figure 4, any carbon substrate that can be converted to a 3-hydroxyalkanoic acids by *P. oleovorans* is potentially capable of supporting cell growth and PHA production. Alkanoic acids [Lageveen et al., 1988; Brandl et al., 1988; Gross et al., 1989; Huisman et al., 1989], alkenoic acids [Huisman et al., 1989; Fritzsche et al., 1990c], alkanes [Lageveen, 1986], and alkenes [Lageveen et al., 1988] have been most frequently used because they are easily available, and PHAs were prepared in good yields from these carbon sources.

Comparison of the results of PHA synthesis by *P. oleovorans* using various types of organic substrates as the carbon source showed that the physical and chemical structures of the organic substrates are critical in PHA production. For example, the n-alkanoic acids from acetic acid to pentanoic acid all supported cell growth but did not support PHA production from *P. oleovorans*. Higher n-alkanoic



acids, up to hexadecanoic acid, supported both cell growth and PHA production [Huisman et al., 1989]. In a study of PHA production using 1-alkenes, no PHA was obtained from cells grown with hexene and dodecene, but hexane and dodecane supported PHA production. These results show that there are certain limits of chain length for a carbon substrate to support both cell growth and PHA production, and the limits vary according to the type of the carbon source. As an example, the strong effect of spatial structure of the carbon substrates was demonstrated in a study of PHA production using 5-, 6-, and 7-methyloctanoic acid as the carbon sources which will be discussed later [Fritzsche et al., 1990b].

In Section III. C, the relative amounts of repeating units within a particular PHA produced from a specific carbon source was constant regardless of the presence of another carbon source or of somewhat different growth conditions. A conclusion was made that novel PHAs may be prepared only by growing *P. oleovorans* with carbon sources having different and unusual structures. Therefore, it was decided to evaluate previously untested carbon substrates that could support PHA production for that purpose. To this end, *P. oleovorans* was cultured in growth media containing various types of carbon substrates, each at an initial concentration of 10 mM. The cells were observed by optical microscopy to determine the presence of PHA granules. In this section, these carbon substrates are classified based on ability to support cell growth and to produce PHA.



## 2. Classification of Carbon Substrates

When exposed to new carbon substrates, *P. oleovorans* may either grow with PHA production, or may grow without PHA production, or may not grow. What is observed with the optical microscope for these three cases is schematically presented in Figure 30. The compounds examined as potential carbon sources in the present study, and also those reported in other studies, are classified in Table 11 as one of the three groups based on these microscopy observations. The compounds belonging to group "a" support cell growth and PHA production. Those belonging to group "b" support cell growth but do not support PHA production. Those belonging to group "c" do not support cell growth and, thus, cannot support measurable PHA production.

n-Alkanoic acids and n-alkanes supported PHA production by *P. oleovorans* only when they have more than 5 and less than 17 carbon atoms. The reason why *P. oleovorans* does not produce PHAs with alkanoic acids shorter than hexanoic acid may be related to the nature of the polymerase in that bacterium but the reason why *P. oleovorans* does not grow with n-heptadecanoic acid and longer alkanoic acid might be related to either the very poor solubility of such long alkanoic acids in aqueous media or transport of those compounds into the cell. The growth medium prepared with octadecanoic acid, which was intended to be at a

concentration of 3 mM, was a suspension of undissolved particles of octadecanoic acid in an aqueous solution of inorganic nutrients. There was no noticeable dissolution of octadecanoic acid when the mixture was agitated vigorously, even for periods longer than 24 hours. Nevertheless the cells exposed to this medium were alive after approximately 40 hours as indicated by the fact that the cells were moving although there was no noticeable increase of cell density as observed by optical microscopy. The result with octadecanoic acid may be comparable with the results from oleic acid, elaidic acid, and  $\gamma$ -linolenic acid because they all have the same number of carbon atoms. The solubility of oleic acid was much higher than that of octadecanoic acid and even though the medium prepared with oleic acid was also a suspension, the undissolved oleic acid remained as a liquid because the melting transition temperature of oleic acid is approximately 30 °C. Nevertheless, the yield of PHA was extremely low. From 12 l of culture grown with a 10 mM concentration of oleic acid, less than 50 mg of PHA was isolated. This polymer will be described in Section III.F.

Even though n-alkanes are readily available compounds, only those from hexane to dodecane were examined for PHA preparation. As n-alkanes are oxidized to n-alkanoic acids in the cells, it is highly probable that the range of n-alkanes supporting PHA production might be the same as that of n-alkanoic acid provided that there is no problem caused by high melting temperature.



The results with n-alkenes were quite different from those with n-alkanes and n-alkanoic acids. Hexene and dodecene were reported not to support PHA production while hexane, hexanoic acid, dodecane, and dodecanoic acid did support PHA production. Therefore, the presence of unsaturated groups in these alkenes apparently prevented polymer formation. The difference between hexane and hexene may be explained by the close proximity of the double bond to the  $\beta$  position and its effect on either monomer preparation or polymerization.

It seems to be that the presence of functional groups has a more critical effect on the PHA production than on the cell growth because compounds belonging to group "b", which contain a very polar substituent can support cell growth. The effect of distance between functional substituent and the bioreactive groups—carboxylic group on cell growth could be seen from the growth experiments using  $\omega$ -bromoalkanoic acids. The growth curves for *P. oleovorans* with 6-bromohexanoic acid (6BRHA), 8-bromooctanoic acid (8BROA), and 11-bromoundecanoic acid (11BRUA) are presented in Figure 31. There was no detectable increase of optical density for the growth with 6BRHA for 40 hours whereas the growth rate was fastest with 11BRUA. The optical density of a culture grown with 6BRHA increased to approximately 0.6 after 100 hours. The optical density in the stationary phase was also highest for the growth with 11-bromoundecanoic acid.



These results show that the location of the bromine in the carbon substrate affected the growth. Because OA and undecanoic acid were equally good carbon sources, one may conclude that 11BRUA allowed better growth than 8BROA because of the distance between the bromo group and the carboxylic end of the molecule.

A comparison of the results with 7-methyloctanoic acid, 6-methyloctanoic acid, and 5-methyloctanoic acid suggests that steric hindrance of a carbon substrate can also disturb either monomer formation or polymerization [Fritzsche et al., 1990c]. The fact that the presence of a methyl group at position 5 or 6 of the OA prohibited polymer production, and the fact that the minimum length required for alkanes and alkanolic acids to be polymerized is six carbons suggest that enzymes for monomer formation or the polymerase may interact with the substrates at those positions. For the latter, the presence of a methyl branch could prevent the proper contact between either the monomers or the polymer and the enzyme. The different results obtained with 4-phenylbutyric acid (PBA) [Leppelier, 1989], PVA, and 4-cyclohexylbutyric acid (CHBA) as the substrate may be explained similarly. PBA did not support PHA production but CHBA and PVA did. The structures of these substrates are shown in Figure 32. 4-Cyclohexylbutyric acid may have a molecular shape similar to n-heptane as far as the enzymes for monomer preparation and polymerization are concerned. However, 4-phenylbutyric acid may not have a molecular shape similar to n-heptene in

that regard because the phenyl ring is planar. Its shape may be closer to that of n-hexene, which does not support PHA production. Lastly, PVA may have a shape similar to n-heptene, which supports PHA production.

Some of the carbon substrates classified as group "a" gave extremely low yields of cells and PHAs. Examples of such are cyclohexylbutyric acid, tridecanoic acid, oleic acid, methyl octanoate, and methyl decanoate. Twelve liter cultures grown with these carbon substrates gave PHA yields between 20 and 100 mg, and it was difficult to observe PHA granules in the cells grown with these substrates. The production of PHA when methyl octanoate was used as the carbon source was only confirmed by extracting the PHA from the biomass. Based on these observations, it is highly probable that some of the carbon substrates classified in group "b" may support production of PHA in such a small amount that no PHA granule was large enough to be observed in the cell. Such substrates are dodecene, sebacic acid monoesters, and esters of UND:.

Unusually long lag phases were observed during growth experiments with many of the substrates belonging to group "b" and also some of those belonging to group "a". For example, the lag phase for growth with 4-cyclohexylbutyric acid was longer than 3 days, and the lag phases for growth with UND: and with PVA were approximately 20 hours. The lag phases for growth with methyl octanoate and with methyl decanoate were approximately two days. However, once



started, growth was rapid. Therefore, the long lag phases might be related either to the transport of the carbon substrates into the cells or the slow development of active mutants.

The organic substrates belonging to group "c" were either too small, too large or too polar, and the presence of more than one polar group appears to be unfavorable for growth. For example, none of the aminoalkanoic acids examined supported cell growth.

It is quite interesting that sebacic acid did not support cell growth while the monoesters of sebacic acid did.

At present, it is very difficult to derive general rules relating the chemical and physical properties to cell growth and PHA production. There seems to be certain limitations of molecular size, and the minimum and maximum chain lengths which allow growth and PHA production vary according to the type of carbon substrates. Also, a longer distance between the carboxylic acid and the substituent group seems to be favorable for cell growth. The spatial structure of the chain at the 5th and 6th carbon seems to be important as well.



## E. PHAs Prepared from Long n-Alkanoic Acids

### 1. Introduction

PHAs produced from *P. oleovorans* contained relatively long side chains which were determined by the length of the carbon source with which the cells were grown. The monomer compositions of PHA-HEX, PHA-HEP [Gross, R. et. al, 1989], PHA-OCT, and PHA-NON are shown in Table 12. PHA-OCT was the most crystalline ( $\Delta H_m = 8.3$  cal/g) and PHA-HEX was reported to be amorphous while PHA-HEP had a little crystallinity ( $\Delta H_m = 1.3$  cal/g). The degree of crystallinity of PHA-OCT and PHA-NON was determined to be 20 to 30 % by wide angle X-ray diffraction; WAXD [Marchessault et al., 1990].

Polymers containing long side chains have common features which originate from ordering of side chains. These polymers crystallize in sheet-like layers as shown in Figure 33. The side chains extend from the polymer backbone. Because of this arrangement, these polymers are called 'comb-like' polymers. The fiber repeat length,  $d_3$  in Figure 33, depends on the interaction between the side chains and the conformation of the main chain while the interplanar spacing,  $d_4$  in Figure 33, is constant regardless of the type of main chain and the degree of interaction between side chains. The melting transition and glass transition temperatures of comb-like polymers decrease as the length of the side chain increases but when it becomes longer than a

critical length these transition temperatures start to increase. The critical length of the side chain depends on the nature of the polymer backbone and should be longer for a polymer containing a stiffer backbone. For example, the glass transition and melting transition temperatures of polyacrylates, PAs, and polymethacrylates, PMAs, containing alkyl side chains shorter than C8-C10 units were decreased as the length of the side chains were increased. However, the transition temperatures of PAs and PMAs containing longer side chains increased as the length of the side chain increased [Plate and Shibaev, 1974].

WAXD studies on PHA-OCT, PHA-NON, and PHA-DEC [Marchessault et. al 1990] showed that these polymers had the same features as comb-like polymers. Two possible  $2_1$  helices were proposed for these PHAs as shown in Figure 34. In these WAXD studies, the PHA samples were stretched to enhance biaxial orientation.

Recently, Huisman et. al reported PHA production by *P. oleovorans* using alkanolic acids from butyric acid to octadecanoic acid as carbon sources [Huisman et al., 1989]. They reported that n-alkanoic acids longer than hexadecanoic acid did not support cell growth. Independently, the preparation of PHAs from alkanolic acids longer than NA had been investigated in the present thesis work. The objectives of the present study were to prepare PHAs containing long alkyl side chains in order to investigate the effect of the length of side chains on the properties of the PHAs and to



understand how *P. oleovorans* polymerizes long alkanolic acids by investigating the repeating unit compositions of the PHAs obtained.

## 2. Preparation of PHAs from n-Alkanolic Acids Longer Than Nonanoic Acid

The growth media containing higher n-alkanoic acids were suspensions of undissolved particles of the acids in aqueous solution of inorganic nutrients. The undissolved particles were present in the culture throughout the growth so that it was impossible to measure the optical density of the culture. Therefore, the optimal harvesting time could not be determined by monitoring the growth curve. From the growth experiments using shorter chain alkanolic acids for which water solubility was not a problem, it was usually observed that the color of the culture turned pale green or the odor became very unpleasant when the growth reached the stationary phase. From these observations, it was possible to harvest the cultures grown with the longer alkanolic acids when either the color of the culture turn green or the smell of the culture became unpleasant. However, significant amounts of undissolved particles of carbon sources were remaining when cultures were harvested in this manner. The carbon source particles remaining could not be separated from cellular materials so that the the biomass yield could not be determined.



The lowest PHA yield was obtained from cells grown with tridecanoic acid. From a 12 L of culture grown with 5 mM tridecanoic acid, approximately 50 mg of PHA was obtained. All of the other alkanoic acids, from undecanoic acid to hexadecanoic acid, gave good amounts of PHAs, approximately 1 gram from 12 L of culture.

The repeating unit compositions of PHAs prepared from decanoic acid to hexadecanoic acid were calculated from GC peak areas of methanolized polymers, and the data obtained are collected in Table 13. The gas chromatograms of the methanolized samples of these PHAs are shown in Figure 35. The presence of ion fragments with mass number of 103 and 74 in the mass spectrum was examined for confirmation of presence of repeating units longer than 3-hydroxydodecanoate unit. The data in Table 13 and Figure 35 show that the largest repeating units in these PHAs were 3-hydroxyalkanoates containing the same number of carbons as the carbon source. This result differs from the result previously reported by Huisman and coworkers. They reported that there was no monomer unit longer than 3-hydroxydodecanoate in the PHAs isolated from *P. oleovorans* regardless of the carbon source. In Figure 36, the total ion chromatogram (TIC) and mass spectra of a methanolized sample of PHA obtained from tetradecanoic acid, PHA-TET, are shown. The TIC was obtained by injecting 6  $\mu$ L of the methanolized samples into the GC/MSD and activating mass spectrometer 13.5

minutes after injection for sufficient sample to obtain mass spectrum.

The data in Table 13 also shows that the main repeating unit in these PHAs was either 3-hydroxyoctanoate unit (C8) or 3-hydroxynonanoate unit (C9) regardless of the carbon source. The C8 unit was the main repeating unit in the PHAs prepared from alkanolic acids which had an even number of carbon atoms and were higher than heptanoic acid, while the C9 unit was the main repeating unit in the PHAs prepared from alkanolic acids which had an odd number of carbons and were higher than OA. The preference of *P. oleovorans* for forming C8 and C9 units was very strong considering that the C8 unit is presumably formed from hexadecanoic acid only after four deacetylation reactions.

In general, a 3-hydroxyalkanoic acid can be utilized either for polymer production or for acetate production, and a 3-hydroxyalkanoic acid shorter than the original 3-hydroxyalkanoic acid by two carbons is produced as a product of the deacetylation reaction. This shorter 3-hydroxyalkanoic acid can be utilized either for polymer production or for acetate production. If the 3-hydroxyalkanoate is either longer than 3-hydroxynonanoic acid or shorter than 3-hydroxyoctanoic acid, deacetylation takes place as the main reaction. The destiny of n-alkanoic acids is presented in a flow diagram in Figure 37.

Heptadecanoic acid and longer carboxylic acids were reported not to support cell growth [Huisman et al., 1989].



The data in Table 13 shows that PHA-TET, PHA obtained from dodecanoic acid (PHA-DOD), and PHA obtained from decanoic acid (PHA-DEC) have quite different repeating unit compositions from each other but PHA-TET and PHA obtained from hexadecanoic acid, PHA-HEXD, have similar repeating unit compositions. These results shows that the ratio of deacetylation to polymerization of the 3-hydroxyalkanoate intermediates depends on the initial length of the alkanolic acid when they are shorter than C14.

The melting transition temperature and the heat of fusion of PHAs prepared from various alkanolic acids are collected in Table 14. As mentioned earlier, the glass transition and melting transition temperatures of polymers containing alkyl side chains decrease until the side chain length reaches a critical length, after which the transition temperatures start to increase. PHB has a higher melting transition temperature and a higher glass transition temperature than all other PHAs obtained from n-alkanoic acids. Two parameters, length of the side chains and inhomogeneity of repeating units, are responsible for the lower transition temperatures of the latter. It is difficult to separate the effects of these two parameters on the melting transition temperatures. PHA-OCT and PHA-NON have higher  $T_g$ 's and  $T_m$ 's than PHA-HEX and PHA-HEP. In addition, PHA-OCT has higher  $T_m$  than PHA-NON, which might be explained by the higher homogeneity of repeating unit composition of this PHA than that of PHA-NON (Table 12). The monomer



compositions of PHA-HEX and PHA-HEP in Table 12 show that the higher of  $T_m$  of PHA-NON than those of PHA-HEX and PHA-HEP is not the effect of inhomogeneity of repeating unit composition. Therefore, the higher  $T_m$  of PHA-NON than that of PHA-HEX or PHA-HEP is most likely the effect of the length of the side chains, that is, the critical length for the side chains of PHAs to increase  $T_m$  is likely C5 or C6. The PHAs produced from longer alkanolic acids might have lower transition temperatures because they have more types of repeating units in significant amounts than PHA-NON or PHA-OCT as seen in Table 12 and 13.

The presence of repeating units with longer side chain in these PHAs was shown by the d-spacings. The d-spacings for the PHAs prepared from OA to hexadecanoic acid are collected in Table 15 (see Figure 33 for  $d_1$ ,  $d_3$ ,  $d_4$ ). The d-spacings for the PHA-OCT, PHA-NON and PHA-DEC corresponded to the values obtained in other studies closely [Gross et al., 1989; Marchessault et al., 1990]. The  $d_1$  value increased as the side chain of the PHA became longer which may indicate side chains were fully extended from the backbone. The fiber repeat length,  $d_3$ , was not affected by the side chain length.

A conformational analysis by Marchessault and coworkers suggested that the possible minimum fiber repeat length for a  $2_1$  helix of poly- $\beta$ -hydroxypropionate is 4.5 Å [Cornibert and Marchessault, 1975]. The fiber repeat lengths of the PHAs listed in Table 15 are approximately 4.6 Å which is quite

close to this minimum showing that the main chains of PHAs rotate to allow the side chains to be positioned as closely as possible.

The DSC thermogram of the PHA-DOD showed a significant shoulder, which appeared after the main melting transition peak. The DSC thermograms of PHA-HEXD and PHA-DOD are shown in Figure 38 and 39. The unusual feature of PHA-DOD is that it contains a significant amount, 16.5 mole%, of 3-hydroxydodecanoate units, C12, and these C12 units may induce side chain crystallization. The WAXD pattern of a stretched PHA-DOD sample differed from those of others. The shape of the ring corresponding to the  $d_1$  spacing was close to full circle while those from other PHA samples were arcs. These observations suggest a possibility that there might be more than one crystalline form in PHA-DOD because of the side chain crystallization.

The low value of  $\Delta H_m$  of PHA-TRI may be explained by the fact that this PHA had 8 different repeating units which would greatly disturb crystallization. All of the PHAs produced in this study were transparent when cast as films even after a significant period of annealing at room temperature and none of them showed birefringence as observed by polarized microscopy.

$^{13}\text{C}$  NMR spectra of PHAs prepared in this study are presented in Figures 40 to 43.



## F. PHAs Containing Unsaturated Groups

### 1. Introduction

Polymers containing unsaturated units are of great interest because they can be modified to form functional polymers by various reactions. PHAs containing unsaturated repeating units were first prepared from *P. oleovorans* in a study on the oxidation of alkenes by *P. oleovorans* [Lageveen, 1986] using n-hexene, n-octene, and n-dodecene as carbon sources for PHA production. As discussed in Section III.D., alkenes shorter than heptene and alkenes longer than undecene did not support PHA production by *P. oleovorans*. These results are quite different from those obtained when alkanoic acids were used as the carbon source.

The repeating unit compositions of PHAs prepared from hexene, nonene, and decene are presented in Table 16, which shows that these PHAs contained significant amounts of saturated units (45 %, 61 %, and 54 %, respectively) as well. The authors of that study proposed a mechanism to explain the incorporation of the saturated units into these PHAs as shown in Figure 44 [Lageveen et al., 1989]. The mechanism proposed that both ends of alkenes be oxidized by *P. oleovorans* to form epoxides, 3-hydroxyalkanoic acid-SCoA, (A), and 3-hydroxy- $\omega$ -alkenoic acid-SCoA, (B). Both (A) and (B) can be utilized for PHA production. However, no evidence supporting this mechanism was provided in that study nor was it



determined whether a single homopolymer was produced or whether two or more PHAs were produced.

Most recently, PHAs produced from 3-hydroxy-7-octenoic acid, 7OCT:, and 3-hydroxy-6-octenoic acid, 6OCT:, by *P. oleovorans* was studied in our laboratory [Fritzsche et al., 1990c]. The fractions of unsaturated repeating units in PHA-7OCT: and PHA-6OCT: were 74 % and 94 %, respectively. These values are significantly higher than those in the PHAs obtained directly from alkenes. The higher fraction of unsaturated units in PHAs produced from 3-hydroxyalkenoic acids than in PHAs produced from alkenes can be explained as shown in Figure 45.

According to the scheme in Figure 45, the carboxylic acid end of the 3-hydroxyalkenoic acid can be polymerized directly leading to the formation of polymers with unsaturated units, but the olefinic group could be converted to a 3-hydroxyalkanoic acid group and polymerized to form a polymer with saturated repeating units. The rate of polymerization through the 3-hydroxycarboxylic acid group,  $k_p$ , would be higher than the product of the rates of formation of 3-hydroxyalkanoic acid,  $k_o$ , and the polymerization rate of 3-hydroxyalkanoic acid,  $k_p$  (see Figure 45). For alkenes, therefore, both ends could go through several reaction steps to form either 3-hydroxyalkenoic acid or 3-hydroxyalkanoic acid, so the amount of unsaturated units in the PHA would be determined by the relative rates of those reactions. From the results of

PHA production from alkenes, we can conclude that the overall polymerization rates through both ends,  $k_o \cdot k_p$  and  $k_o' \cdot k_p'$ , are similar.

The formation of saturated units in the PHAs produced from 7OCT: and 6OCT: cannot be explained by the mechanism shown in Figure 44. The possible products of the oxidation reactions of these acids are shown in Figure 46. The final products from 3-hydroxy-7-octenoic acid and 3-hydroxy-6-octenoic acid would be 3,6-dihydroxy-1,8-dicarboxylic acid, (A), and 5-hydroxy-7-oxo-octanoic acid, (B), respectively. (A) and (B) would not likely support the growth of *P. oleovorans* considering that the chemically similar substrate, sebacic acid, did not. Also, it is not likely 3-hydroxyalkanoate units would be formed from either (A) or (B). The saturated units in PHA-7OCT: and PHA-6OCT:, therefore, are probably formed by reduction of the unsaturated units, and the lower fraction of saturated units in the PHA-6OCT:, 6 %, than PHA-7OCT:, 6 %, suggests that reduction is affected by the structure of the alkenoic acid.

The structures of PHAs prepared from mixtures of two carbon substrates was of great interest. The resulting polymer might be random copolymers, physical mixtures of two different polymers which are produced from each substrate, or block copolymers. In the previous section, the PHA produced from a mixture of OA, and NA, was investigated using GC/FAB mass spectrometry which revealed that it was a random copolymer. Another possible method of investigating the



types of PHAs produced from mixed carbon sources is comparing the physical properties of the two PHAs prepared separately from each carbon source, and blends of two separately prepared PHAs with the PHA produced from the mixed carbon source. If the two separately prepared PHAs have significantly different properties so that they are not miscible each other, it should be possible to distinguish physical mixtures of two PHAs from a random copolymer, both of which contain repeating units from two carbon sources, by comparing their physical properties such as transition temperatures.

The type of the PHAs obtained from mixtures of NA and OA could not be elucidated by comparing their physical properties with the blends of PHA-NON and PHA-OCT because PHA-NON and PHA-OCT are miscible each other. Therefore, for the investigation of the types of PHAs produced from mixed carbon sources by comparing physical properties as discussed above, two carbon sources yielding immiscible PHAs should be used for PHA production.

An amorphous polymer might be immiscible with PHA-NON considering that PHA-NON has approximately 20 % of crystallinity. PHAs containing unsaturated units were selected as the model polymer because it was expected that unsaturated units would disturb crystallization and cause lower degree of crystallinity than PHA-NON.



## 2. Preparation of PHAs Containing Unsaturated Repeating Units.

As a preliminary study for producing PHAs containing unsaturated units by *P. oleovorans*, 7-octenoic acid alone was examined as a carbon source. 7-Octenoic acid was expected to support PHA production because octene and OA did. From 12 liters of a culture grown with 7-octenoic acid, approximately 5 g of biomass containing 10 % (w/w) of a PHA were obtained. The IR spectrum of the isolated PHA, PHA-OCT:, showed olefinic C-H stretching and C=C stretching peaks at  $3300\text{ cm}^{-1}$  and  $1600\text{ cm}^{-1}$ . The NMR spectrum of PHA-OCT: showed that this PHA contained less than 40 % unsaturated repeating units. This amount was much lower than the fraction of unsaturated repeating units PHA-7OCT:, which contained 74 % unsaturated units. This lower fraction of unsaturated units could be explained in a similar way as shown in Figure 45. That is, *P. oleovorans* has to convert octenoic acid to 3-hydroxyoctenoic acid while it can polymerize 3-hydroxyalkenoic acid polymerize directly.

10-Undecenoic acid, UND:, was examined for PHA production because the PHA produced from this compound, PHA-UND:, was expected to be less crystalline than PHA-OCT: since the PHA produced from undecanoic acid, PHA-UND, had a lower melting transition temperature and heat of fusion than the PHA-OCT. This lower  $T_m$  and  $\Delta H_m$  might be explained that PHA-UND contains more types of repeating units than PHA-OCT.

The growth curve for *P. oleovorans* grown with UND: in a 12 L fermentation is presented in Figure 47(D). The induction time was approximately 20 hours, which was as long as the time taken for the growth with only NA to reach the stationary phase (Figure 47(a)). When the lag phase is ignored, the time taken for the growth to reach the stationary phase with UND: is similar to that taken for the growth with NA. PHA granules were observed in the cells grown with UND: even from a very early stage in the growth.

The proton NMR spectrum of PHA-UND: shown in Figure 48 indicates that this PHA contained 99 mole% of unsaturated units, and there was no measurable amount of saturated units detected by the gas chromatographic analysis. This amount of unsaturated unit is higher than any other PHAs reported. The  $^{13}\text{C}$  NMR spectrum and IR spectrum of PHA-UND: are shown in Figures 49 and 50. GC/MSD and pyrolysis GC/FAB MS showed the presence of 3-hydroxyundecenoate units, C11:, 3-hydroxynonenoate units, C9:, and 3-hydroxyheptenoate units, C7:, in the PHA-UND:. The C9: unit was the main repeating unit in this PHA. This result correlates with the previous observation that the C9 unit was the main repeating unit in the PHAs obtained from cells grown with n-alkanoic acids longer than OA and having an odd number of carbons. Therefore, the preference for repeating units containing 9 or 8 carbons may be a general feature of PHA production by *P. oleovorans*.



PHA-UND: was a very viscous polymer at room temperature. The DSC thermogram of this polymer showed an endothermic transition at approximately 35 °C with a heat of fusion of 1 cal/g, which was very low compared to those of PHA-NON and PHA-OCT. The glass transition temperature was approximately -50 °C. These results suggested that 10-undecanoic acid would be an ideal carbon source for investigation of the type of PHAs produced from mixed carbon sources.

The growth curves for *P. oleovorans* grown with various mixtures of NA and UND: are presented in Figure 47. The growth curve for *P. oleovorans* with a 2:1 mixture of OA and UND: is not shown, but it was almost the same as that for a 2:1 mixture of NA and UND:. Figure 47 shows the shape of the growth curves are almost the same regardless of the carbon source. This result is to be expected because the growth rate during linear growth was determined primarily by the aeration rate, which was fixed as 2 L/min (Section III.B).

Figure 47 shows that the growth curve shifted to the right as the fraction of UND: in the carbon source was increased. This shift could be interpreted as a result of a longer induction time when the mixed carbon sources contained more UND:.

The biomass yield, PHA yield, and PHA content in the biomass obtained from *P. oleovorans* grown with various mixtures of NA and UND: are given in Table 17. The biomass yield was significantly affected by the presence of UND:, but



the PHA content was constant when UND: was present in the carbon source.

The repeating unit compositions of PHAs produced from mixtures of NA or OA and UND: are shown in Figure 51. The total mole fraction of unsaturated repeating units in PHAs obtained from mixtures of NA and UND: is plotted with respect to the fraction of UND: in the carbon source in Figure 52. The mole fractions of the unsaturated repeating units in these PHAs were higher than the mole fractions of UND: in the carbon source mixtures. For example, a 2:1 mixture of NA and UND: gave a PHA containing approximately 40 mole% unsaturated units. This difference might be explained on the basis that NA was used more for growth than for PHA production. This result suggested that when a carbon source that gives a lower PHA yield (poor carbon source) is fed to *P. oleovorans* in the presence of NA as cosubstrate, the resulting PHA contains repeating units from the poor carbon source in a higher fraction than the fraction of the poor carbon source in the carbon source mixture.

When OA was used as the cosubstrate, the mole% of unsaturated units was almost the same as the mole% of UND: in the carbon source mixture. The PHA obtained from cells grown with a 2:1 mixture of OA and UND: contained 33 mole% unsaturated repeating units, and the PHA obtained from 95:5 mixture contained 5 mole% unsaturated units [Gagnon, 1990]. The biomass and PHA yields obtained from cells grown with a 2:1 mixture of OA and UND: were also lower than those

obtained from cells grown with a 2:1 mixture of NA and UND:. Therefore, OA was not as useful as NA as cosubstrate to improve the incorporation of a poor carbon source into the PHA. Other examples of different results obtained from NA and OA were discussed in other sections.

The IR spectrum, proton NMR spectrum, and  $^{13}\text{C}$  NMR spectrum of a PHA produced from a 2:1 mixture of NA and UND: are shown in Figures 53, 54 and 55.

In Section III.C, it was demonstrated that the relative amounts of repeating units from NA (C7, C9, C11) and UND: (C7:, C9:, C11:) in the PHAs prepared from mixtures of these carbon sources were constant regardless of the presence of each other. This result might be obtained if the polymers obtained from cells grown with mixed carbon sources were mixtures of two PHAs produced independently from each carbon source.

It was expected that NA and UND: might be utilized at different rates by *P. oleovorans* because of the very different induction times and the constant relative amounts of repeating units derived from each acid in the polymer obtained from mixtures of these acids. To investigate this possibility, the amount of each of these alkanolic acid which remained in the medium was determined with respect to growth time. Five of the gas chromatograms obtained in this study were shown in Figure 56 and the calculated results are presented in Figure 57. The data in Figure 57 shows that NA and UND: were utilized by *P. oleovorans* at almost the same



rate. These results suggested that the cells in the inoculum, which were grown with NA, apparently were able to metabolize UND: for PHA production. Therefore, it is not clear what causes the unusually long induction time when *P. oleovorans* was grown solely with UND:. One possible explanation would be that the transportation of UND: into the cells requires the presence of NA. That is, *P. oleovorans* might need to produce energy or enzymes required to take UND: into the body from NA, which means the UND: is taken into the cells by active transport mechanism. However, further investigation is required to prove this suggestion.

The repeating unit compositions of PHAs isolated from cells grown with an equimolar mixture of NA and UND: were determined with respect to growth time. The gas chromatograms of the methanolized products of several different samples are shown in Figure 58. The repeating unit composition change determined by gas chromatographic analysis is reported in Table 18, and the total fraction of olefinic units in the PHA as determined by NMR spectroscopy is shown in Figure 59. Table 18 and Figure 59 reveal that there is no significant compositional change with respect to growth time. These results show that *P. oleovorans* did not show any preference for NA during polymerization.

In Figure 60 the biomass and PHA yields are plotted against growth time. The PHA content decreased as *P. oleovorans* degraded and consumed PHA granules after approximately 33 hours. The fact that the repeating unit



composition of the PHA isolated after 33 hours was identical to those of the PHAs isolated at earlier times suggests that either depolymerization took place randomly or the PHA had a random distribution of repeating units.

Molecular weights of both the crude extract and the purified PHA isolated from the biomass harvested at different times were constant and were not affected by purification and growth time. These results correspond to those discussed in Section III.B. and C where NA was used as the sole carbon source. The relative amounts of repeating units from NA (C7, C9, and C11 units) and from UND: (C7:, C9, and C11 units) were also constant regardless of growth time as listed in Table 19.

All the experimental results except the constant ratios of C7, C9, and C11 units and C7:, C9:, C11: units, regardless of growth time, suggested that the PHA produced from an equimolar mixture of NA and UND: is a random copolymer. More convincing data proving that this PHA is a random copolymer were obtained from a pyrolysis GC/FAB MS study and by the comparison of properties of the PHA produced from mixtures of NA and UND:, PHA-NON•UND:, with the polymer blend of PHA-NON and PHA-UND:.

In Figure 61 are presented, the DSC thermograms for the melting transitions and glass transitions of PHAs produced with mixtures of NA and UND: and those for blends of PHA-NON and PHA-UND:. Figure 61(a) and (b) contain data for the melting transitions and (c) for the glass transitions. The

melting transitions designated by a, b, c, and d in Figure 61(a) are for the blends of PHA-NON and PHA-UND: in ratios of 3:1, 1:2, 1:1, and 1:0. The melting transitions designated by a, b, c, and d in Figure 61(b) are for the PHAs obtained from growth mixtures of NA and UND: in ratios of 0:1, 1:2, 1:1, and 1:0. The glass transitions designated by a, b, c, and d, in Figure 61(c) are for PHA-NON, PHA from 2:1 mixture of NA and UND:, PHA-UND:, and 1:1 (wt:wt) mixture of PHA-NON and PHA-UND:.

The melting transition temperatures of the PHAs obtained using mixed carbon sources changed smoothly with respect to the mole fraction of the unsaturated repeating units in the polymer. However, the melting transition temperatures of physical mixtures or blends of PHA-NON and PHA-UND: was constant and corresponded to the melting transition temperature of PHA-NON. PHA-UND: has a very low degree of crystallinity and crystallizes very slowly, so the melting transition of PHA-UND: was not detected in Figure 61(a).

Also two glass transitions were detected from the 1:1 (by weight) blend of PHA-NON and PHA-UND: as shown in Figure 61(c). The two glass transition temperatures corresponded to those of PHA-NON and PHA-UND:, but PHA-NON•UND: had only one glass transition between those of PHA-NON and PHA-UND:. These results showed that PHA-NON and PHA-UND: were not compatible as was expected and that *P. oleovorans* produced random copolymers when it was grown with mixtures of NA and UND:, and the constant ratio of



repeating units derived from a single carbon source regardless of the presence of another carbon source is definitely not an indication that the resulting product is a physical mixture of two different polymers.

The result of the WAXD study on a stretched sample of the PHA produced from a 2:1 mixture of NA and UND: showed that  $d_1$  is 20.4 Å and the other  $d$  spacings were almost identical to those of PHA-NON. This longer  $d_1$  spacing of this PHA than that of PHA-NON indicates that 3-hydroxyundecenoate units, C11:, in this PHA are fully extended from the polymer backbone and contribute to the intermolecular distance.

The results described in this section suggested that the PHAs produced from mixtures of NA and a poor carbon source could contain a significant amount of repeating units from the poor carbon source. In the following sections most of PHAs studied were prepared based on this assumption. Another apparent general feature of PHA production by *P. oleovorans* is that random copolymers are generally produced when mixtures of carbon sources were used, but in Section III.G. an example is given in which mixtures of two PHAs were obtained from mixed carbon sources.

### 3. Sequential Feeding

It was of considerable interest to determine what type of polymer was obtained from cells grown by feeding NA and UND: in sequence. Based on the results described above, that



random copolymers were obtained from mixtures of these carbon substrates, it was probably that at least some of the polymer obtained by feeding NA and UND: in sequence would be block copolymers.

When a new carbon source is fed to cells containing a PHA produced from another carbon source, there are several possible pathways for further polymerization, including (a) the monomer from the second carbon source is added to the PHA previously produced from the initial carbon source, or (b) the monomer from the second carbon source is added to the PHA which is being formed when the second carbon source was added, or (c) another new polymer is formed. That is, it is not known if (a) a PHA already formed is capable of further polymerization, or (b) whether it is possible that when a second carbon source is added depolymerization could take place to maintain a constant molecular weight regardless of growth time. If either (a) or (b) occurs, at least some of the PHA molecules produced by *P. oleovorans* by feeding NA and UND: in sequence would be a block copolymer.

The molecular weights of PHAs obtained from cells grown by feeding with NA and UND: in sequence and harvested at different growth times were constant. The number average molecular weights of the PHAs produced were approximately 60,000 and the polydispersity index was approximately 2.0. This constant molecular weight suggests polymerization and depolymerization could take place simultaneously if block copolymers are to be produced.

The growth curve obtained from this study is shown in Figure 62. The relative amount of PHA-UND: in the PHA was calculated by multiplying PHA yield and fraction of unsaturated units,  $(\text{PHA yield}) \cdot (\text{fraction of unsaturated units})$ , and the relative amount of PHA-NON was obtained by  $(\text{PHA yield}) \cdot (1 - \text{fraction of unsaturated units})$ . The biomass yield, PHA yield, fraction of unsaturated units determined by gas chromatographic analysis, relative amount of PHA-UND:, and relative amount of PHA-NON are plotted in Figure 63 against growth time. The maximum PHA yield was obtained when the growth reached the deceleration phase. The data in Figure 63 show that the fraction of unsaturated units in the PHA increases while PHA-UND: and PHA-NON decrease with respect to time. These results suggest that PHA-NON is digested earlier than PHA-UND:.

The DSC thermogram of the PHA obtained in Figure 64 which contains two melting transitions indicates this PHA was not a random copolymer.

An experiment was conducted to determine whether this PHA was either a physical mixture of PHA-NON and PHA-UND: or a block copolymer. For this study, 1:1 (w:w), 2:1, and 1:2 blends of PHA-NON and PHA-UND: and also the PHA produced by sequential feeding were crosslinked using diborane which reacts selectively with olefin groups. The crosslinked polymers were swelled with excess chloroform and the amounts of polymer extracted were measured. The results listed in Table 20 show that the amount of soluble fraction obtained



from the crosslinked product of the PHA obtained from cells grown by sequential feeding was significantly lower than that obtained from the crosslinked product of blends of PHA-NON and PHA-UND:. These results suggested that most of PHA molecules produced by feeding carbon sources in sequence might be block copolymers obtained by polymerization and depolymerization which could take place simultaneously. Further investigation is necessary to clarify these possibilities experimentally.

The total ion chromatogram (TIC) and mass spectra of olefinic repeating units in the PHA obtained from cells fed with NA and UND: sequentially are presented in Figure 65.

A preliminary study of epoxidation of PHA-UND: showed approximately 67 % of olefin groups were epoxidized after 6 hours of reaction with metachloroperbenzoic acid under the experimental conditions described in Chapter II. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of the epoxidized product of PHA-UND: are shown in Figures 66 and 67 which show the presence of epoxides. The product was crosslinked after several days when it was exposed to the air at room temperature. No further investigation was carried out to modify the olefin groups.



## G. PHAs Containing Brominated Units

### 1. Introduction

It was described in Section III.F. that the mole fractions of unsaturated repeating units in the PHAs produced by *P. oleovorans* grown with mixtures of NA, and UND: were higher than the mole fractions of UND: in the carbon sources even though UND: gave significantly lower biomass yield and PHA yield than NA when used as single carbon source. These results suggested the possibility that PHAs enriched with the repeating units derived from poor carbon substrates could be produced by co-feeding *P. oleovorans* with the poor substrates and a good cosubstrate, one which supports high biomass and PHA yields. Carbon substrates that support cell growth without PHA production were classified as type "b" in Section III.D., and it was of interest to determine if those substrates would be incorporated into PHAs if a cosubstrate is present. This section describes the study of the polymer produced from mixtures of a series of  $\omega$ -bromoalkanoic acid and either NA or OA.

Recently, it was shown that a PHA containing chlorinated units could be prepared by growing *P. oleovorans* with a mixture of octane and 1-chlorooctane [Doi et al., 1990]. The  $^{13}\text{C}$  NMR study of this polymer showed that this PHA was a random copolymer containing C6, C8, 8-chloro-3-hydroxyoctanoate, and 6-chloro-3-hydroxyhexanoate

units. The weight percent of chlorine in the polymer was 16.7 % as determined by elemental analysis. This PHA was reported to be amorphous, but it was not reported if a PHA was obtained from cells grown with only 8-chlorooctane.

## 2. Production of PHAs Containing Brominated Units

The growth curves of *Pseudomonas oleovorans* with 6-bromohexanoic acid (6BRHA), 8-bromooctanoic acid (8BROA) or 11-bromoundecanoic acid (11BRUA) were presented in Figure 32. The growth rate of *P. oleovorans* was fastest with 11BRUA and slowest with 6BRHA, which shows that the greater the separation between the bioreactive group (COOH) and bromine the more favorable it is for cell growth. However, none of the cells grown with these bromoalkanoic acids contained PHA granules, so equimolar mixtures of NA and each of  $\omega$ -bromoalkanoic acids were examined for PHA production.

The growth curves of *P. oleovorans* with these mixtures are presented in Figure 68, which shows that the growth was actually faster when the carbon source contained the shorter bromoalkanoic acid, in contrast the growth results when the bromoalkanoic acids were used as single carbon source. The cultures grown with these mixtures turned brown in the late stationary phase, and the increase of the optical density of the culture grown with an equimolar mixture of NA and 11BRUA in the late stationary phase was associated with the appearance of this brown color.



Data for the biomass and PHA yields from cells grown with these mixtures are presented in Table 21. The highest PHA and biomass yields were obtained from cells grown with an equimolar mixture of NA and 11BRUA. The elemental analysis and NMR spectroscopic analysis showed that all of these PHAs contained significant amounts of bromine. The PHA obtained from cells grown with an equimolar mixture of NA and 11BRUA contained the highest amount of brominated units (Table 21), showing again that the longer separation between the carboxylic acid group and the bromine was more favorable for polymer production.

The NMR spectrum of the PHA prepared from cells grown with an equimolar mixture of NA and 6BRHA is presented in Figure 69. The peak areas of  $H_d$  and  $H_b$  in Figure 69 were used for the determination of the mole fraction of brominated units as follows:

$$x = \frac{A(H_d)}{2 \cdot A(H_b)} \cdot 100 \quad (\%)$$

in which  $x$  is the mole percent of bromine containing units and  $A(H_i)$  is the area of the peak for proton  $i$

The gas chromatogram of a methanolized sample of the PHA produced from an equimolar mixture of NA and 6BRHA is shown in Figure 70. A new peak marked as "X" is detected in addition to C7, C8, and C11 units. The mass spectrum of peak "X" did not have ion fragments of 103 and 74, which are



characteristic ones for 3-hydroxyalkanoates. GC/AED analysis showed this peak had no bromine, either. These results indicate that this peak was not  $\omega$ -bromo-3-hydroxyalkanoate unit but a product of unknown reaction occurred during methanolysis.

The GC/AED chromatograms of the PHAs produced from equimolar mixtures of NA and either 6BRHA, 8BROA, or 11BRUA are shown in Figures 71, 72, and 73. Figures 71 and 72 show peak "X" was formed from the PHAs produced from mixtures of NA and 6BRHA, and NA and 8BROA. As will be shown later, the same peak was detected in a methanolized sample of the PHA produced from mixtures of OA and 8BROA.

As mentioned earlier, the PHA produced from a mixture of octane and 8-chlorooctane was reported to contain 3-hydroxy-6-chlorohexanoate units [Doi et al., 1990], which would be formed as a result of deacetylation of 8-chloro-3-hydroxyoctanoate. Considering the new peak, X, was commonly found in methanolized samples of PHAs produced from carbon source mixtures containing 6BRHA and 8BROA, it is likely that X was produced by an unexpected (and unknown) reaction of 3-hydroxy-6-bromohexanoate during acid-catalyzed methanolysis. The presence of bromine in the solvent peaks in Figures 71 and 72 supported this suggestion; but no further effort was made to identify that peak.

A similar new peak, X', was detected from the methanolized sample of the PHA produced from an equimolar mixture of NA and 11BRUA (X' in Figure 73). The retention

time of X' was longer than that of X by approximately 0.6 minutes which is within a reasonable range for a retention time difference for a homologous compound containing one more methylene group than X. Therefore, X' is most likely a product of the unknown reaction of 3-hydroxy-7-bromoheptanoate during methanolysis.

Figures 72 and 73 clearly show that the PHAs produced from equimolar mixtures of NA and either 8BROA or 11BRUA contained brominated repeating units. The main repeating units containing bromine in these PHAs were 3-hydroxy-8-bromooctanoate and 3-hydroxy-9-bromononanoate.

The results described above show that bromoalkanoic acids are incorporated into polymers when NA was present as cosubstrate, so many of the carbon substrates belonging to type "b" could also be potential carbon sources for PHA production. Some of other type "b" substrates were examined, and the results are reported in Section III.I.

The PHAs described above were very soft and sticky, especially, the PHA produced from an equimolar mixture of NA and 11BRUA. The DSC thermograms of the PHAs produced from NA and 8BROA and from NA and 11BRUA mixtures are shown in Figure 74. The PHA prepared using the 11BRUA mixture had a lower heat of fusion and a lower melting transition temperature than the PHA prepared using the 8BROA mixture. The glass transition temperature, melting transition temperature, and heat of fusion of the PHA produced from an equimolar mixture of NA and 6BRHA were -28.7 °C, 48.7 °C, and



2.4 cal/g, respectively, which are very close to the values for the PHA obtained from the NA and 8BROA mixture. As mentioned above, the PHA which contained chlorinated units was reported to have no melting transition [Doi et al., 1990], so the presence of brominated units instead of chlorine units might have had a less effect on decreasing the degree of crystallinity.

All of these PHAs had one glass transition temperature and one melting transition temperature which indicates that they were either random copolymers or mixtures of two miscible polymers, which were produced from NA and bromoalkanoic acid independently.

The lower crystallinity of the PHA prepared from mixtures of longer bromoalkanoic acid is presumably related to the presence of longer brominated side chain, but this behavior is different from the results obtained from the PHAs prepared from n-alkanoic acids. That is the PHAs prepared from hexanoic acid (PHA-HEX) or heptanoic acid (PHA-HEP) had much lower degrees of crystallinity than the PHA-OCT. This result may be explained on the basis that the carbon source mixture containing 11BRUA would be expected to yield a PHA consisting more types of repeating units than the others, and this type of randomness would lower the crystallinity.

To examine the relationship between the composition of carbon source and the amount of brominated units incorporated into the PHA, *P. oleovorans* was grown with a 2:1 mixture of NA and 8BROA. The PHA yield and the PHA content obtained



from cells grown with this mixture almost tripled in amount by changing the mixing ratio from 1:1 to 2:1 (Table 21), but the total biomass yield decreased by approximately 10 %, and the mole percent of brominated units decreased to approximately 4 %.

The NMR spectra of PHAs produced from an equimolar mixture and a 2:1 mixture of NA and 8BROA are shown in Figure 75.

The weight percent of bromine in the polymer determined by elemental analysis was less than 2 %. This decrease of the mole percent of brominated units from 25 % to 4 % was too large to be caused by a decrease of the mole percent of 8BROA in the carbon source from 50 % to 33 %. These results suggested there might be a minimum concentration of 8BROA which should be exceeded for 8BROA to be efficiently incorporated into the polymer. However, as will be discussed later, the experimental results from mixtures of OA and bromoalkanoic acid did not support this proposition.

The PHA yield from a 12 L culture prepared using 120 mmole of NA was approximately 0.3 g per liter, and the PHA yield from a 12 L culture prepared a 2:1 mixture of NA (80 mmole) to 8BROA (40 mmole) was approximately 0.2 g per liter. This result shows that the PHA yield was proportional to the amount of NA in the growth medium and was not affected by the presence of 8BROA.

A growth experiment in which *P. oleovorans* was fed with NA and 11BRUA consecutively was carried out. If

*P. oleovorans* grown with NA first was able to polymerize 11BRUA alone, the polymer obtained from cells grown initially with NA and then 11BRUA should contain a significant amount of brominated units. For this experiment, *P. oleovorans* was grown with a 12 L medium containing NA in an initial concentration of 5 mM. When the growth with NA reached the deceleration phase, a neutral aqueous solution of 60 mmole of 11-bromoundecanoic was added to the culture, and the total amount of the carbon source used in this sequential feeding experiment was the same as the amount of the carbon source used in the experiment in which an equimolar mixture of these carboxylic acids was fed.

The growth rate was resumed at the same level as soon as 11BRUA was added to the culture. This result showed that *P. oleovorans* utilized 11BRUA as soon as it became available. The biomass yield at the second stationary phase was not significantly different from that obtained from a culture grown with an equimolar mixture of these carbon sources, but the PHA yield was almost double that obtained from the culture grown with an equimolar mixture of these carbon sources. The PHA yield (0.18 g/L) was close to one half of the PHA yield obtained from cells grown with 10 mM NA. This PHA yield indicated that NA supplied to the growth medium initially in the concentration of 5 mM was used for PHA production as efficiently as when NA was supplied in the concentration of 10 mM and that the PHA-NON stored in the cells was not consumed until the second growth with 11BRUA



was completed. This observation corresponds to the observation made in our laboratory that PHA granules accumulated in cells did not disappear when an excess amount of carbon source was present in the growth medium [Wolf et al., 1990b].

The result that the PHA yield obtained from the culture grown with an equimolar mixture of NA and 11BRUA was lower than the PHA yield obtained from cells grown by feeding these carbon sources consecutively indicates that 11BRUA suppressed production of PHA from NA when *P. oleovorans* was exposed to these substrates at the same time. Also, it suggests that NA and 11BRUA should be present at the same time for *P. oleovorans* to polymerize 11BRUA.

*P. oleovorans* exposed to a growth medium containing an equimolar mixture of OA and 11BRUA did not grow for 48 hours. This result was very unexpected result and the experiment was terminated after 48 hours. In contrast, one liter of a culture grown with an equimolar mixture of OA and 8BROA afforded 0.31 g of biomass containing 0.11 g of PHA. Surprisingly again, it was found that this PHA contained only 2 mole% of brominated repeating units (0.98 wt% of bromine), which is much lower than that of the PHA obtained from an equimolar mixture of NA and 8BROA. The gas chromatogram of the methanolized sample of this PHA in Figure 76 shows that again there is the unknown component "X" present as described above.



To investigate the effect of the initial concentration of 8BROA in the carbon source on the incorporation of bromine containing repeating units into the polymer, *P. oleovorans* was grown with a 2:1 mixture of 8BROA to OA. The biomass and PHA yields from the cells grown with this mixture were 0.37 g/L and 0.08 g/L. The mole percent of brominated units and the weight percent of bromine were approximately 4 % and 2 %, respectively. Even though the amount of brominated units was still low, it is remarkable that these values were almost doubled the amount in the PHA obtained from an equimolar mixture of these carbon sources. These results suggest that the low amount of bromine in the PHAs produced from mixtures of OA and 8BROA might not be related to the carbon source composition.

These unexpected different behaviors of *P. oleovorans* when it was fed with mixtures of either NA or OA and the  $\omega$ -alkanoic acids was quite surprising considering the result from our previous study on preparation of PHA from *P. oleovorans* using an equimolar mixture of OA and NA as the carbon source (Section III.C) [Ballisteri et. al, 1990]. That PHA produced in that case contained about the same amount of repeating units from each carbon source (46 mole percent of repeating units produced from OA and 54 mole percent of repeating units produced from NA). The polymer was a random copolymer showing that *P. oleovorans* did not prefer one of these carboxylic acids either for growth or for polymerization. As will be discussed in following sections,

other examples were also found showing different behavior of *P. oleovorans* for mixtures of OA and NA with another carbon source.

Changes in the weight percent of bromine in the PHA, the biomass yield, and the PHA yield during growth were determined with a culture grown with an equimolar mixture of NA and 8BROA. The results plotted in Figure 77 show that the PHA yield decreased after 25 hours, but the weight percent of bromine in the PHA remained unchanged. That is, the weight percent of bromine in the PHA was not affected by PHA degradation. The gas chromatographic analysis and NMR spectroscopic analysis of the PHA also showed that the composition of repeating units from NA remained constant throughout the growth. The ratio of the amount of 3-hydroxyheptanoate (C7) unit to the amount of 3-hydroxynonanoate (C9) unit was 21:79 (calculated by GC peak areas), which was the same as that found in other PHAs prepared from cells grown with carbon sources containing NA. The mole percent of brominated units in the PHA was 25 % and remained constant.

The molecular weight of the PHAs obtained from cells harvested after different growth times was constant. The number average molecular weight was approximately 50,000 and the polydispersity index was approximately 2.0. The  $^{13}\text{C}$  NMR spectra of PHAs prepared in this study are presented in Figures 78, 79, 80. For the assignment of peaks in Figures 78 and 79, the PHA obtained from an equimolar mixture



of NA and 6BRHA was assumed to contain C7, C9, 3-hydroxy-4-bromobutyrate, and 3-hydroxy-6-bromohexanoate units, and the PHA obtained from an equimolar mixture of NA and 8BROA was assumed to contain C7, C9, 3-hydroxy-6-bromohexanoate, and 3-hydroxy-8-bromooctanoate units. The NMR spectra in Figures 78 and 79 correspond to these assumptions.

The pattern of the  $^{13}\text{C}$  NMR spectrum of the PHA obtained from an equimolar mixture of NA and 11BRUA was complicated and it was not possible to assign a carbon atom to each peak by theoretical calculations of chemical shifts. Further investigations such as 2-dimensional NMR spectroscopy, would be required for the assignment.

## H. PHAs Containing Phenyl Units

### 1. Introduction

A PHA containing repeating units with phenyl groups was first obtained from *P. oleovorans* grown on PVA as the sole carbon source [Fritzsche et al., 1990a]. This PHA was a homopolymer of 3-hydroxy-5-phenylvalerate units, HPV. The DSC thermogram of poly-3-hydroxy-5-phenylvalerate, PHPV, taken between -100 °C and 100 °C, showed a glass transition at approximately 18 °C and a very small endothermic transition at approximately 70 °C. This glass transition temperature is much higher than those of the PHAs produced from n-alkanoic



acids. The small endothermic transition was presumably for a melting transition but a WAXD pattern of this polymer showed only diffuse halo even though this polymer was cloudy at room temperature and at a temperature higher than 70 °C. Also, no birefringence was for this polymer on the hot stage on a polarized microscope between 20 and 100 °C. The PHPV was more rubber-elastic at room temperature than other PHAs produced from n-alkanoic acids. That is, a piece of PHPV stretched and released returned close to its original shape fast while other PHAs such as PHA-NON and PHA-OCT were deformed to an significant extent.

The high glass transition temperature and the high rubber elasticity of PHPV made it highly desired that HPV units be incorporated into other PHAs produced from n-alkanoic acids to improve their elastic properties at room temperature. As PHPV was less sticky than other PHAs, it was also expected the PHAs containing HPV units would be less sticky.

In previous sections it has been shown that PHAs produced from cells grown with mixtures of two carbon sources are random copolymers and it was concluded that this behavior might be a general feature of PHA production by *P. oleovorans*. Therefore, random copolymers containing HPV units were expected to be produced by *P. oleovorans* when grown on mixtures of PVA and either, OA, or NA. The properties of the PHAs produced by growing *P. oleovorans* on these mixtures are described in the following section.

## 2. Preparation of PHAs Containing Phenyl Units

The results obtained on PHA production by *P. oleovorans* grown with various mixtures of PVA and either NA or OA are presented in Table 22. The harvesting time in Table 22 is the time taken for growth to reach the stationary phase after inoculation. The growth curve of *P. oleovorans* with 10 mM PVA is presented in Figure 81, which shows that the induction period was approximately 20 hours and it took another 30 hours for the growth to reach the stationary phase.

Table 22 contains data for the time taken to reach the stationary phase, which was shorter when PVA was a cosubstrate with NA than with the latter alone. This result differs from that obtained when UND: and NA were cosubstrates for *P. oleovorans*. For each of those two substrates alone, the induction time and the time taken to reach the stationary phase were both longer with UND: than with NA, and for mixed substrates of these two compounds the time to reach the stationary phase was increasingly longer when the fraction of UND: was increased (Section III.F).

The earlier cessation of cell growth when PVA was present as a cosubstrate may be caused by the production of 3-hydroxy-3-phenylpropionic acid, which could be formed by deacetylation of 3-hydroxy-5-phenylvaleric acid. That is, it might be expected that the former would not support cell growth because 4-phenylbutyric acid did not [Leppelier,



1989]. This possibility is shown schematically in Figure 82, although the presence of 3-hydroxy-3-phenylpropionic acid in the growth medium was not investigated.

Table 22 lists the biomass yield and PHA yield, both of which decreased significantly as the ratio of NA to PVA in the carbon source was increased from 1:0 to 2:1. This result is to be expected because of the low biomass and PHA yields obtained when only PVA was used as the sole carbon source.

The data in Table 22 also shows that the differences between biomass yield and PHA yield from cells grown solely on either NA or OA are not significant. Nevertheless, an equimolar mixture of PVA and OA gave significantly lower biomass yield and PHA yield than did an equimolar mixture of NA and PVA. These results indicate that the ability to utilize OA and NA by *P. oleovorans* are apparently different when present as cosubstrates. Similar results were obtained from other combinations of carbon sources as described in Section III.G and III.I.

The repeating unit compositions of the PHAs obtained using various substrate mixtures are presented in Table 23. When PVA was used as either the substrate or as the cosubstrate the 3-hydroxy-5-phenylvalerate unit (HPV) was the only unit in the polymer containing a phenyl group as determined by gas chromatographic analysis of the methanolized polymers and  $^{13}\text{C}$  NMR spectroscopy. The  $^{13}\text{C}$  NMR spectrum of the PHA produced from a 2:1 mixture of NA and PVA is shown in Figure 83. The mole fraction of HPV units



determined by NMR spectroscopy increased smoothly with respect to the mole fraction of PVA in the carbon source mixture as shown in Figure 84. The number average molecular weight of the polymers produced were all approximately 50,000 and the polydispersity index was approximately 2.0, regardless of the composition of the carbon source.

The relative amounts of repeating units derived from NA and OA in PHAs were calculated by ignoring the presence of HPV units. As seen in Table 24, the ratio of 3-hydroxypentanoate units, C5, to 3-hydroxyundecanoate units, C11, were constant regardless of the presence and the amount of PVA in the polymer. Also, the relative amount of C5, C7, and C9 units in the PHA obtained from cells grown with an equimolar mixture of NA and PVA was not affected by the growth time as shown in Table 25. The constant ratio of repeating units derived from a carbon source regardless of the presence of another carbon source has been observed throughout this thesis research work. The molecular weights of the PHAs isolated from cells harvested at different times were also constant, and the purification procedure did not affect the molecular weight distribution.

The visual appearance of the PHA obtained from mixtures of PVA and NA changed according to the harvesting time. That is, the PHA isolated from cells harvested during exponential phase was transparent, and the PHA obtained became increasingly more cloudy with increasing growth time at which the cells were harvested. This result suggested that the PHA

obtained from these substrate mixtures might not be random copolymers.

The relative amounts of NA and PVA remaining in a culture grown with an equimolar mixture of these two substrates was also determined as a function of growth time, and the results are shown in Figure 85. The fraction of NA remaining in the medium decreased to 0.6 after 8 hours, but PVA had not started to be consumed at that time. This selective consumption of NA by *P. oleovorans* was reflected in the mole fraction of HPV units in the PHA. The amount of HPV units in the PHAs increased with growth time as presented in Figure 86. The gas chromatograms of some of the samples for the analysis of NA and PVA remaining in the culture in Figure 87 show that NA disappeared earlier than PVA. It is remarkable that a new peak appeared at 7.71 minutes and became bigger with time. This unknown peak might possibly be methyl-3-hydroxy-3-phenylpropionate which was mentioned earlier.

In Figure 88, this biomass yield, PHA yield, PHA-NON yield, and PHPV yield are shown as a function of growth time. PHA-NON yield and PHPV yield were calculated by assuming that these PHAs were mixtures of pure PHA-NON and PHPV as follows:

• Weight of PHA-NON =

$$\frac{\text{PHA}_{\text{wt}}}{\text{MW}_{\text{PHPV}} \cdot C + \text{MW}_{\text{PHA-NON}} \cdot (1-C)} \cdot (1-C) \cdot \text{MW}_{\text{PHA-NON}}$$

• Weight of PHPV =  $\text{PHA}_{\text{wt}} - \text{PHA-NON}_{\text{wt}}$



where,  $PHA_{wt}$  = total weight of PHA obtained

$MW_{PHPV, PHA-NON}$  = average molecular weight of PHPV  
or PHA-NON

C = fraction of PHPV in the polymer product

The yields of both of these polymers, PHA-NON and PHPV, changed continuously with time. The PHA-NON yield, the fraction of NA remaining in the medium, and the optical density of the culture grown with a 5 mM NA medium are shown as a function of growth time in Figure 89. The growth plot presented in Figure 89 was obtained from a separate growth experiment. The growth reached the stationary phase as NA was depleted. These results are similar to those obtained for growth solely with NA. Therefore, it appears likely that *P. oleovorans* consumed NA and PVA independently for energy and polymer production.

None of the PHAs listed in Table 22 were transparent over the temperature range from room temperature to 70 °C, which is higher than the melting transition temperature of both PHA-NON and PHA-OCT (45 °C and 55 °C, respectively). The DSC thermogram of the PHAs produced from a 2:1 mixture of OA and PVA and from a 1:2 mixture of NA and PVA are shown in Figures 90 and 91. Both thermograms show two glass transition temperatures.

All of these results indicate that the PHAs produced by *P. oleovorans* on cosubstrates of PVA and either NA or OA were not random copolymers but were mixtures of two separate PHAs.



Solvent fractionation of the two components was carried out on the PHA obtained from cells grown with a 2:1 mixture of NA and PVA. The mole fraction of HPV units in this PHA as determined by NMR was 12.6 %. From 1 gram of this PHA, approximately 0.8 g of PHA-NON rich fraction and 0.2 g of PHPV-rich fraction were recovered. The PHA-NON rich fraction contained less than 5 mole% of HPV units, and the PHPV rich fraction contained about 60 mole% of HPV units. These results clearly demonstrate that the PHA produced is a mixture of two PHAs.

The possible effect of degradation of PHA molecules in the late stationary phase was examined. In previous sections in which the production of random copolymers were discussed, the repeating unit compositions of the PHAs were found not to be affected by degradation. In this experiment, degradation was facilitated by increasing the aeration rate after sufficient amount of polymer was produced. That is, the cells were grown under normal conditions for the first 10 hours and then the aeration or agitation rates were increased from 2 L/min and 100 r.p.m. to 6 L/min and 200 r.p.m., respectively. Visual observations by light microscope revealed that a significant number of PHA granules disappeared during this two hour period of increased air supply. The fermentation results from this experiment are shown in the fifth row of Table 22. There was a drastic decrease in PHA yield and PHA content after this procedure was followed, but the amount of cellular materials excluding

PHA, (biomass yield)-(PHA yield), increased from 0.49 g/L to 0.7 g/L. These results showed clearly that under these conditions *P. oleovorans* produced cellular materials as well as energy at the expense of the PHA initially produced. The mole fraction of HPV units slightly increased during the PHA degradation, which suggested PHA-NON was degraded earlier than PHPV. These observations correspond to the results discussed earlier.

The production of two different PHAs by *P. oleovorans* grown on mixtures of PVA and NA was an unexpected result because both of these substrates support cell growth and PHA production when used as sole carbon sources. These results would tend to suggest that there are two different polymerase enzymes present which can simultaneously produce two different polymers. If so, whether these two polymerase systems are present in the same cell or in separate cells remain to be determined.

In an experiment carried out to determine the amount of HPV units formed with respect to growth time (by gas chromatographic analysis of methanolized samples of PHAs obtained from cells in different growth phases) a very unexpected observation was made. The gas chromatograms are shown in Figure 92. In first three chromatograms, a peak corresponding to the C8 unit was detected, and the peak increased initially but then disappeared. The TIC and mass spectrum of this peak shown in Figure 93 confirms that this was a C8 unit. The reproducibility of this observation was



not examined, but this observation was very unusual and unexpected and should be explored further.

## I. New and Non-natural PHAs

### 1. Introduction

In previous sections it has been described that some poor carbon sources, which support cell growth either without PHA production or with production of only small amounts of PHA, can be incorporated into the PHAs produced by *P. oleovorans* in high yields when a good carbon source such as OA and NA was present as the cosubstrate. The mole fraction of repeating units produced from the poor carbon source was usually higher when NA was present as cosubstrate than when OA was present as cosubstrate. The PHAs produced from such mixtures were either random copolymers or mixtures of two polymers produced independently from each carbon source.

Considering these results, it was to be expected that feeding *P. oleovorans* with mixtures of a carbon substrate classified as group "b" in Section III.D and either OA or NA would afford the production of PHAs which contained repeating units produced from the "group b" carbon substrate. In this section, the results of somewhat preliminary experiment on PHA production from mixtures of carbon sources belonging to group "b" and either NA or OA are described. In earlier



sections it was shown that the PHAs produced with OA as cosubstrate usually contained lower mole fraction of repeating units produced from the poor carbon source than PHAs produced with NA as cosubstrate, therefore, NA was mainly used as cosubstrate in this study.

## 2. PHAs Containing Nitrile Groups

11-Cyanoundecanoic acid, 11CNA, supported cell growth but did not support PHA production by *P. oleovorans* so an equimolar mixture of NA and 11CNA was first examined as the carbon source, and the growth plots for this mixture are shown in Figure 94. Growth stopped after approximately 15 hours and the final optical density was approximately 2.5 A.U. One liter of the culture afforded 920 mg of biomass containing 180 mg of PHA.

The  $^1\text{H}$  NMR spectrum of the PHA produced indicated that there were 32 mole% of cyano-repeating units in the PHA, as shown in Figure 95, and the  $^{13}\text{C}$  NMR spectrum also revealed the presence of nitrile groups, as shown in Figure 96. The absorption at  $2220\text{ cm}^{-1}$  in the IR spectrum in Figure 97. is assigned to the CN stretching mode. The elemental analysis showed that there was 2.62 weight percent of nitrogen in this PHA, and the gas chromatogram of the methanolized sample showed the presence of new repeating units. The mass spectrum of these repeating units had ion fragments with  $m/z$  values of 103 and 74, as shown in Figure 98. The presence of

nitrogen in some of these new repeating units was confirmed by GC/AED analysis, as shown in Figure 99.

The changes of biomass yield, PHA yield, and PHA content in the biomass with respect to growth time were determined from a culture grown on an equimolar mixture of NA and 11CNA, and the results are plotted against the growth time in Figure 100. Both biomass yield and PHA yield increased with growth time but the highest PHA content was obtained from the cells harvested after 11 hours. The weight percent of nitrogen and the area percent for the peak of cyano-containing repeating unit in the gas chromatograms of methanolized samples of PHAs obtained at different growth times (Figure 101) are plotted in Figure 102 against growth time. This figure shows that the fraction of cyano-containing repeating units increased with growth time. These results were similar to those obtained from the study of preparation of PHAs from mixtures of PVA and either NA or OA, from which mixtures of two PHAs were obtained.

The effect of the composition of the carbon source on the repeating unit composition of the PHA produced was investigated by growing *P. oleovorans* on both a 7:5 and a 2:1 mixture of NA to 11CNA. The growth with a 7:5 mixture stopped after 17 hours, and the final optical density was 2.1 A.U. One liter of the culture grown on this mixture afforded 9.8 g of biomass and 0.25 g of PHA, which contained 25 mole% of cyano-containing repeating units and 2.31 weight percent of nitrogen. The growth with a 2:1 mixture stopped



after 16 hours, and the final optical density was 2.0 A.U. One liter of the culture grown on this mixture afforded 0.8 g of biomass and 0.29 g of a PHA with 17 mole% of cyano-containing repeating units and 0.68 weight percent of nitrogen. These results showed that there was not a drastic decrease of the amount in cyano-containing repeating units as a result of the decreased fraction of 11CNA in the carbon source mixture.

All the PHAs prepared in this study were transparent, soft and sticky, which indicated the presence of low degrees of crystallinity. The DSC thermograms of these PHAs are shown in Figure 103. The melting transition temperatures were 54 °C, 46 °C, and 46 °C, and the heats of fusion were 2.9, 0.42, and 0.17 cal/g, respectively, for the PHAs containing 17, 25, 32 mole percent of cyano-containing repeating units, respectively. Only one glass transition and one melting transition were seen in each case which indicated that these PHAs were either random copolymers or blends of two miscible polymers.

The increasing amount of cyano-containing repeating units in the PHA produced from an equimolar mixture of NA and 11CNA with respect to growth time is unusual because the repeating unit compositions of other PHAs having random distribution of repeating units were not dependent on the growth time. However, the ratio of C7, C9, and C11 units of 22:70:8 in the cyano-containing PHAs, as determined by gas chromatography, was constant regardless of the composition of



carbon source and the growth time. This ratio is almost identical to that of the PHA-NON.

The effect of changing the cosubstrate from NA to OA on the incorporation of cyano-repeating units was examined by growing *P. oleovorans* on an equimolar mixture of OA and 11CNA. The growth on this mixture was stopped after 13 hours with a final optical density of 1.50 A.U. One liter of the culture grown with this mixture afforded 0.48 g of biomass and 89 mg of PHA containing 15 mole% of cyano-repeating units and 1.36 weight percent of nitrogen, which was much lower than those in the polymer produced from an equimolar mixture of NA and 11CNA. This result again shows that OA gives a lower incorporation of a poor carbon source in its PHA, and also gives lower biomass and PHA yields than NA when used as cosubstrate. However, the amount obtained was still much higher than the amount of brominated units in the PHA prepared from an equimolar mixture of OA and should compare to 11BRUA.

### 3. PHAs Containing Ester Groups

*P. oleovorans* could conceivably utilize esters of alkanolic acids either by hydrolyzing ester group to yield an alkanolic acid, which it could then metabolite, or it could oxidize the terminal alkyl group. The possible products from esters of alkanolic acids are shown in Figure 104. Substrates (A) and (B) in Figure 104 could be utilized as energy and

carbon sources for growth and PHA production. The other compounds in Figure 104 might not support cell growth because *P. oleovorans* does not grow on sebacic acid [Section III.D].

Methyl caprylate supported cell growth and PHA production by *P. oleovorans*, as discussed in Section III.D. The growth plot of *P. oleovorans* with this organic substrate is shown in Figure 105. The cells were harvested before the growth reached the stationary phase, and the optical density of the culture was 0.7 A.U after 43 hours, when it was harvested. One liter of the culture afforded 420 mg of biomass and 70 mg of PHA, which contained 25 mole% of repeating units containing the methyl ester group as determined from the NMR spectrum shown in Figure 106. The IR spectrum of this PHA is shown in Figure 107. DSC analysis showed that this PHA had a glass transition at -24 °C and a melting transition at 53 °C with  $\Delta H_m$  of 0.54 cal/g. The DSC thermograms is shown in Figure 108. The total ion chromatogram (TIC) of the methanolized sample of this PHA and the mass spectrum of each peak are shown in Figure 109. The peaks with retention times of 15 minutes and 16.9 minutes are presumably formed by new repeating units, but the mass spectrum of these peaks had either a very small peak or no peak at 103 mass number but had significant 74 mass number peaks.

A PHA containing the benzyl ester group in the side chain was prepared using an equimolar mixture of NA and the monobenzyl ester of sebacic acid. The growth with this



mixture stopped after approximately 15 hours with the final optical density of 1.80 A.U. One liter of the culture gave 0.58 g of biomass and 0.15 g of PHA (PHA content 25 wt%). The mole percent of benzyl ester groups was approximately 4 % as determined from the  $^1\text{H}$  NMR spectrum shown in Figure 110. The  $^{13}\text{C}$  spectrum of this PHA showed the presence of aromatic carbons as indicated in Figure 111. In an experiment in which NA and the monobenzyl ester of sebacic acid were fed in equal amounts, 0.84 g of biomass containing 22 wt% of the PHA was obtained from one liter of the culture. This PHA contained approximately 4 mole% of benzyl ester groups. The selective reduction of the benzyl esters in this PHA was attempted by catalytic hydrogenation using a 10 % Pd/C catalyst. After 4 to 6 hours of reaction there was no noticeable decrease in the amount of aromatic groups in the polymer. The reason for this result might be that the pressure of hydrogen was too low when the apparatus shown in Chapter II was used.

One liter of a culture grown on a 1:1 (vol:vol) mixture of nonane and benzyl-10-undecenoate for approximately four days gave 0.33 grams of biomass containing 1.5 weight percent of PHA. This PHA contained approximately 5 mole% benzyl ester groups and 17 mole percent of unsaturated units as determined from the NMR spectrum, which is shown in Figure 112.

A 10 L culture grown on a 1:1 mixture (vol:vol) of nonane and methyl-10-undecenoate for four days gave



approximately 0.7 g of biomass containing approximately 15 mg of PHA. The optical density after four days was 0.67 A.U. This PHA contained approximately 6.7 mole% of repeating units containing the methyl ester and 60 mole% of unsaturated repeating units as determined from the NMR spectrum shown in Figure 113.

#### 4. PHAs Containing Cyclohexyl Groups

As discussed in Section III.D, some of the cells grown on solely with 4-cyclohexylbutyric acid, CHBA, contained granules. The induction time during growth with this substrate alone was approximately 48 hours, and the optical density of the culture was only 0.18 after three days, after which remained constant. Twelve liters of the culture gave only 1 g of biomass and 20 mg of PHA. The TIC and the mass spectrum of the methanolized sample of this PHA as shown in Figure 114 indicate the presence of C4, C7, and C9 units and a new unit with a retention time of 12.4 minutes. The mass spectrum of the new repeating unit had ion fragments with  $m/z$  values of 55 and 83 which were apparently formed from cyclohexyl ion as shown in Figure 114. The C7 and C9 units in the PHA may have come from PHA-NON, which could have been stored in the cell during the preparation of inoculum, but the origin of C4 unit is not clear.

When *P. oleovorans* was grown on an equimolar mixture of NA and CHBA, the growth stopped after 17 hours with a final

optical density of 2.0 A.U.. One liter of the culture grown on this mixture afforded 0.71 g of biomass and 0.11 g of PHA. This PHA contained C7, C9, and 3-hydroxy-4-cyclohexylbutyrate units, but there was no significant amount of C4 units present. The TIC and mass spectrum of the methanolized sample of this PHA are presented in Figure 115. This PHA was soft and transparent even after annealing for several months at room temperature, but the DSC thermogram of this PHA in Figure 116 showed a melting transition at 47 °C with  $\Delta H_m$  of 1.3 cal/g. The d spacings determined by X-ray scattering study were 19.31, 5.0, 4.65, and 4.15 Å for  $d_1$ ,  $d_2$ ,  $d_3$ , and  $d_4$  (See Section III.E), respectively which were almost identical to those of PHA-NON, indicating that the 3-hydroxy-4-cyclohexylbutyrate units in the PHA were not contributing to the interchain distance. This PHA was likely a random copolymer because it was transparent and had only one  $T_g$  and  $T_m$ . The  $^{13}\text{C}$  NMR spectrum of this PHA is shown in Figure 117.

## 5. A PHA Containing Hydroxyl Groups

A PHA containing a small amount of hydroxyl groups, approximately 2 mole%, was obtained from cells grown with a 2.4:1 mixture of NA and 12-hydroxydodecanoic acid, HOD. The growth on this mixture stopped after approximately 15 hours with a final optical density of 1.93 A.U. One liter of this culture afforded 0.75 g of biomass and 0.17 g of PHA, which

contained a trace amount of alcoholic group. The NMR spectrum of this PHA is shown in Figure 118. Presumably, the mole fraction of hydroxyl-containing repeating units should be increased by increasing the fraction of HOD in the substrate mixture. This PHA had a glass transition temperature at  $-35^{\circ}\text{C}$  and a melting transition temperature at  $53^{\circ}\text{C}$  with  $\Delta H_m$  of 4.33 cal/g, as shown in Figure 119.

#### 6. PHAs Produced from Mixtures of Aminoalkanoic Acids and Either NA or Octanoic Acid

One liter of a culture grown on an equimolar mixture of NA and 11-aminoundecanoic acid, 11AMA, gave approximately 0.5 g of biomass and 0.11 g of PHA, while one liter of a culture grown on an equimolar mixture of OA and 11AMA gave 0.19 g of biomass and 0.03 g of PHA. This result is another example of obtaining poor result by using OA as the cosubstrate. Elemental analyses showed these PHAs contained no detectable amounts of nitrogen. However, the different yields of biomass and PHA from these two mixtures suggests that the presence of the cosubstrate affected the growth and PHA production in some manner.



#### 7. PHA Produced from a Mixture of Nonane and Trimethylsilyl Ether of Decanol

One liter of a culture grown on a 1:1 (vol:vol) mixture of nonane and trimethylsilyl ether of decanol gave 0.32 g of biomass and 0.07 g of PHA. The NMR analysis and gas chromatographic analysis showed there was no detectable amount of repeating units containing trimethylsilyl ether group.

#### 8. PHA Produced From a Mixture of NA and 1,12-Dodecanediol

One liter of a culture grown on an equimolar mixture of NA and 1,12-dodecanediol gave 0.85 g of biomass and 0.1 g of PHA. The growth curves are shown in Figure 120. This PHA seems to contain very small amount of hydroxyl groups as indicated in the NMR spectrum shown in Figure 121.

### J. Conclusions and Future Work

The physical and chemical structures of carbon substrates affect both cell growth and PHA production by *P. oleovorans*. A longer separation between the carboxylic acid group and a substituent is more favorable for biosynthesis of PHA by *P. oleovorans*, and the presence of bulky groups in the 5th and 6th carbon of carboxylic acids disturbed both cell growth and PHA synthesis.

The main repeating unit in the PHAs obtained from carboxylic acids shorter than OA was 3-hydroxyalkanoates having the same number of carbons as the carbon source, but it was C8 or C9 in the PHAs obtained from carboxylic acids longer than heptanoic acid. The largest repeating units in PHAs obtained from alkanoic acids shorter than decanoic acid had two more carbon atoms than the carbon source, but the largest unit in PHAs obtained from higher carboxylic acids had the same number of carbon atoms as the carbon source.

The PHA produced by *P. oleovorans* grown with UND: was remarkable because almost 100 % repeating units in the polymer contained unsaturated unit. PHAs containing desired amounts of unsaturated units could be produced by growing *P. oleovorans* with mixtures of UND: and either OA or NA.

The most important finding in this thesis work is that *P. oleovorans* polymerizes a carbon substrate that supports cell growth without PHA production as a sole carbon substrate when either NA or OA was present as cosubstrate. NA and OA gave different results when they were used as cosubstrate with other carbon sources such as bromoalkanoic acids. OA usually gave poorer result in respect to the amount of repeating unit from the poor carbon source.

*P. oleovorans* generally produced random copolymers from mixed carbon sources, however, physical mixtures of more than one PHA were obtained when mixtures of 5-phenylvaleric acid(PVA), and either NA or OA were used as carbon sources.

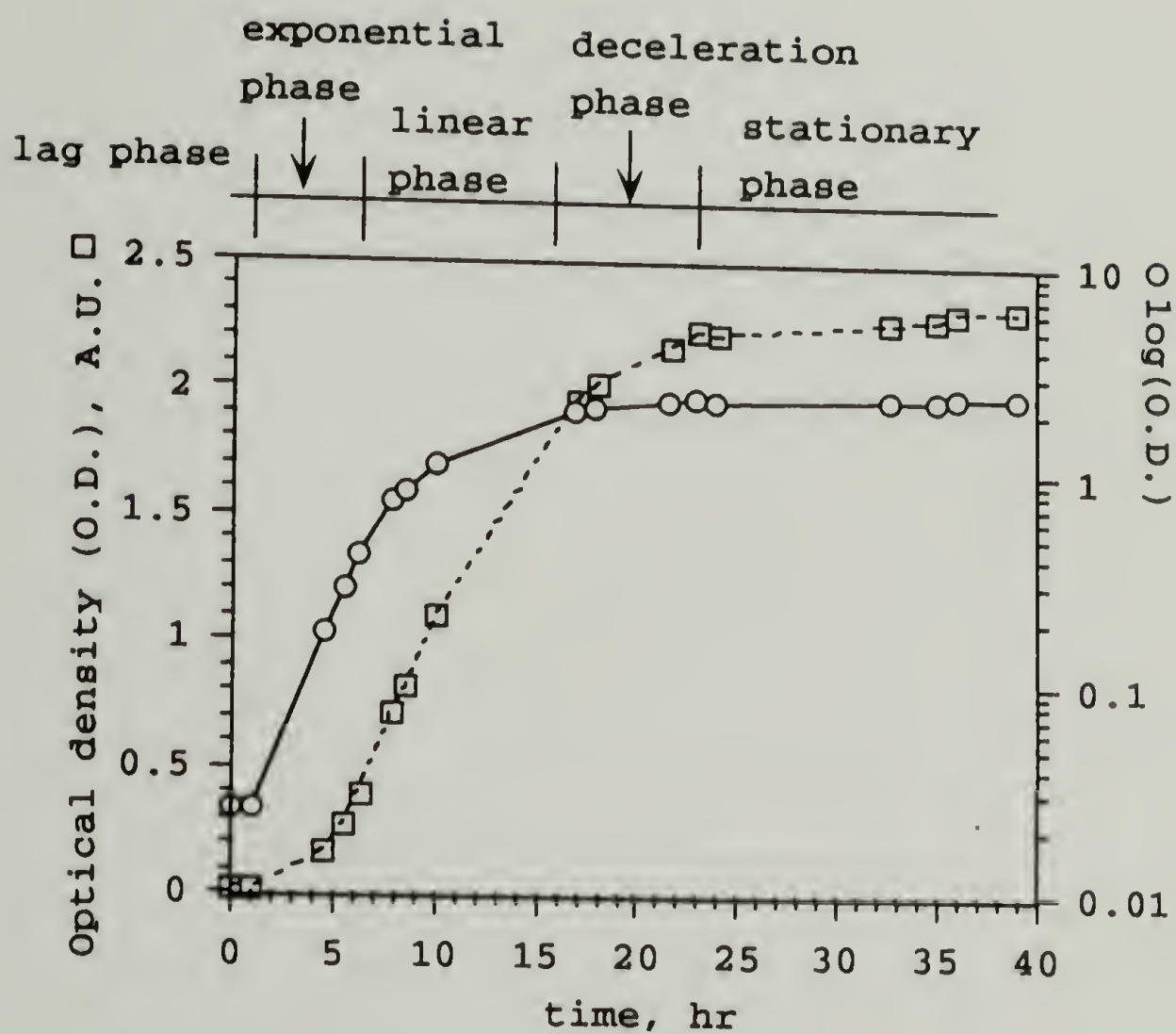


Figure 13. Growth Curve for *P. oleovorans* with 10 mM Nonanoic Acid.



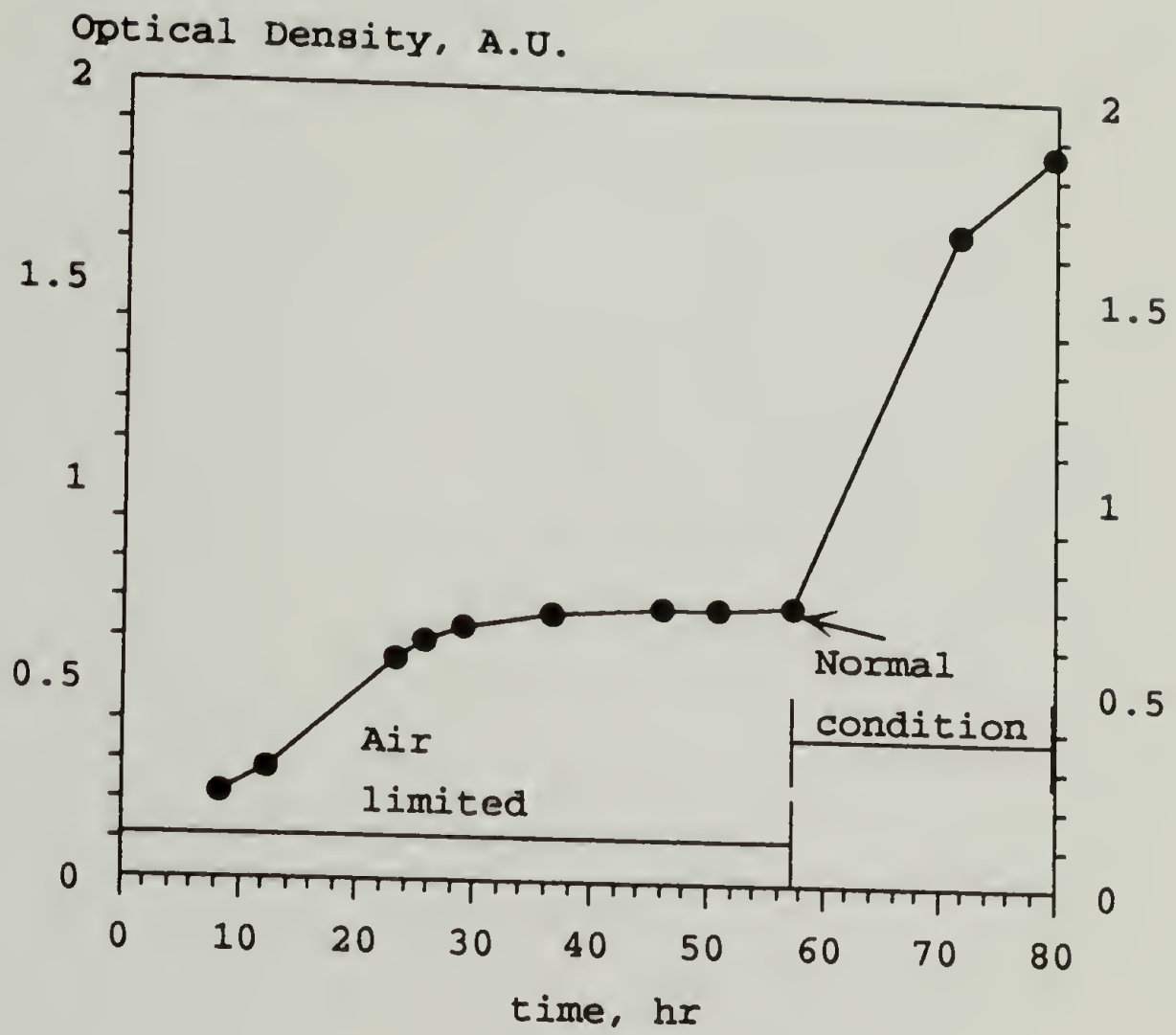


Figure 14. Growth curve for *P. oleovorans* under air limiting conditions.



(a) old culture grown initially under an air limited condition.



(b) old culture grown under normal condition.

Figure 15. Cell morphologies of two different cultures in late stationary phase.

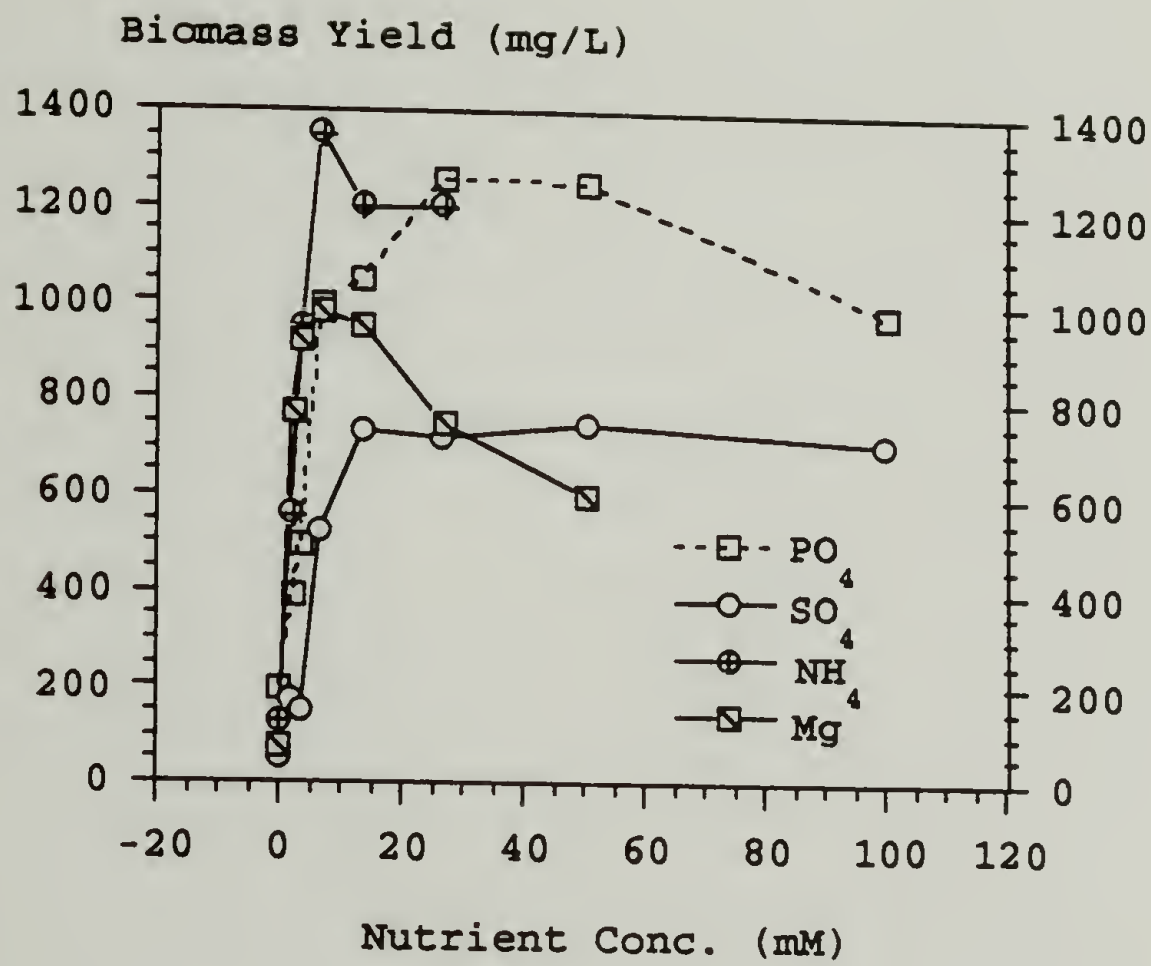


Figure 16. The Effect of Initial Concentrations of Nutrients on Biomass Yield [Wolf et al., 1990b].



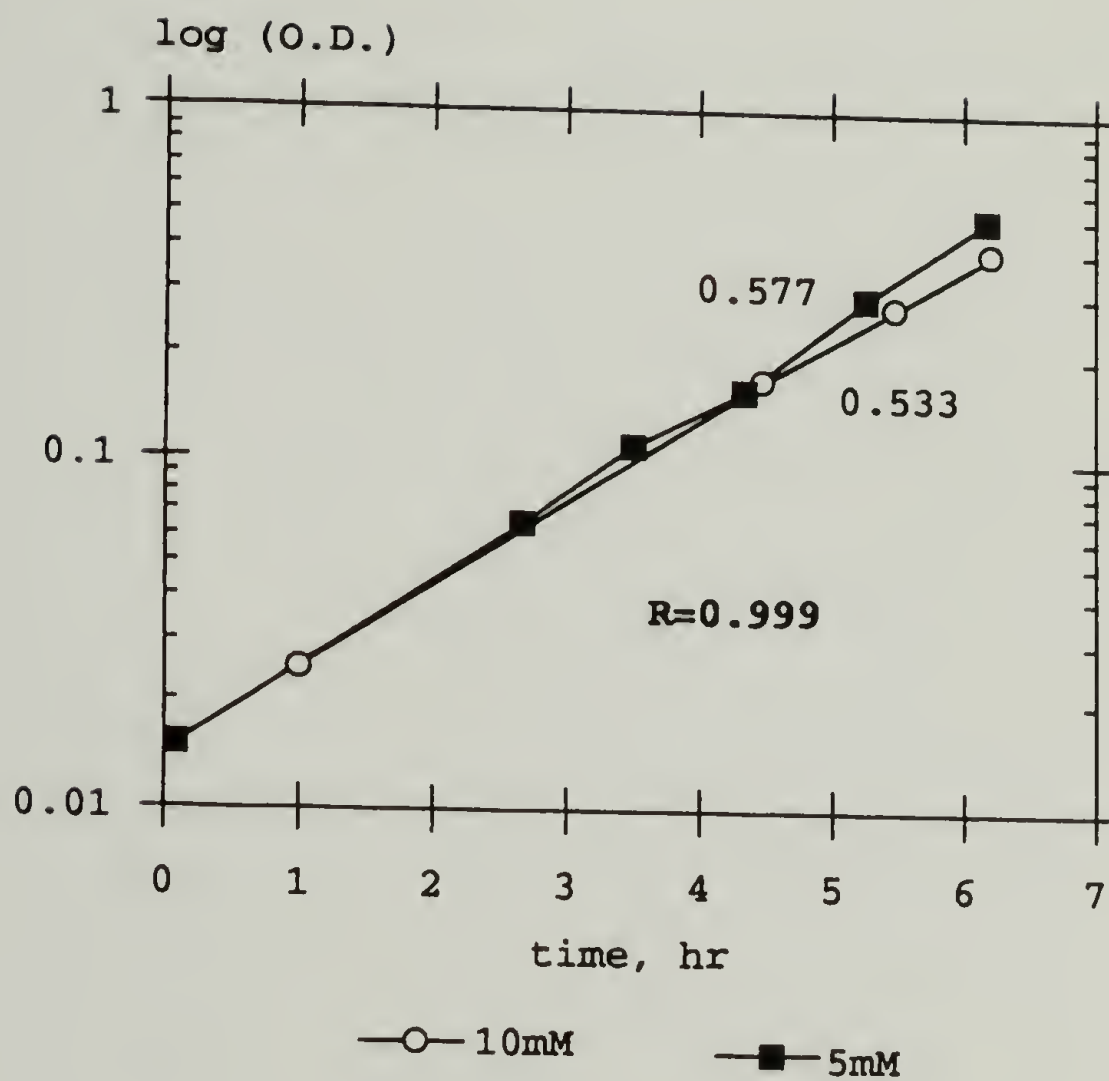


Figure 17. Exponential phase of growth of *P. oleovorans* with 5 mM and 10 mM nonanoic acid.

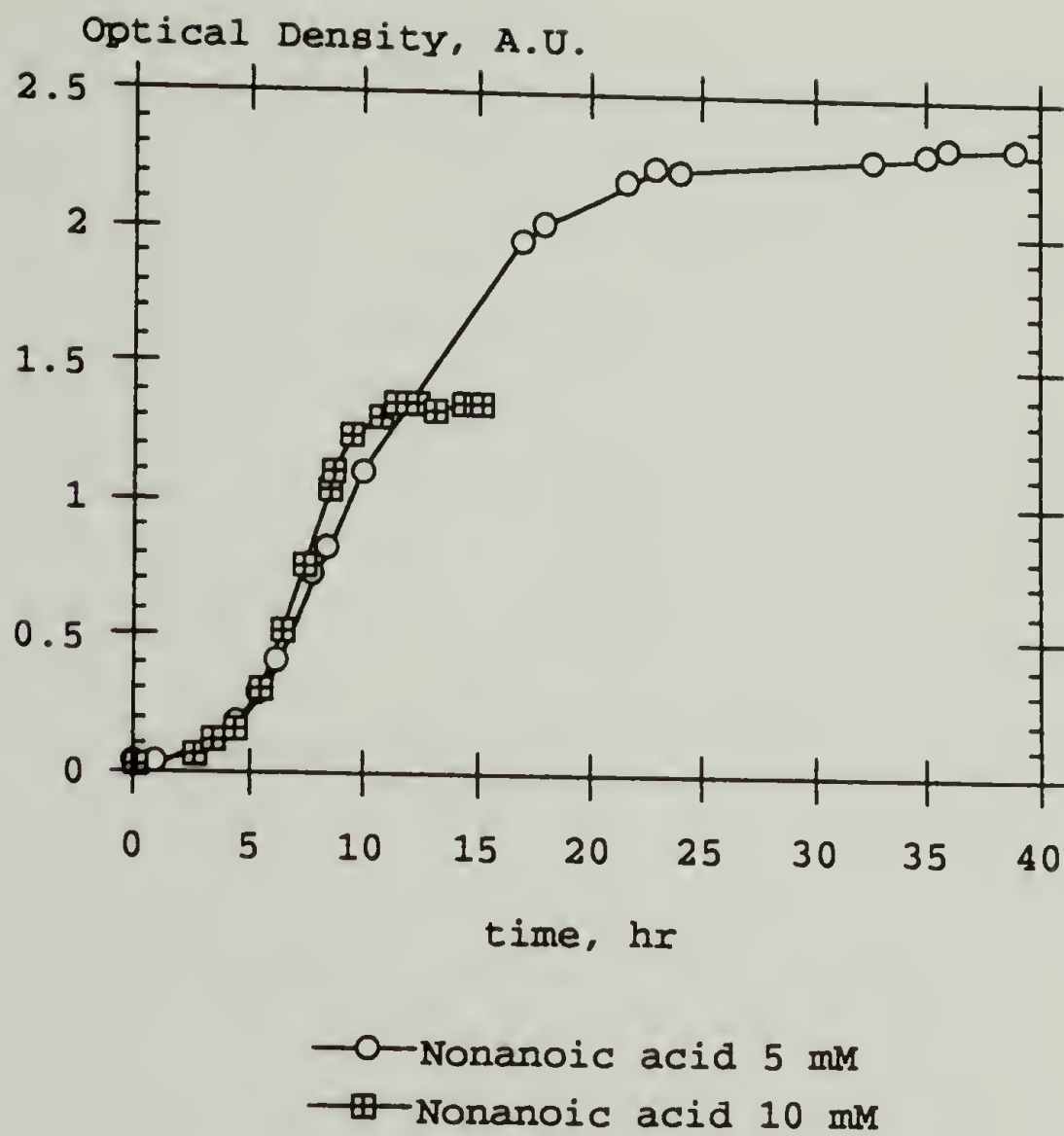


Figure 18. Optical density vs. time for growth of *P. oleovorans* with 5 mM and 10 mM nonanoic acid.

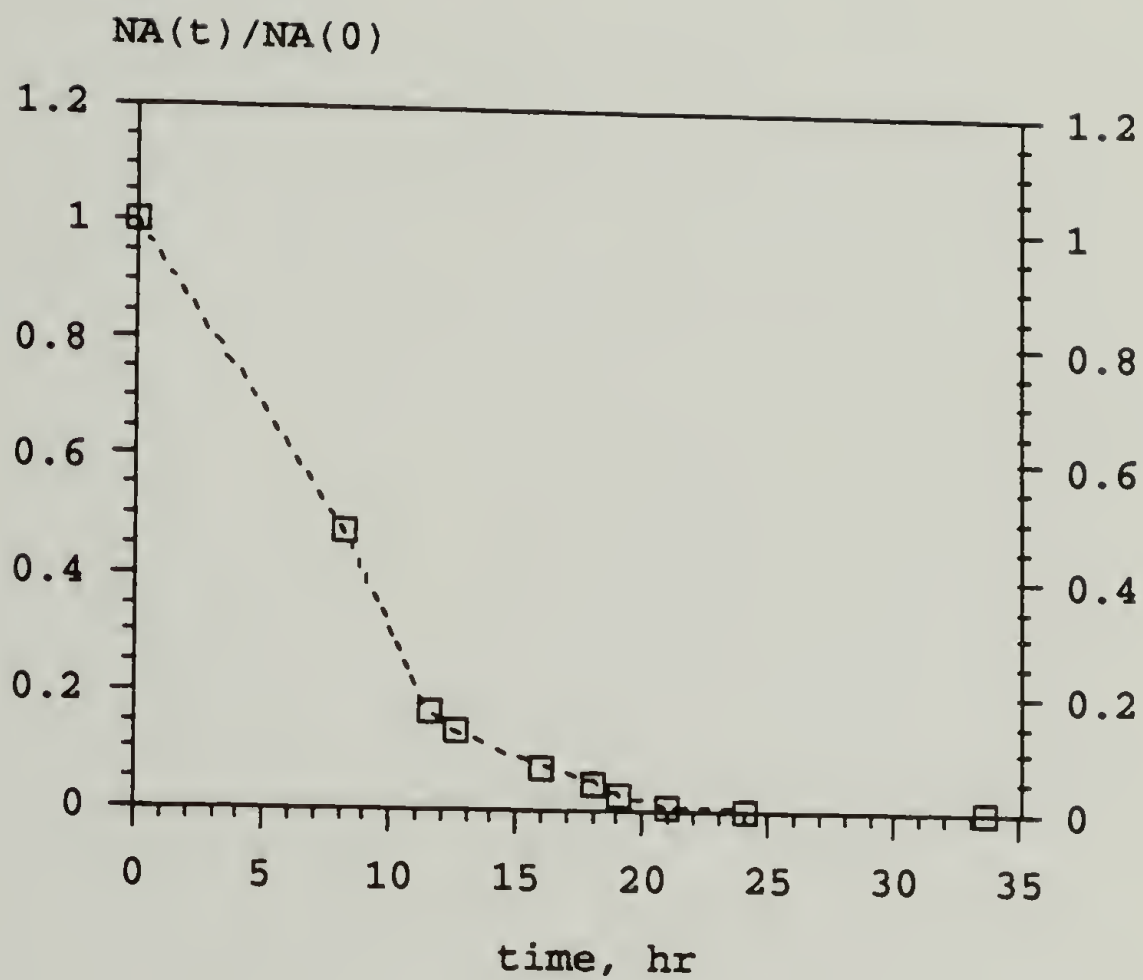


Figure 19. The fraction of nonanoic acid remaining in the medium vs. growth time (10 mM nonanoic acid).



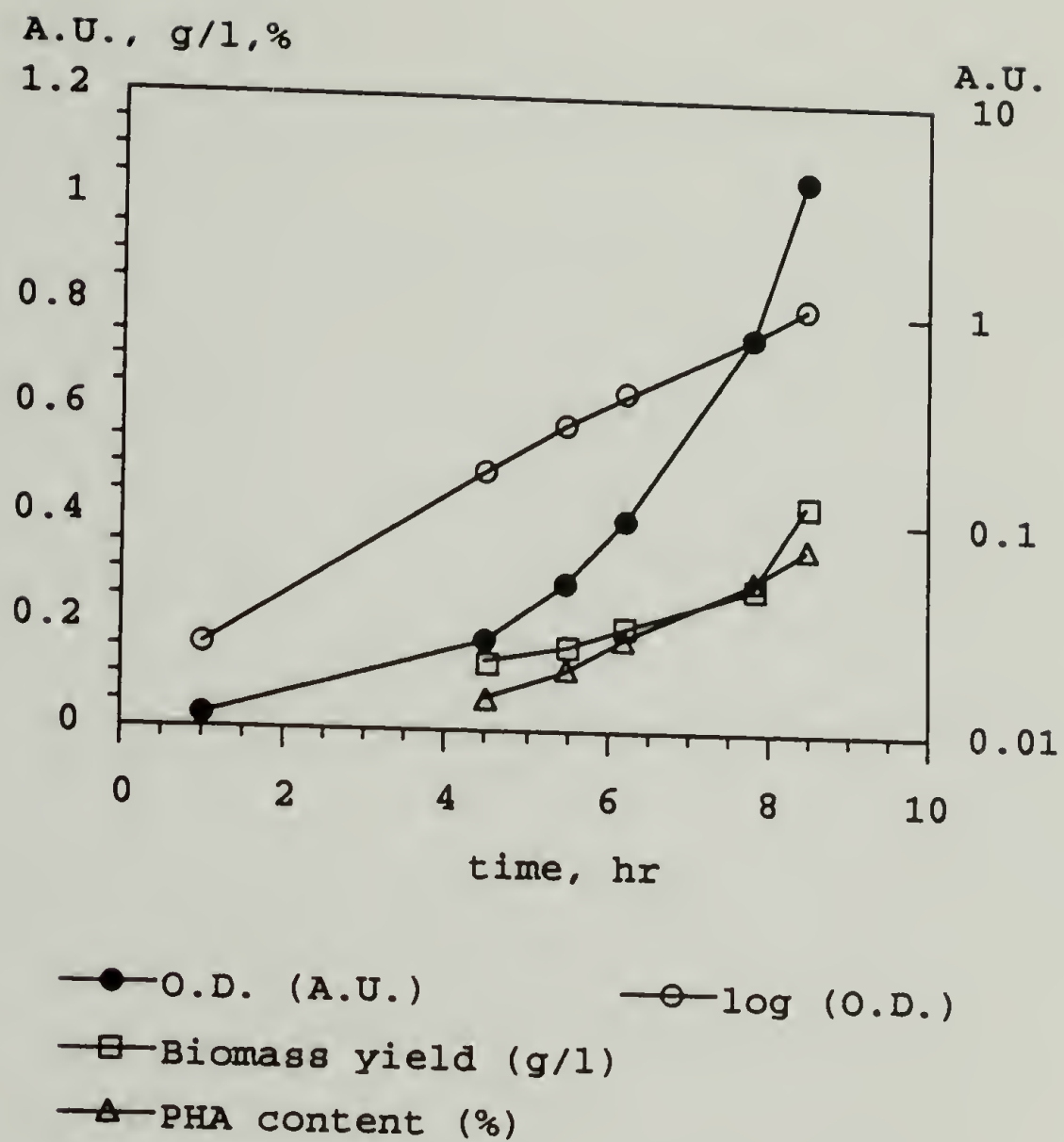


Figure 20. The Biomass Yield and PHA Content vs. Growth Time During Exponential Phase (10 mM Nonanoic Acid).

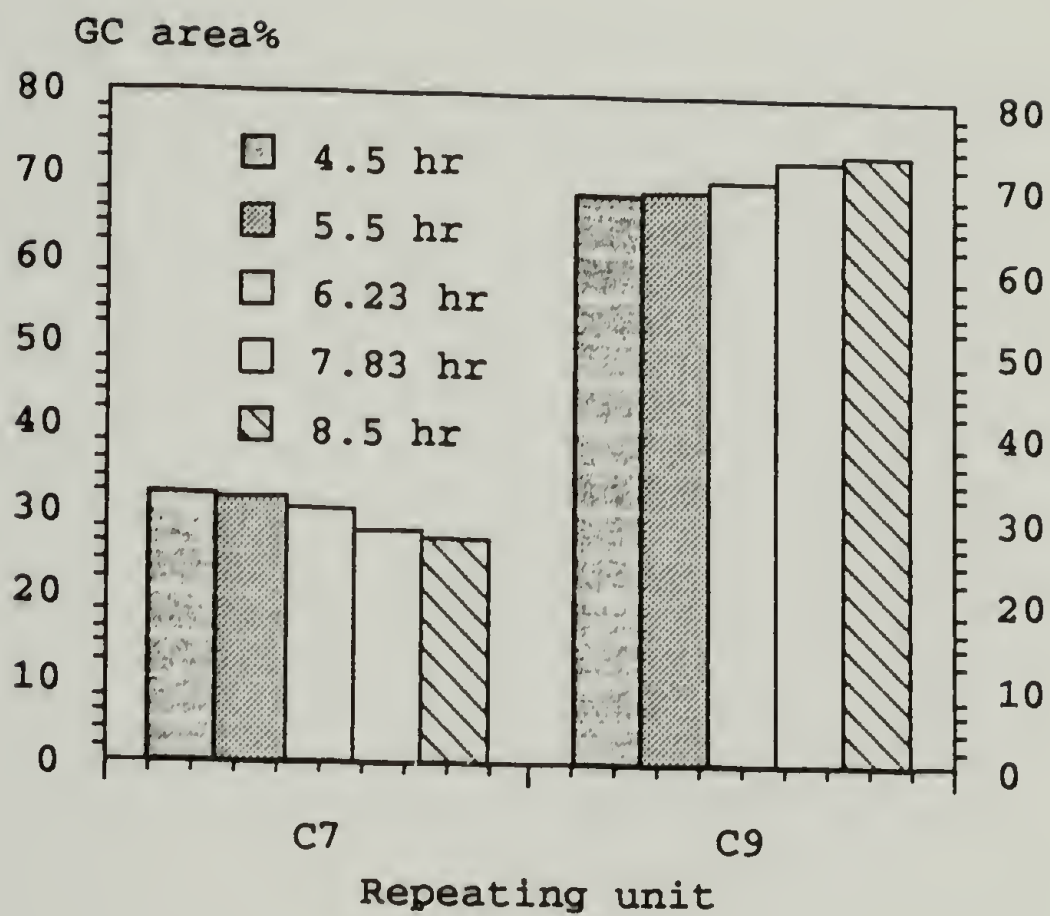


Figure 21. Repeating Unit Composition Change of PHA-NON During Exponential Phase.

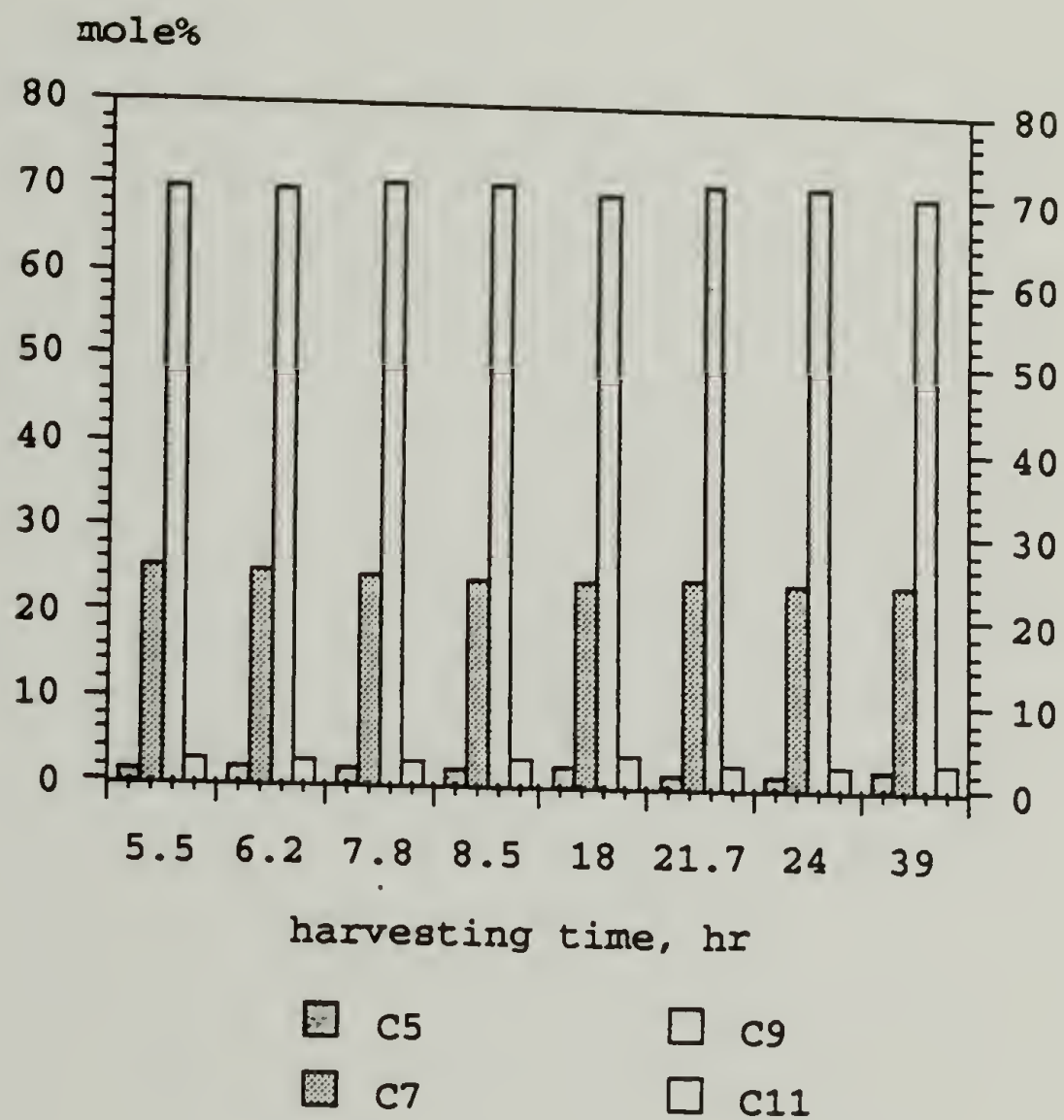


Figure 22. Repeating unit composition change of PHA-NON throughout the growth.



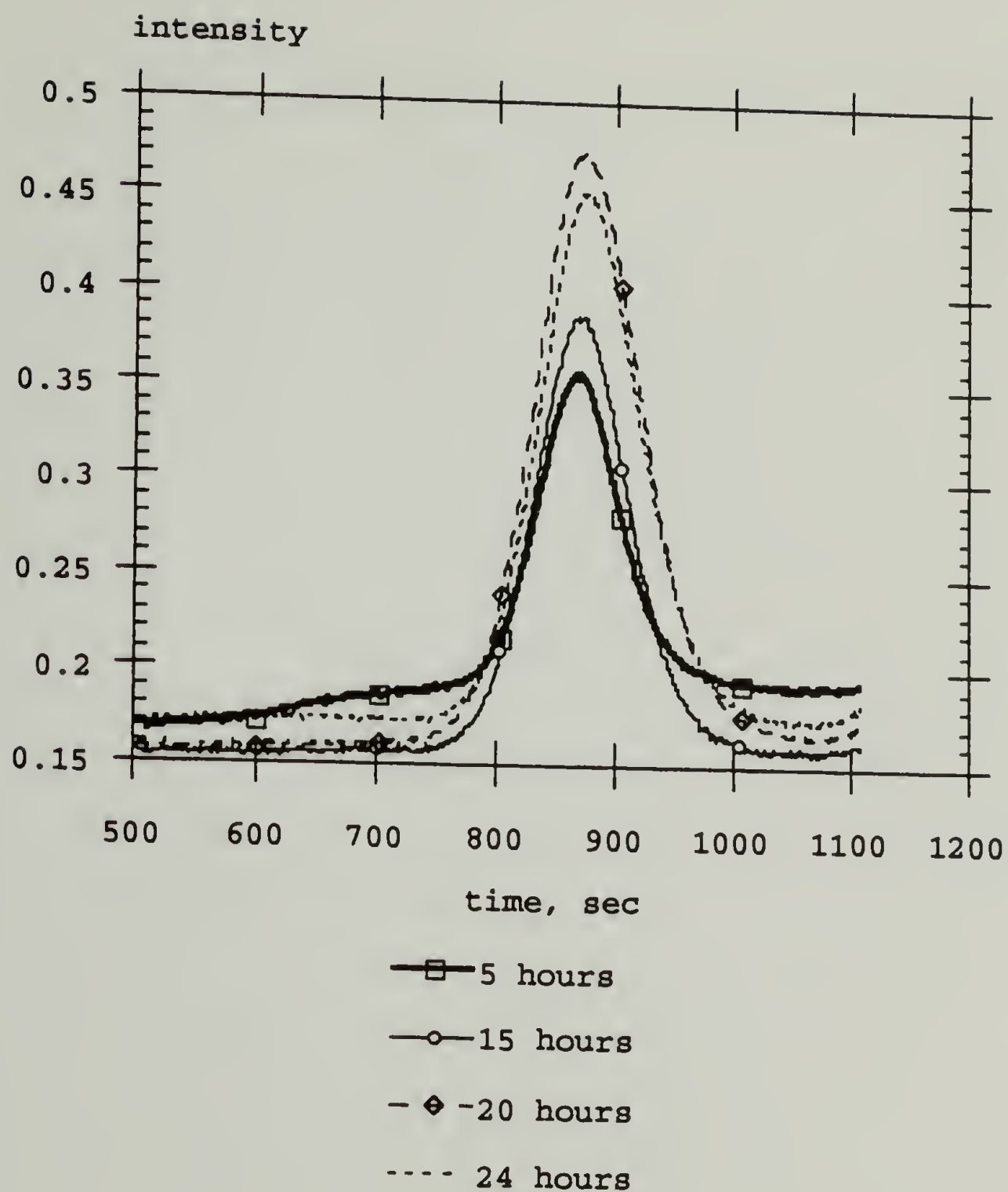


Figure 23. Gel permeation chromatograms of crude extracts from biomasses harvested at different growth times.

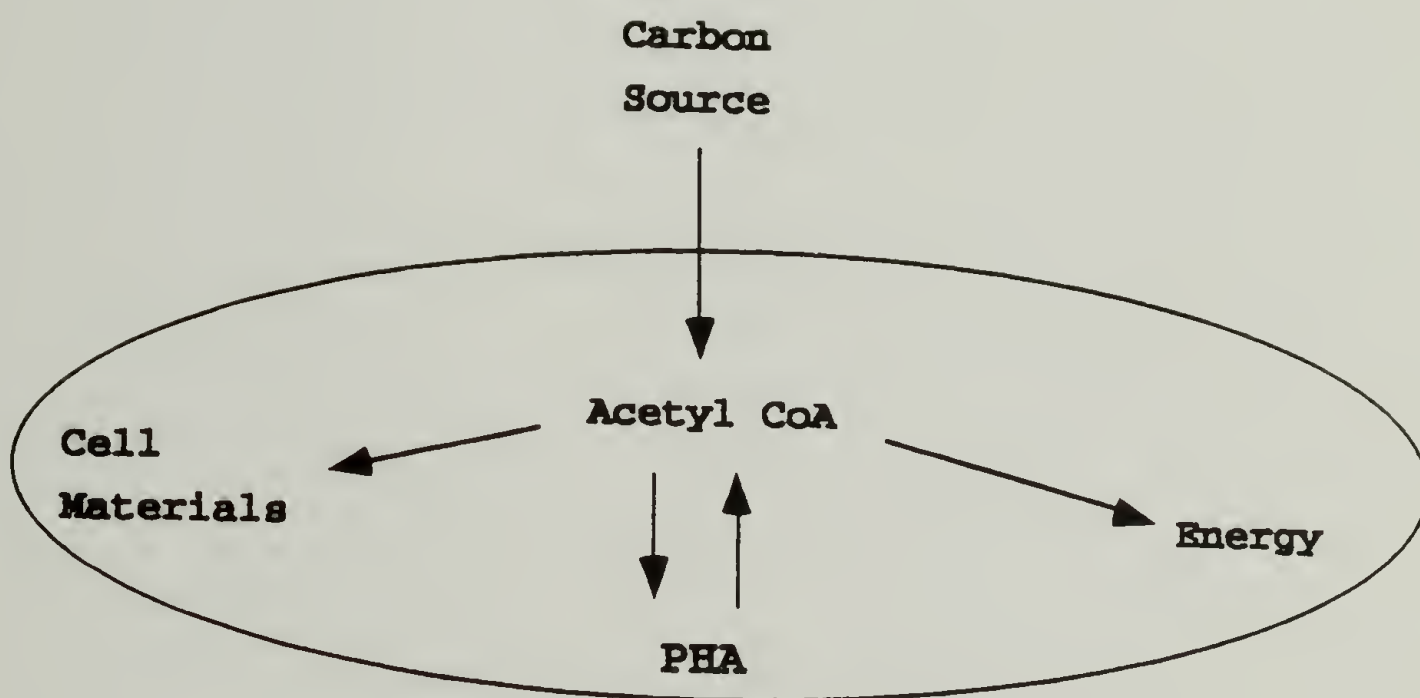


Figure 24. The fate of carbon source.

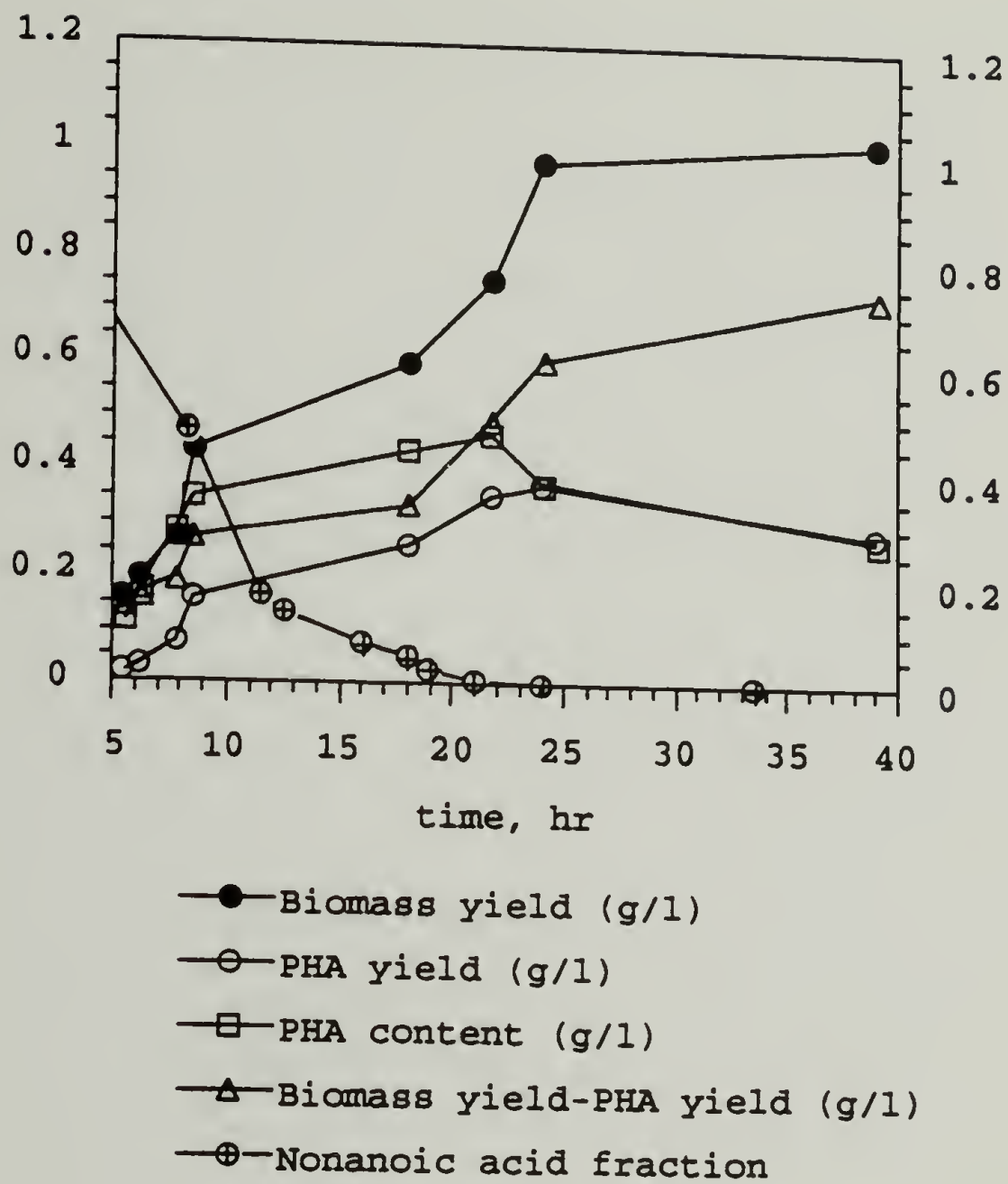


Figure 25. Fermentation results vs. growth time.



Table 9. Biomass yield, PHA yield, and molecular weight distribution of PHAs prepared from repeated batch culture fermentations.

Carbon source <sup>a</sup>	Exp. I.D. <sup>b</sup>	Biomass (g/l) <sup>c</sup>	PHA yield (g/l)	M <sub>n</sub> <sup>d</sup> (x1000)	PDIE <sup>e</sup>
90% NA	A	0.66	0.32	54	1.6
	B	1.1	0.45	56	1.7
	C	0.96	0.36	60	1.7
98% NA	A	1.1	0.43	54	1.6
	B	0.93	0.43	53	1.8
	C	0.96	0.46	55	1.7
99% UND:	A	0.62	0.23	52	1.8
	B	0.66	0.21	54	1.7
98% UND:	A	0.7	0.22	59	1.7
	B	0.8	0.25	57	1.7
NA:UND: (2:1)	A	0.8	0.28	57	1.7
	B	0.86	0.26	58	1.8

a, Initials for Carbon Source

NA-Nonanoic acid

UND:-Undecylenic acid (10-undecenoic acid)

b, Experiment identification

c, Number average molecular weight

d, Polydispersity index

Table 10. Repeating unit compositions of PHAs prepared from repeated fermentations<sup>1,2</sup>.

Carbon source		Amount of repeating units <sup>a</sup>								
	I.D.	C5	C6	C7 (C7:)	C8	C9 (C9:)	C10	C11 (C11:)		
90% Nonanoic acid (NA)	A	1.6	0.7	22.5	5.2	65.6	1.6	2.8		
	B	1.8	0.8	24.7	5.1	64.3	1.3	2.0		
	C	1.7	0.8	23.8	5.5	64.9	1.3	2.0		
98% Nonanoic acid	A	1.9		26.8		69.6		1.7		
	B	1.7		25.3		70.1		2.9		
	C	1.9		26.0		70.3		1.8		
10-Undecenoic acid <sup>b</sup> (UA:)	A	0.8		0.5		0.6				
				(7.2)		(60.9)		(30.0)		
	B	0.7		0.6		0.6				
NA:UA(:) (2:1) <sup>b</sup>				(6.0)		(61.5)		(30.6)		
	A	0.3		11.9	0.9	44.7	NR <sup>c</sup>	NR		
				(3.4)		(26.4)		(11.7)		
	B	0.3		13.6	1.0	46.2	0.6	NR		
				(2.4)		(23.3)		(11.9)		

a, Cn is β-hydroxyalkanoate with n carbon atoms; Cn is β-hydroxy-w-alkenoate with n carbons  
b, Calculated from the peak area of the gas chromatograms  
c, Not resolved

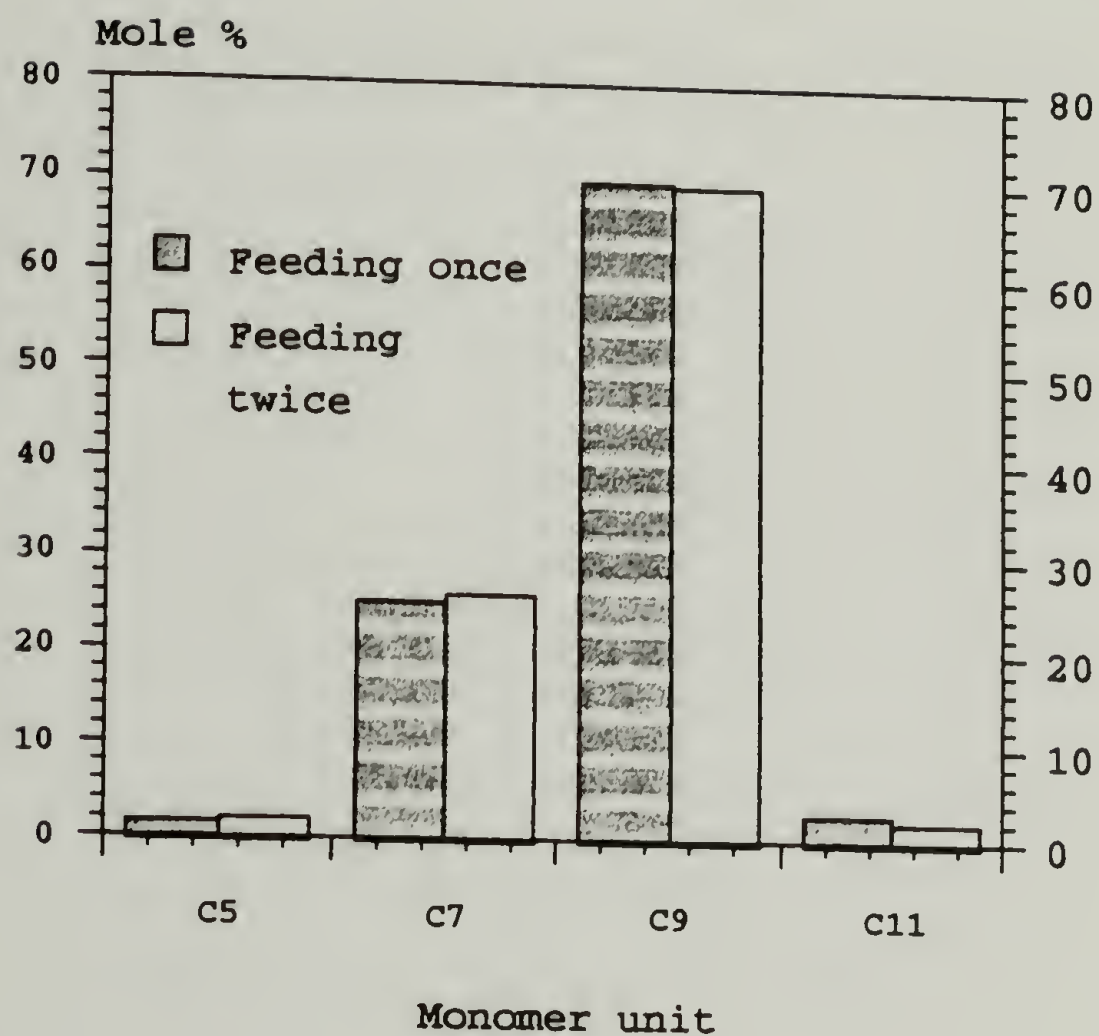
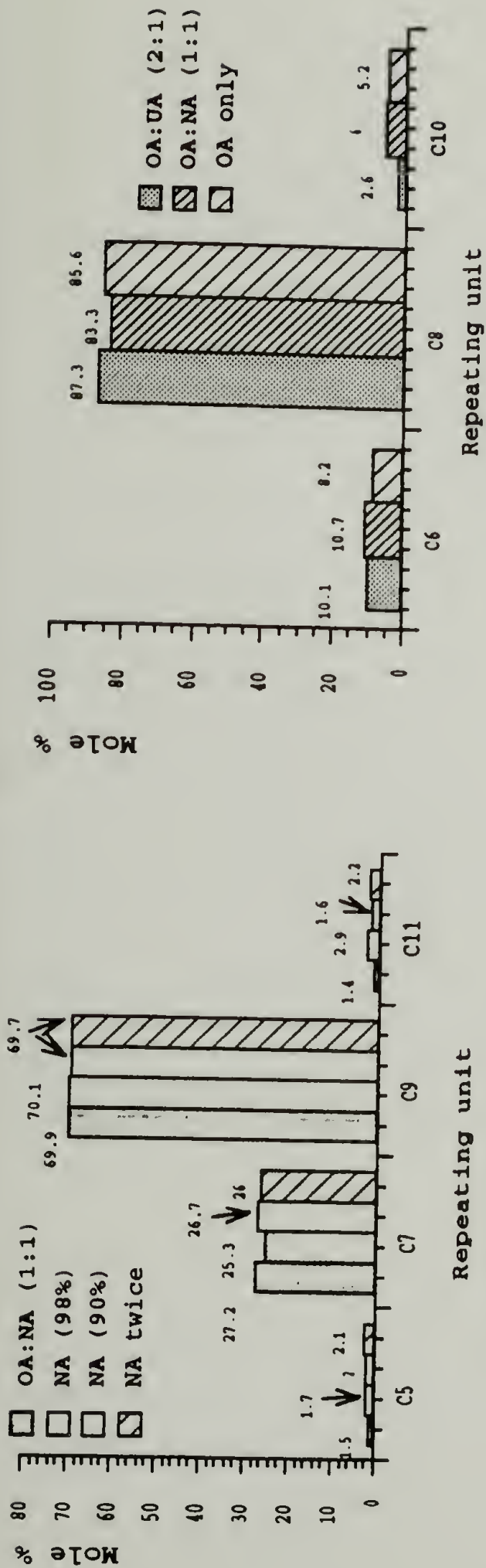
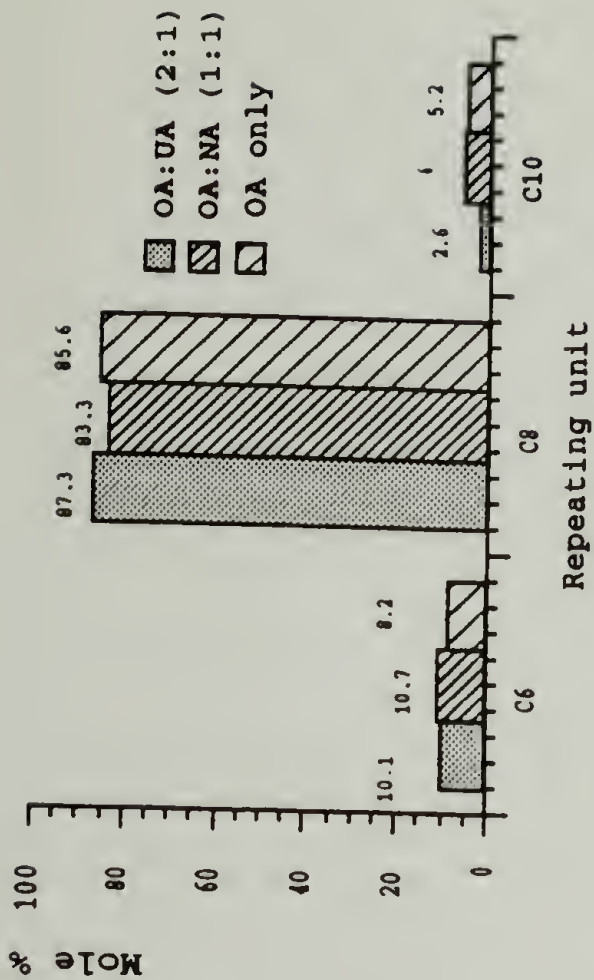


Figure 26. The repeating unit compositions of PHAs prepared by feeding nonanoic acid either once or twice consecutively.

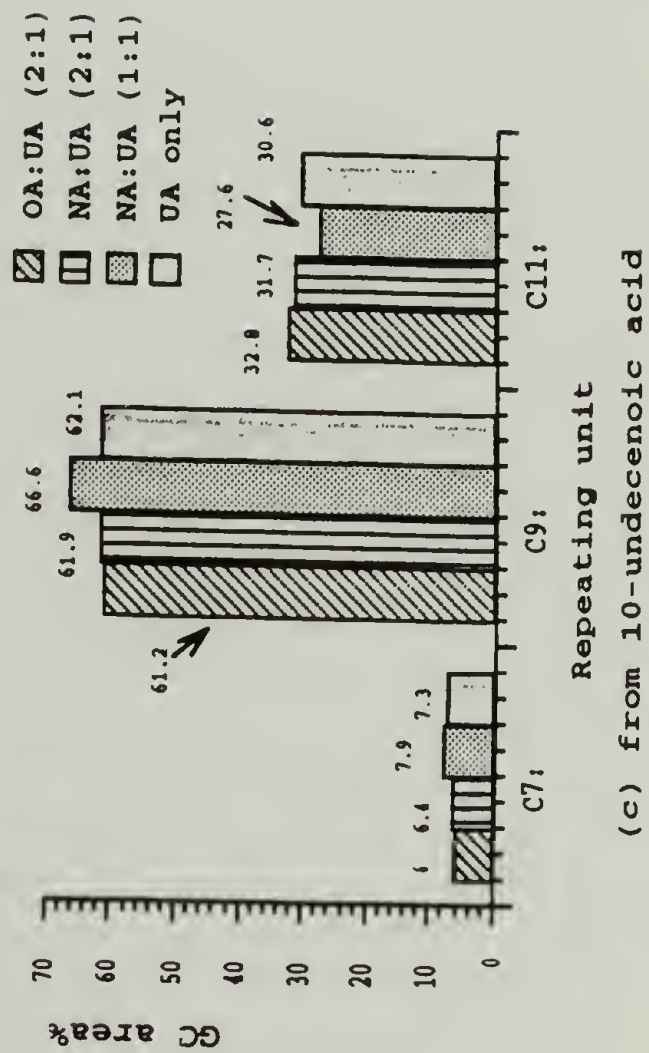




(a) from nonanoic acid



(b) from octanoic acid



(c) from 10-undecenoic acid

Figure 27. Relative amount of repeating units in various PHAs.

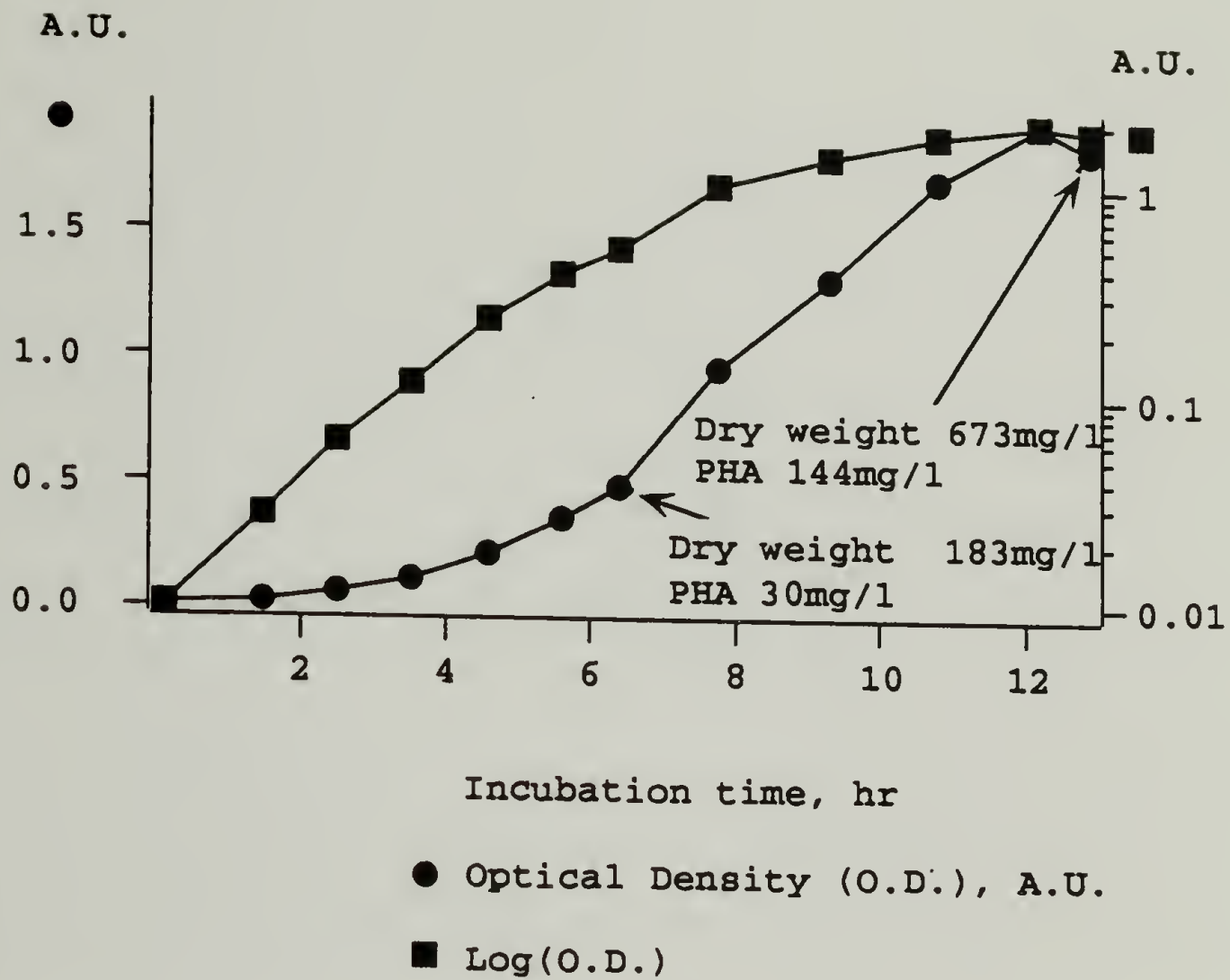


Figure 28. Growth curve for *P. oleovorans* with a medium containing a 1:1 mixture of nonanoic acid and acetic acid.

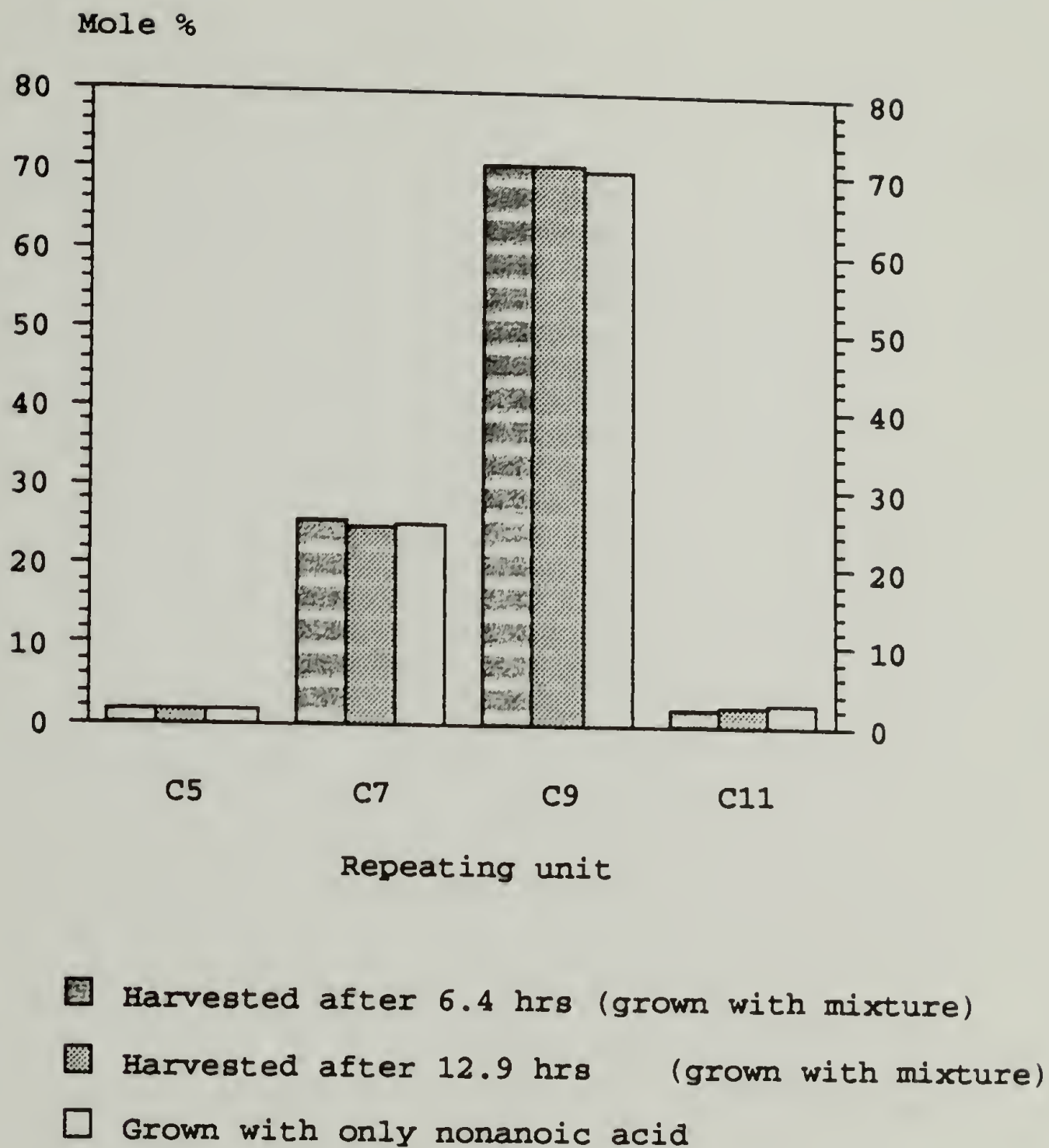
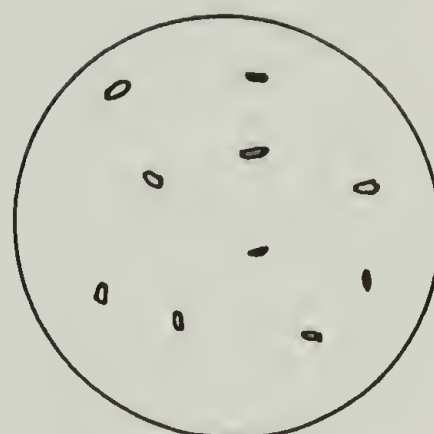


Figure 29. Repeating unit compositions of PHAs produced from either a 1:1 mixture of nonanoic acid and acetic acid or nonanoic acid.

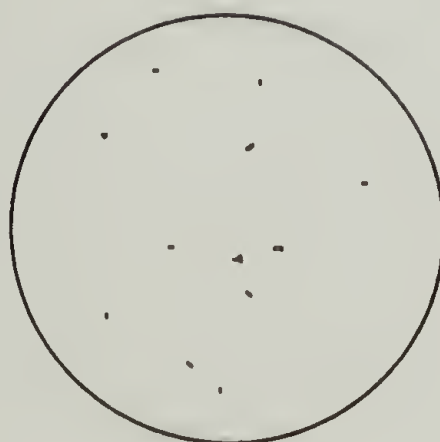




(a) Growth with PHA production



(b) Growth without PHA production



(c) No growth

Figure 30. Cell morphology observed by optical microscopy.

Table 11. Classification of carbon substrates based on the growth characteristics.

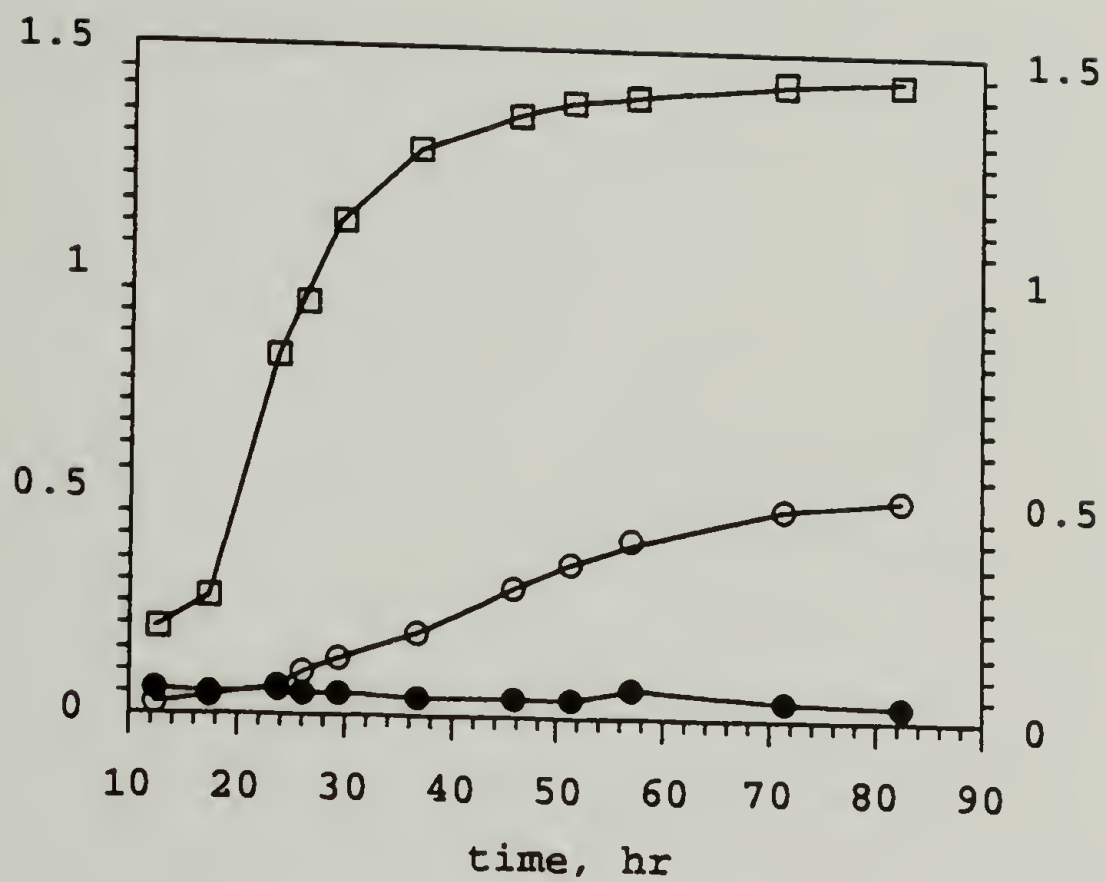
grou	Chemical type	Compounds
p		
a	alkanoic acid	hexanoic acid (C6) to hexadecanoic acid (C16)
		7-methyloctanoic acid, 5-phenylvaleric acid, 8-styrylacrylic acid, cyclohexylbutyric acid
	alkenoic acid	10-undecenoic acid, 7-octenoic acid, 6-octenoic acid, oleic acid <sup>1</sup> , elaidic acid <sup>2</sup> , γ-linolenic acid <sup>3</sup>
	ester	methylcaprylate, methyldecanoate
	alkane	hexane to dodecane <sup>4</sup>
	alkene	heptene, octene, decene
	ester	methyl-10-undecenoate, benzyl-10-undecenoate, sebacic acid monoesters (methyl, benzyl)
	alkene	hexene, dodecene
	alkanoic acid	acetic acid to hexanoic acid
		6-bromomhexanoic acid, 8-bromooctanoic acid, 11-bromoundecanoic acid
b		6-methyloctanoic acid, 5-methyloctanoic acid
		11-cyanoundecanoic acid, 12-hydroxydodecanoic acid
	diol	1,12-dodecanediol
	alkanoic acid	heptadecanoic acid, octadecanoic acid
		4-phenylbutyric acid
c		6-aminohexanoic acid, 8-aminooctanoic acid, 11-aminoundecanoic acid
	diol	1,8-octanediol, 1,6-hexanediol
	thiol	octanethiol
	diacid	sebacic acid
	others	4-octylbenzene sulfonic acid
		4-heptylbenzoic acid

1, cis-9-octadecenoic acid

2, trans-9-octadecenoic acid

3, cis-6,9,12-octadecatrienic acid

4, no data available for longer carbon sources



- 6-bromohexanoic acid
- 8-bromooctanoic acid
- 11-bromoundecenoic acid

Figure 31. Growth of *P. oleovorans* with Various Bromoalkanoic Acids.



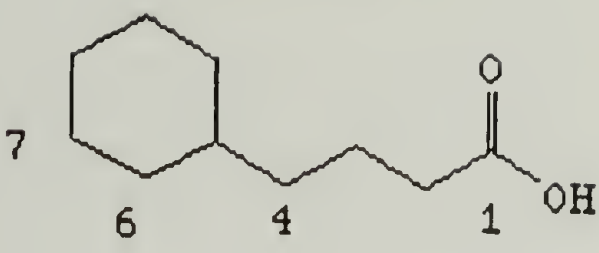
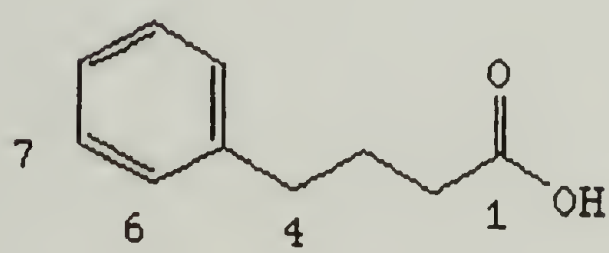
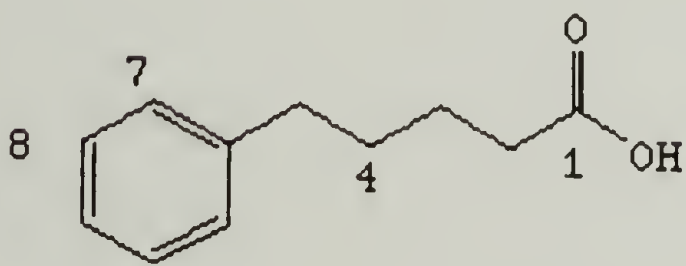
<u>Substrate</u>	<u>Structure</u>	<u>Growth Behavior</u>
I		growht with PHA production, (a)
II		no growth, (c)
III		growth with PHA production, (a)

Figure 32. Growth of *P. oleovorans* with 4-cyclohexylbutyric acid (I), 4-phenylbutyric acid (II), and 5-phenylvaleric acid (III).

Table 12. Repeating unit compositions of PHAs prepared from alkanolic acids shorter than decanoic acid.

Carbon Source	Repeating units, mole%							
	C4	C5	C6	C7	C8	C9	C10	C11
Hexanoic acid	3	<1	72		22		3	
Heptanoic acid		7	<1	86	<1	7		
Octanoic acid	<1		10.7		83.3		6	
Nonanoic acid		1.7		25.3		70.1		2.9

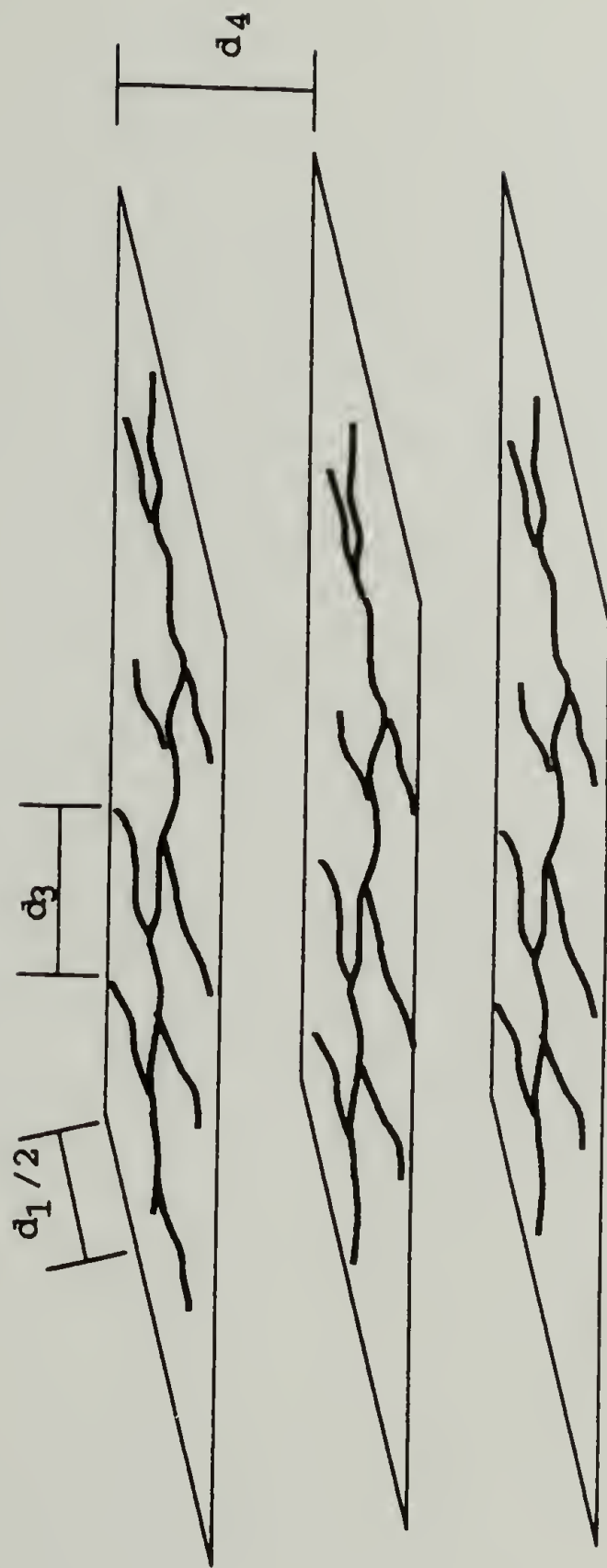


Figure 33. Sheet-like arrangement of polymers with long side chains.



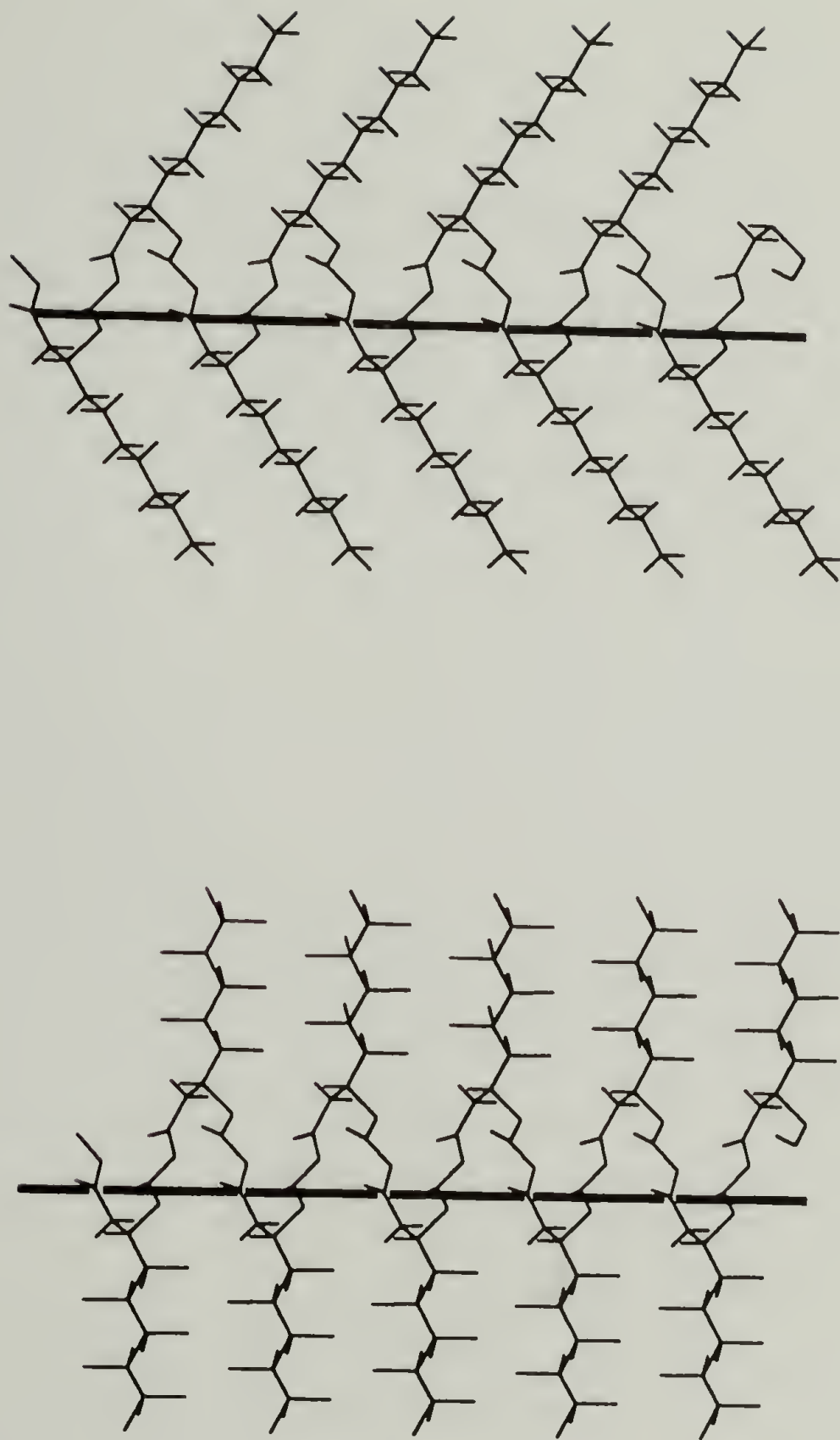


Figure 34. Possible  $2_1$  helices proposed for PHAs produced by *P. oleovorans* [Marchessault et al., 1990].

Table 13. Repeating unit compositions of PHAs prepared from long alkanolic acids.

Carbon		Relative amount of repeating unit											
source		C5	C6	C7	C8	C9	C10	C11	C12	C13	C14		
undecanoic acid <sup>a</sup>		1.9	-	21.8	0.4	50.2	-	25.7	-	-	-		
dodecanoic acid <sup>a</sup>		-	6.6	0.9	40.0	3.2	32.8	-	16.5	-	-		
tridecanoic acid <sup>b</sup>		NR	1.0	22.9	8.6	45.6	5.7	10.0	1.4	5.0	-		
tetradecanoic acid <sup>b</sup>		NR	7.3	-	55.2	-	27.8	-	7.8	-	1.9		
hexadecanoic acid <sup>b,c</sup>		0.2	7.2	0.4	51.2	0.8	28.8	0.3	8.6	NR	NR		

a, mole%

b, GC area ratio

c, there are unidentified peaks with longer retention times than that of C14  
NR, not resolved

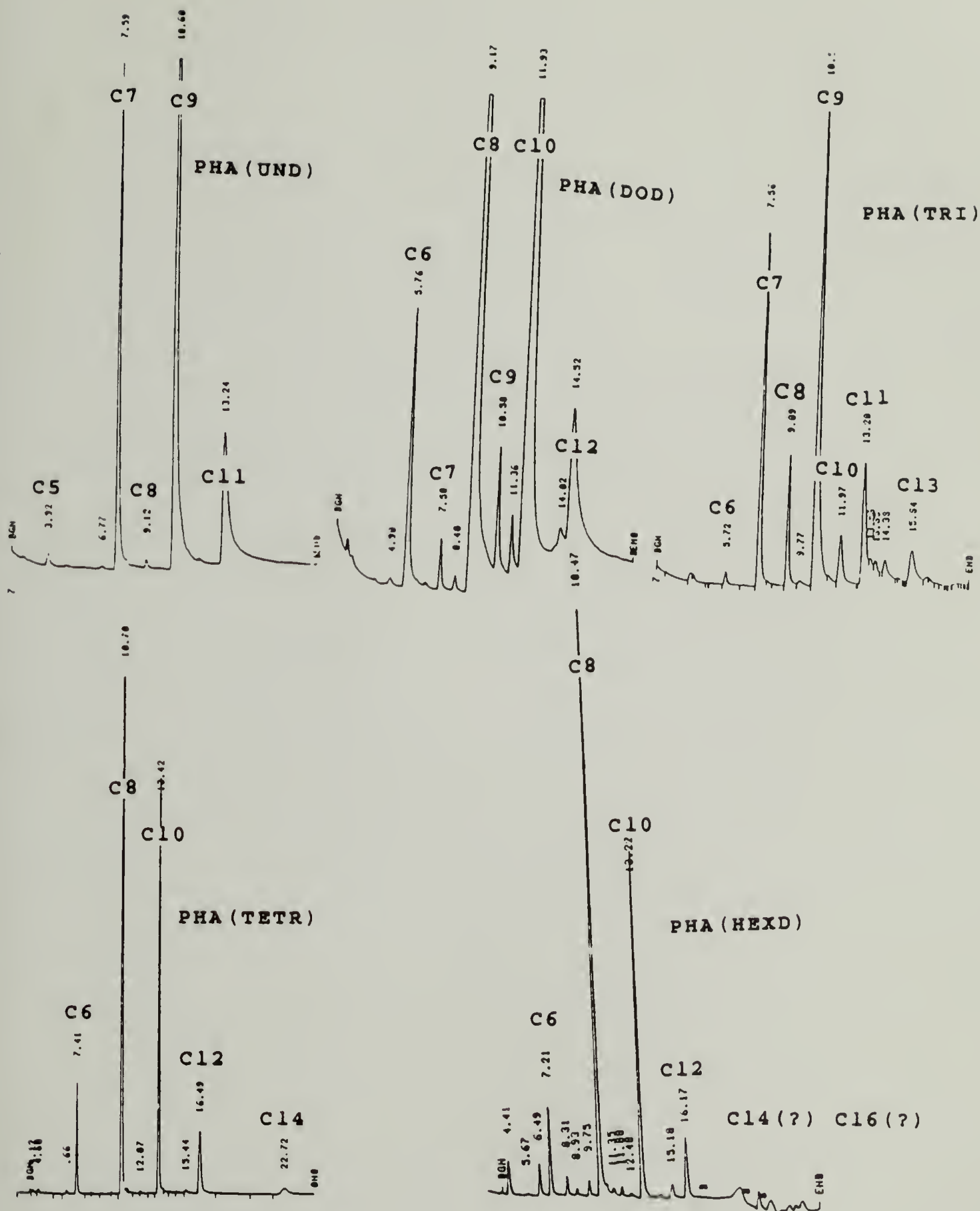
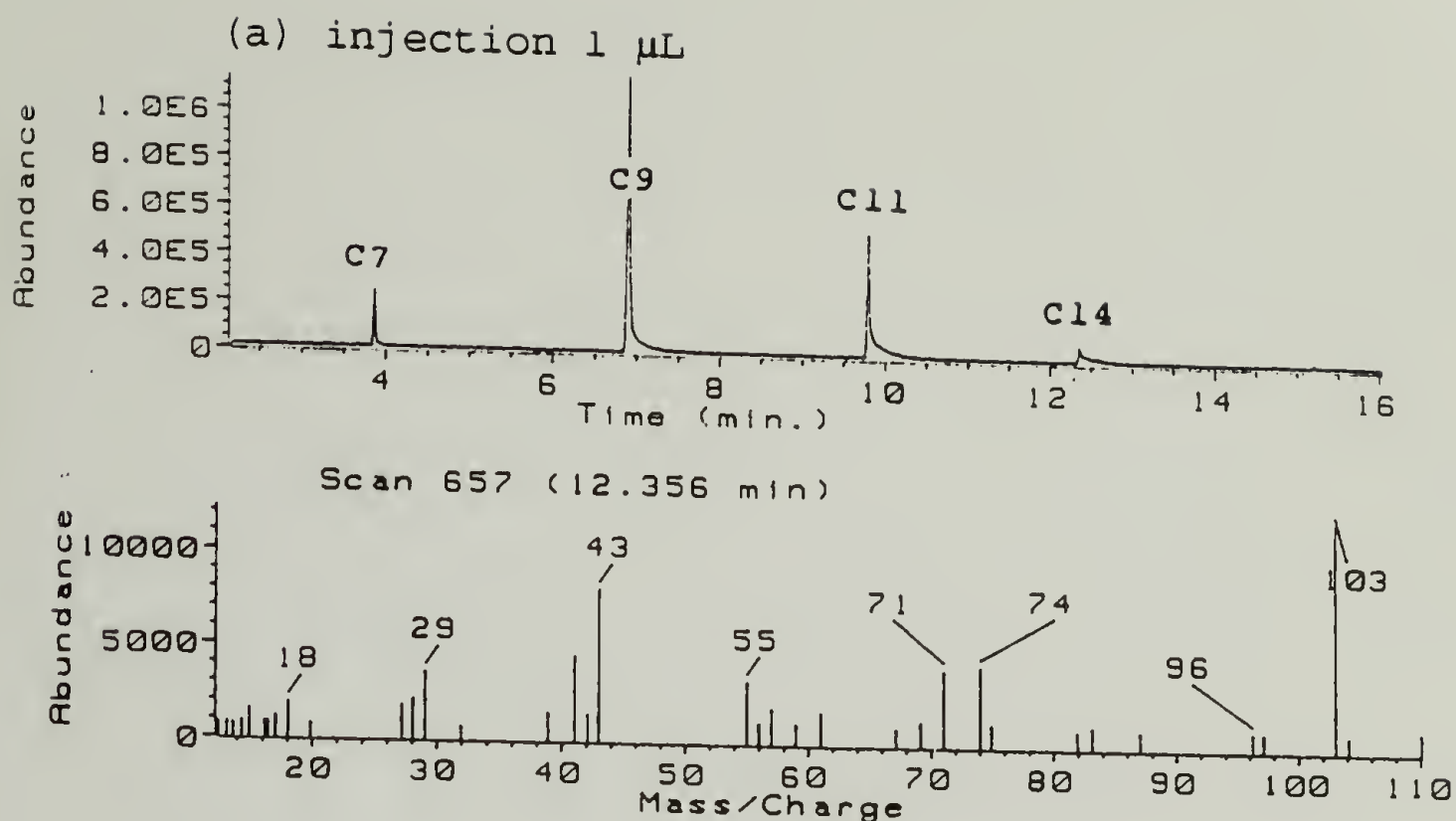


Figure 35. Gas chromatograms of methanolized samples of PHAs produced by *P. oleovorans*.





(b) injection 6  $\mu\text{L}$  and the mass spectrometer was activated 13.5 minutes after injection.

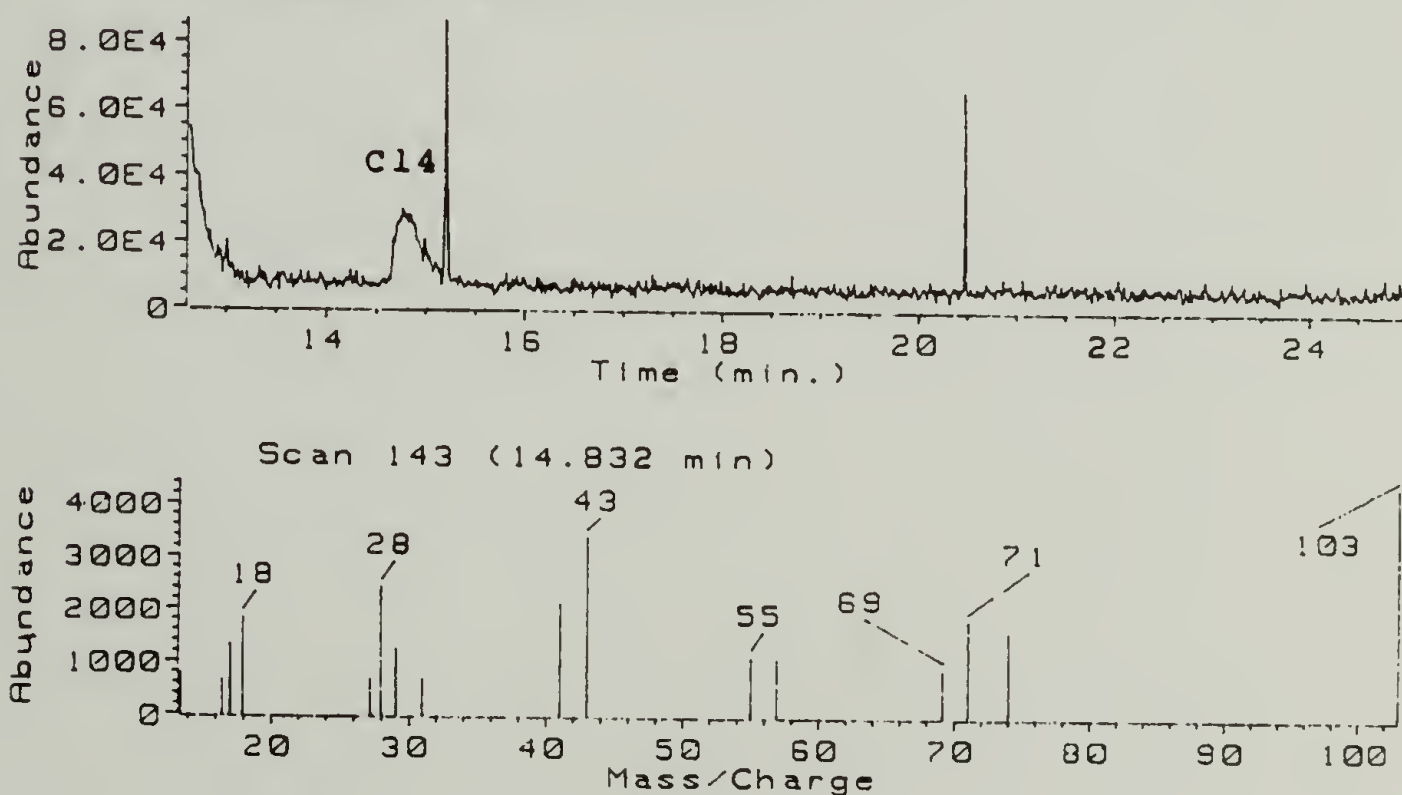


Figure 36. TIC and mass spectrum of C14 units in the methanolized sample of PHA-TET.

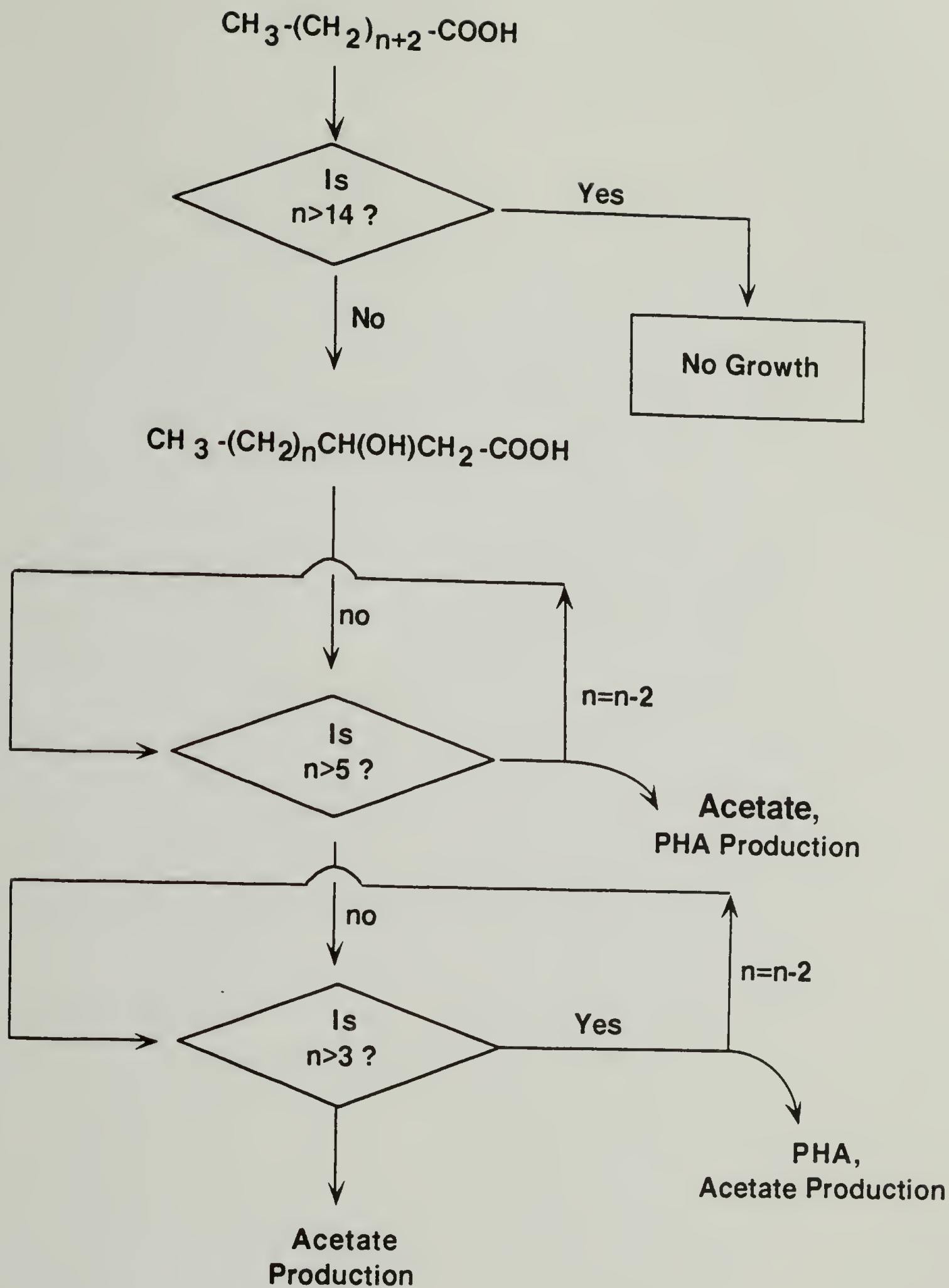


Figure 37. Flow diagram for utilization of n-alkanoic acids by *P. oleovorans*.

Table 14. Melting transition temperatures,  $T_m$ , and heats of fusion,  $\Delta H_m$ , of PHA's.

Carbon source	$T_m$ , °C	$\Delta H_m$ , cal/g
Hexanoic acid <sup>I</sup>	-	-
Heptanoic acid <sup>I</sup>	45.0	1.30
Octanoic acid	56.0	5.34
Nonanoic acid	50.0	4.37
Decanoic acid <sup>I</sup>	54.0	4.90
Undecanoic acid	45.0	3.92
Dodecanoic acid	46.2	4.46
Tridecanoic acid	46.3	1.91
Tetradecanoic acid	43.7	3.19

I, Gross et al., 1989



Table 15. d Spacings of oriented PHAs prepared from various carbon sources.

Carbon Source	d spacing, Å			
	d <sub>1</sub>	d <sub>2</sub>	d <sub>3</sub>	d <sub>4</sub>
Octanoic acid	18.5	4.91	4.66	4.13
Nonanoic acid	19.4	4.98	4.65	4.14
Decanoic acid	19.9	5.05	4.67	4.16
Undecanoic acid	20.5	5.0	4.65	4.15
Dodecanoic acid	20.8	4.88	4.61	4.16
Tetradecanoic acid	20.1	4.95	4.63	4.15

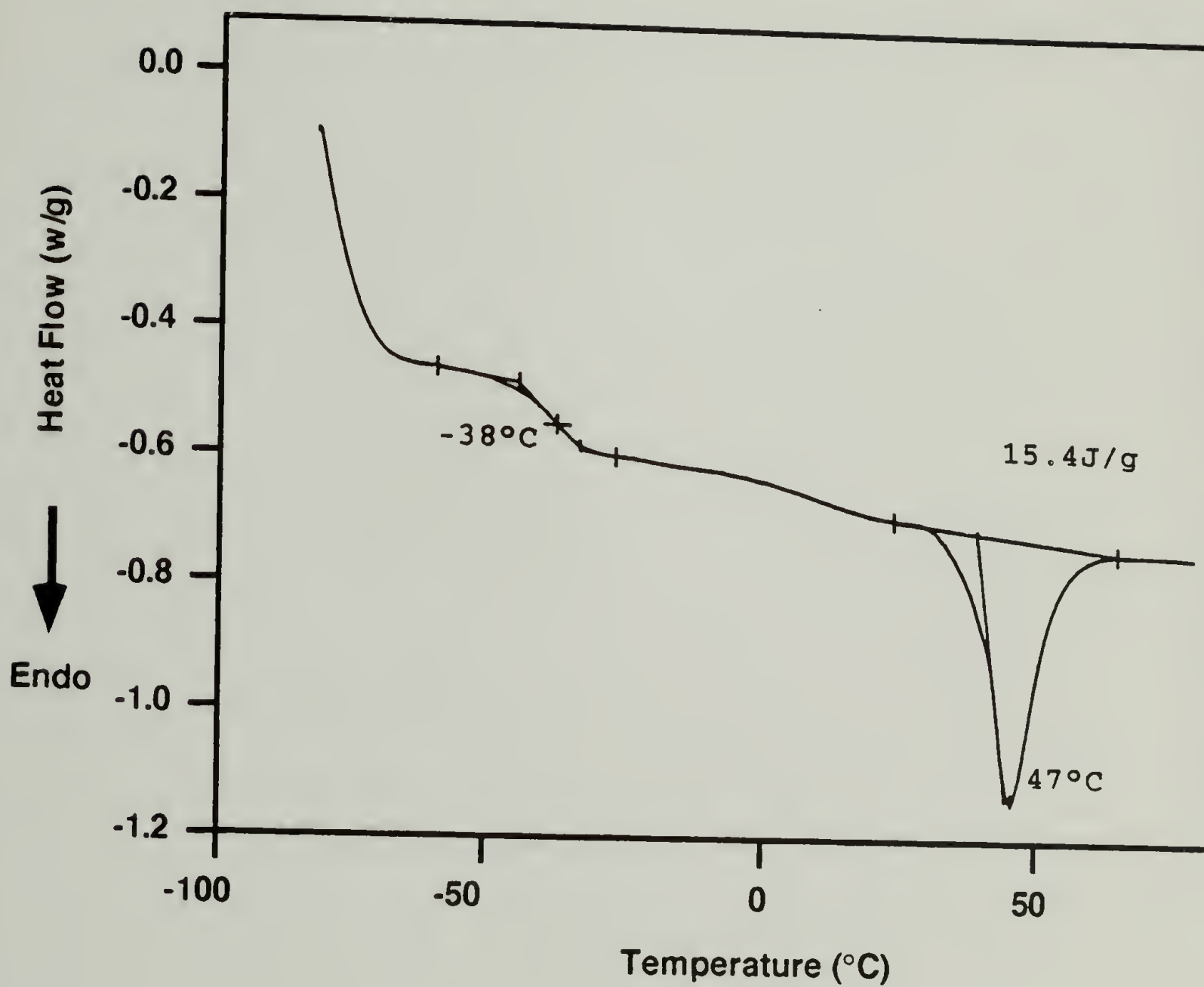


Figure 38. DSC thermogram of PHA-HEXD.

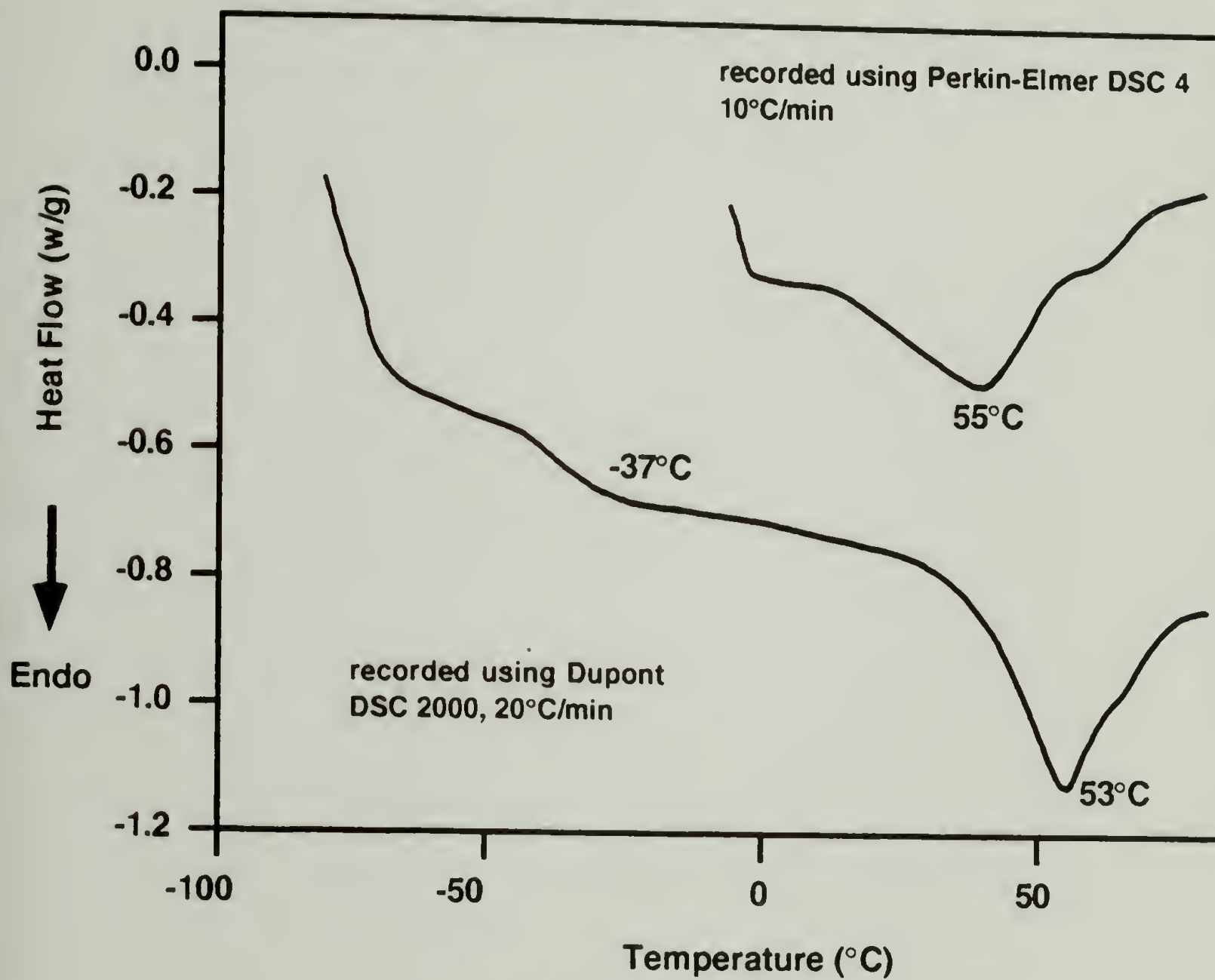


Figure 39. DSC thermogram of PHA-DOD.



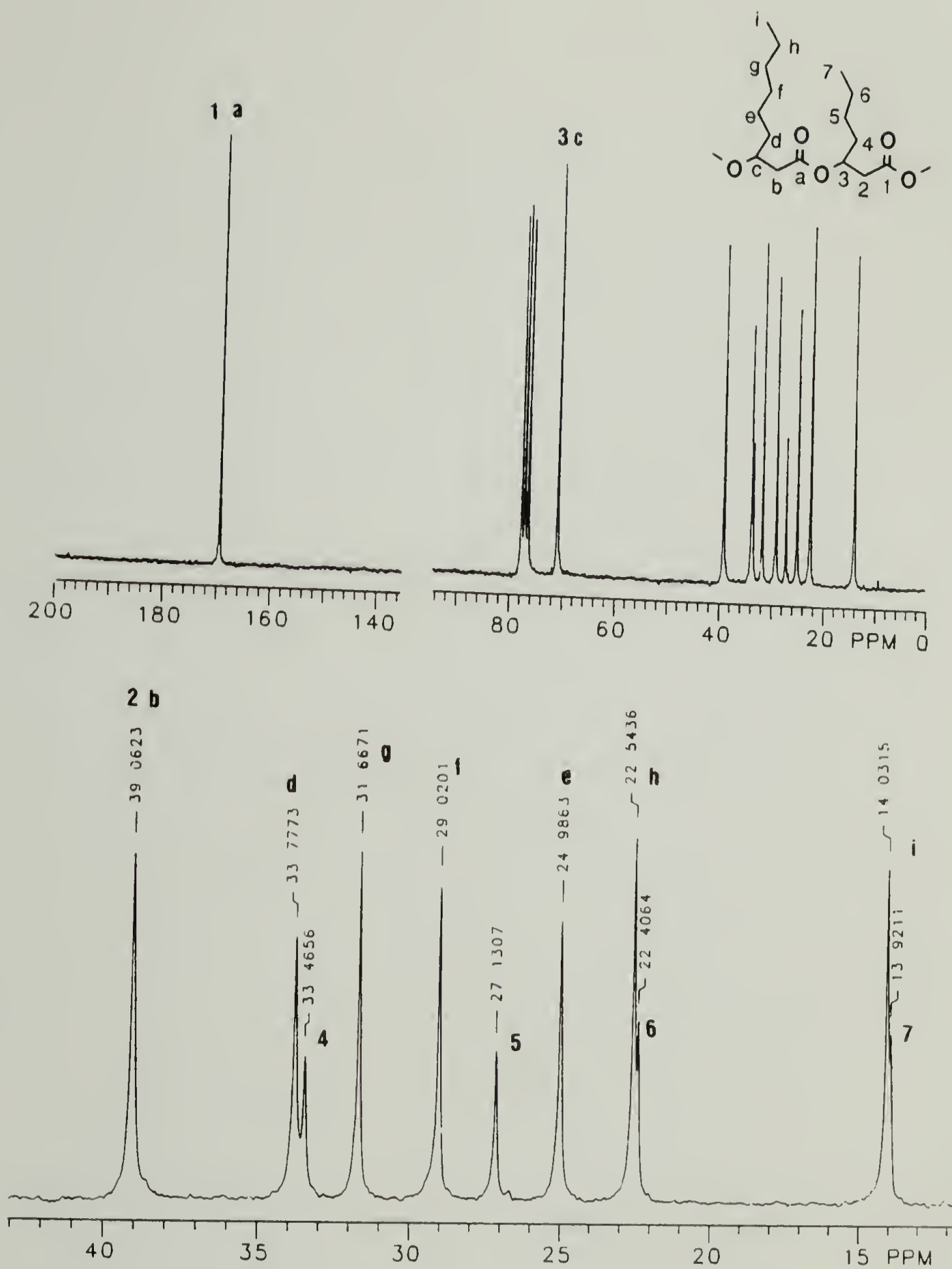


Figure 40.  $^{13}\text{C}$  NMR spectrum of PHA-NON.

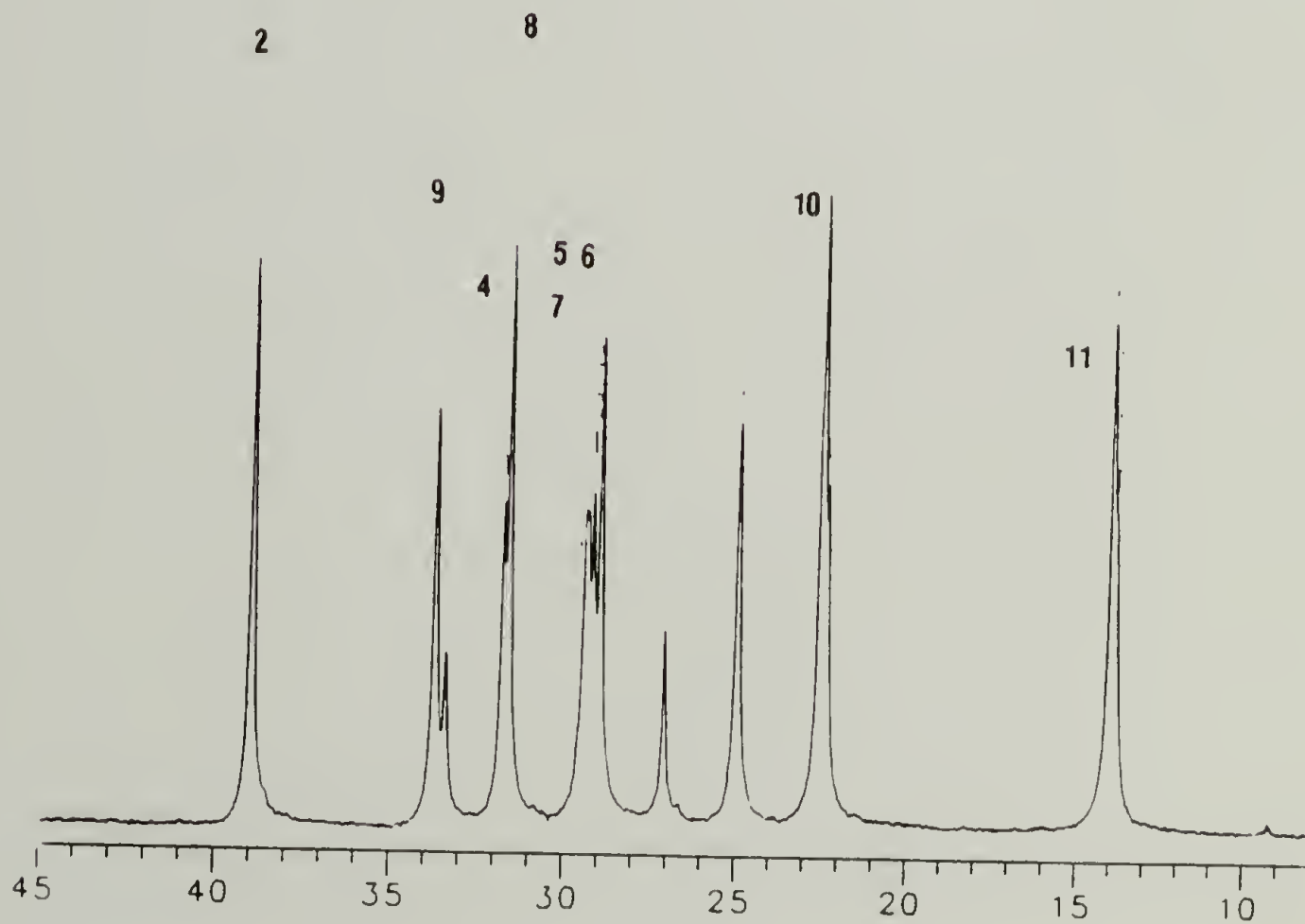
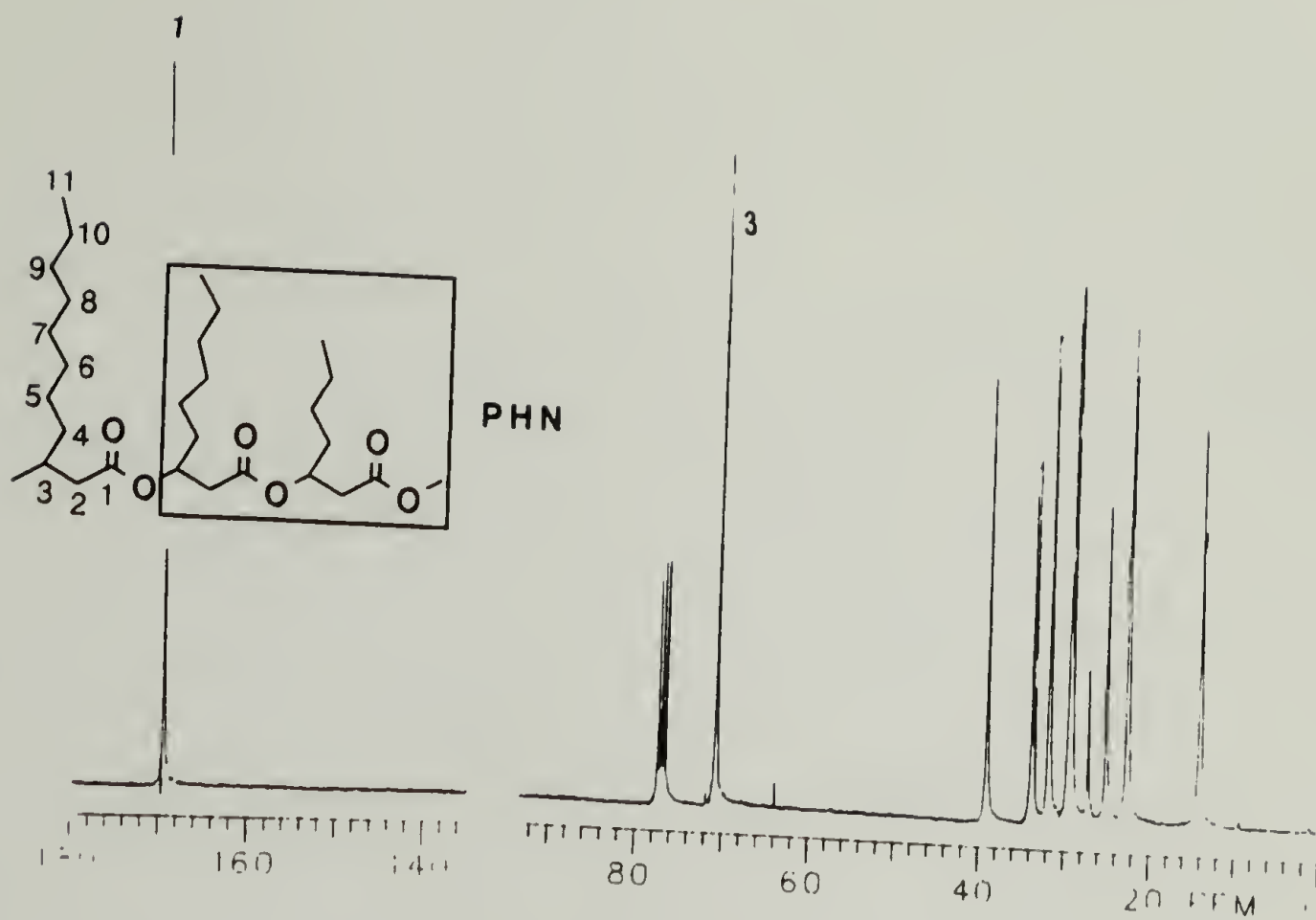


Figure 41.  $^{13}\text{C}$  NMR spectrum of PHA-UND.

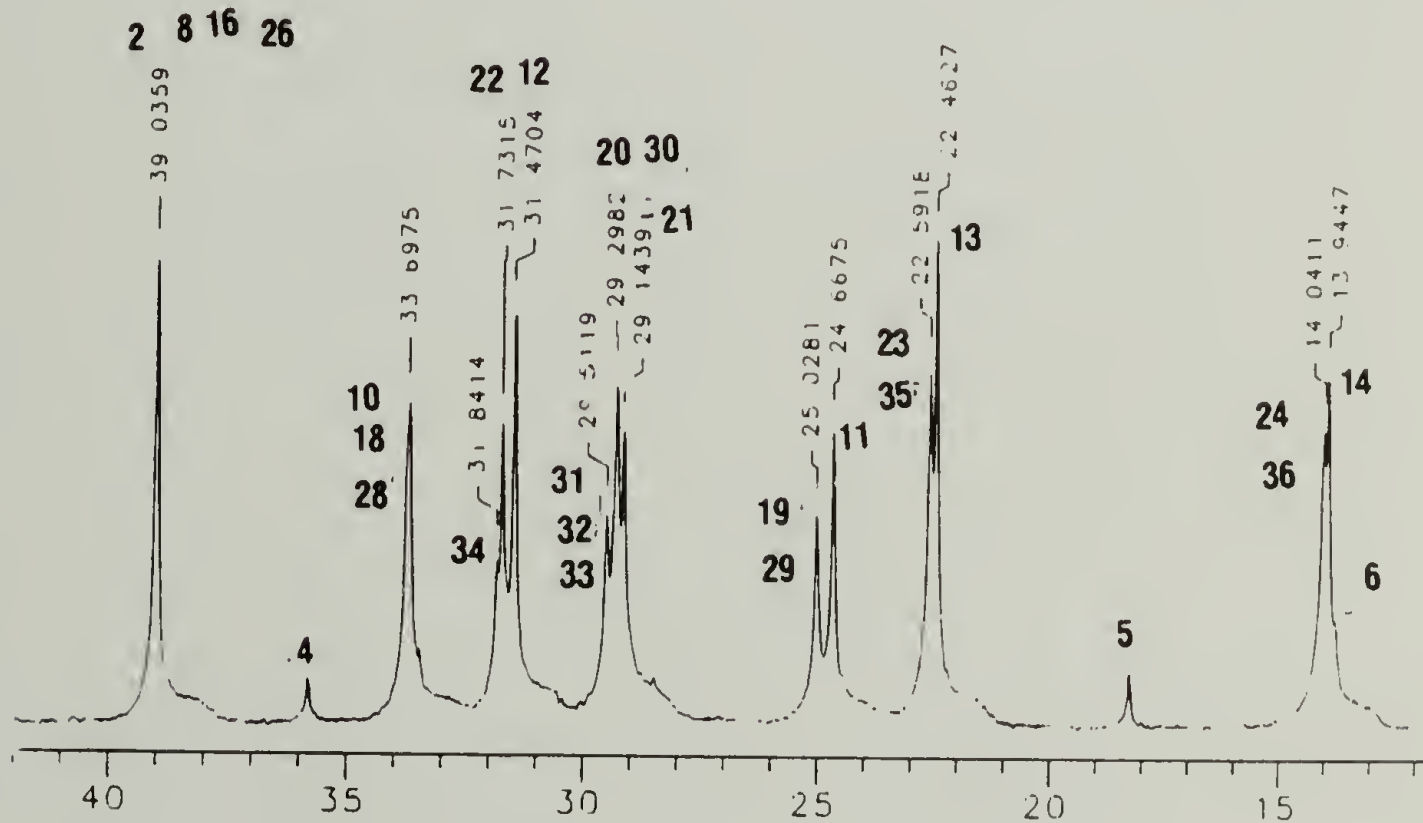
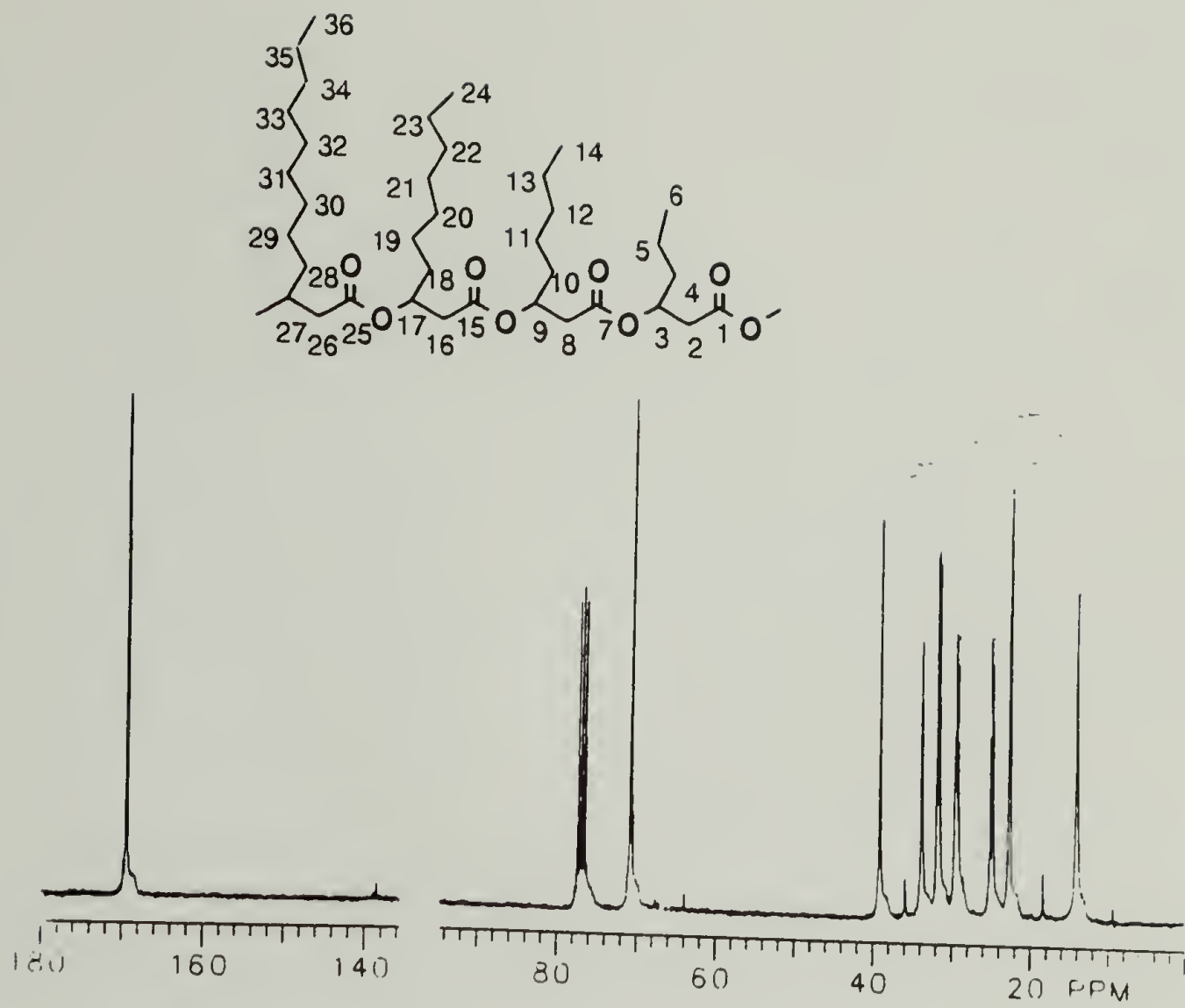


Figure 42.  $^{13}\text{C}$  NMR spectrum of PHA-DOD.



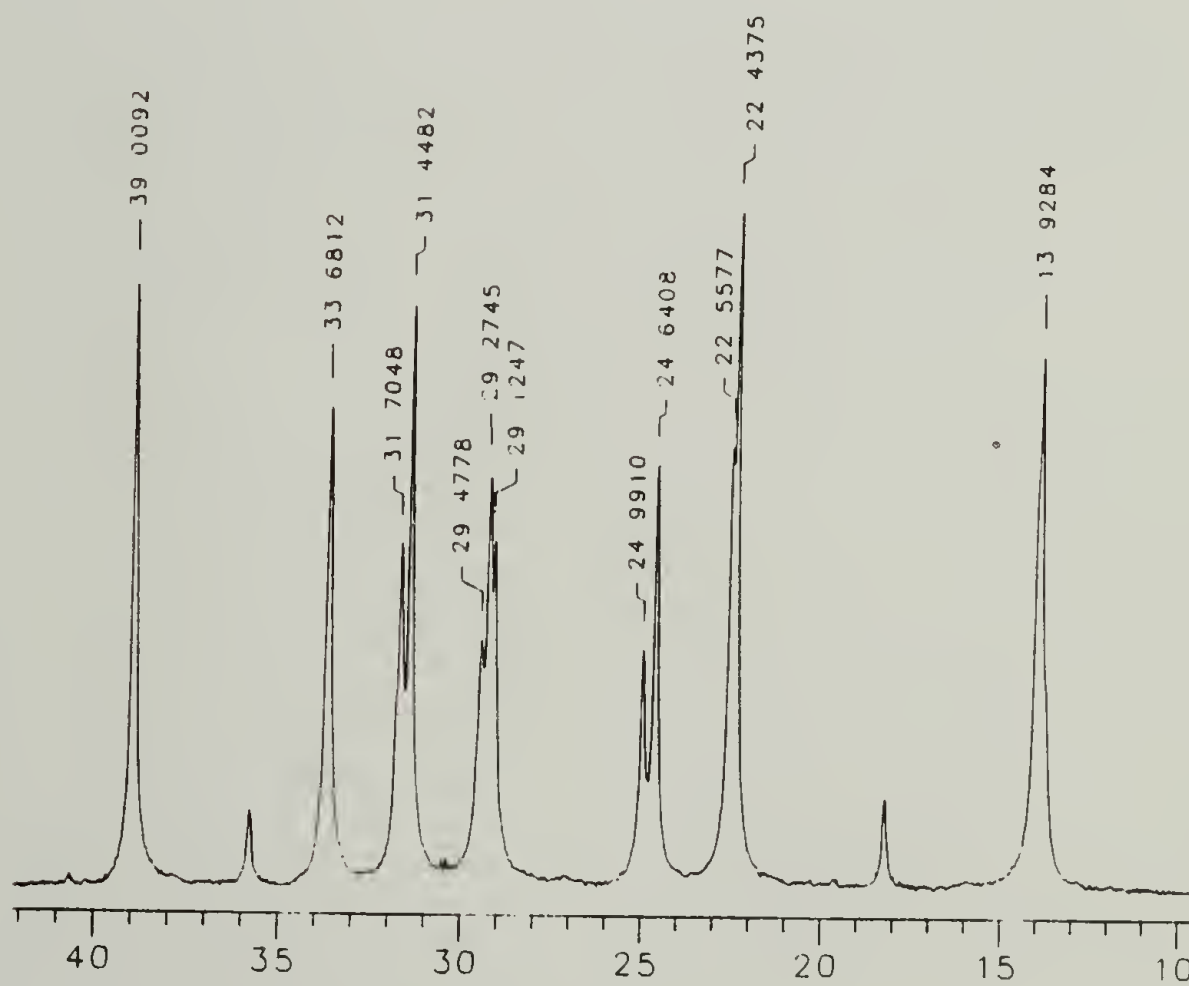
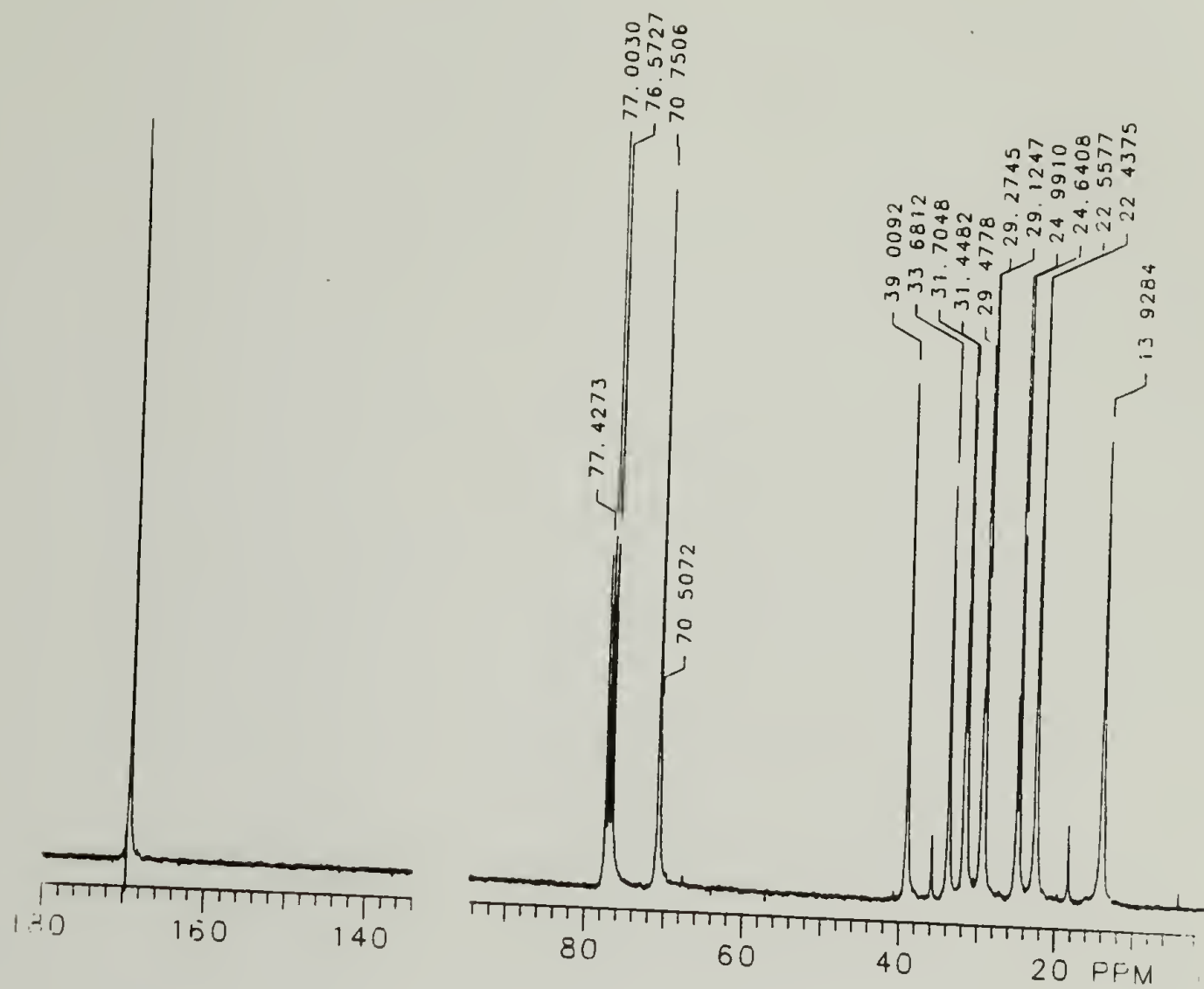


Figure 43.  $^{13}\text{C}$  NMR spectrum of PHA-TET

Table 16. Repeating unit compositions of PHAs produced by *P. oleovorans* grown with 1-alkenes [Lageveen et al., 1988].

Carbon Source	Relative monomer composition of PHA <sup>I</sup> , GC area%							
	C6	C6:	C8	C8:	C9	C9:	C10	C10:
Octene	0.06	0.06	0.39	0.49				
Nonene					0.61	0.39		
Decene			0.33	0.34			0.21	0.12

I, C<sub>n</sub>-saturated units with n carbons

C<sub>n</sub>:-unsaturated units with n carbons

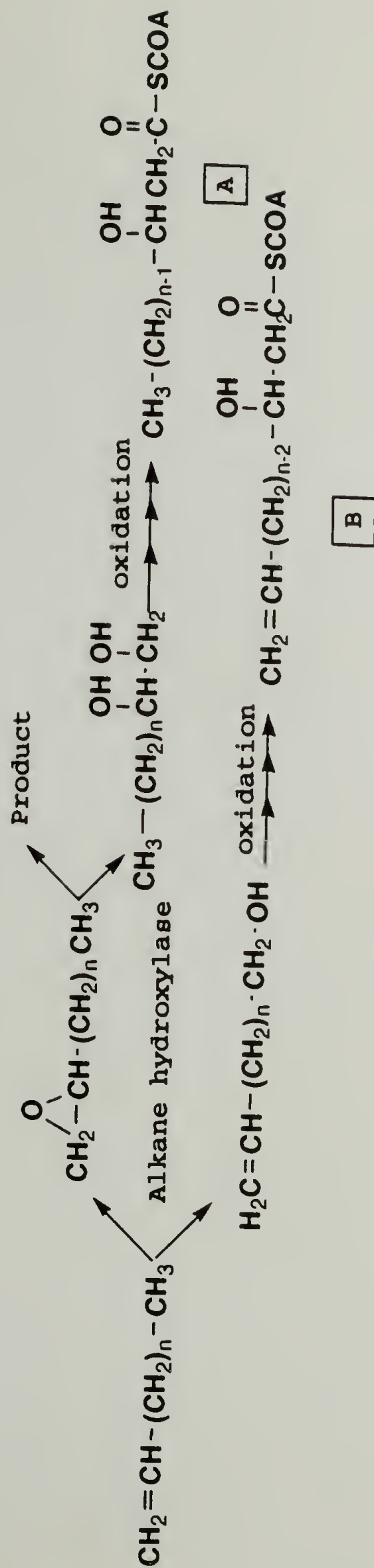


Figure 44. Postulated reactions of 1-alkenes in *P. oleovorans* leading to epoxides and PHA.



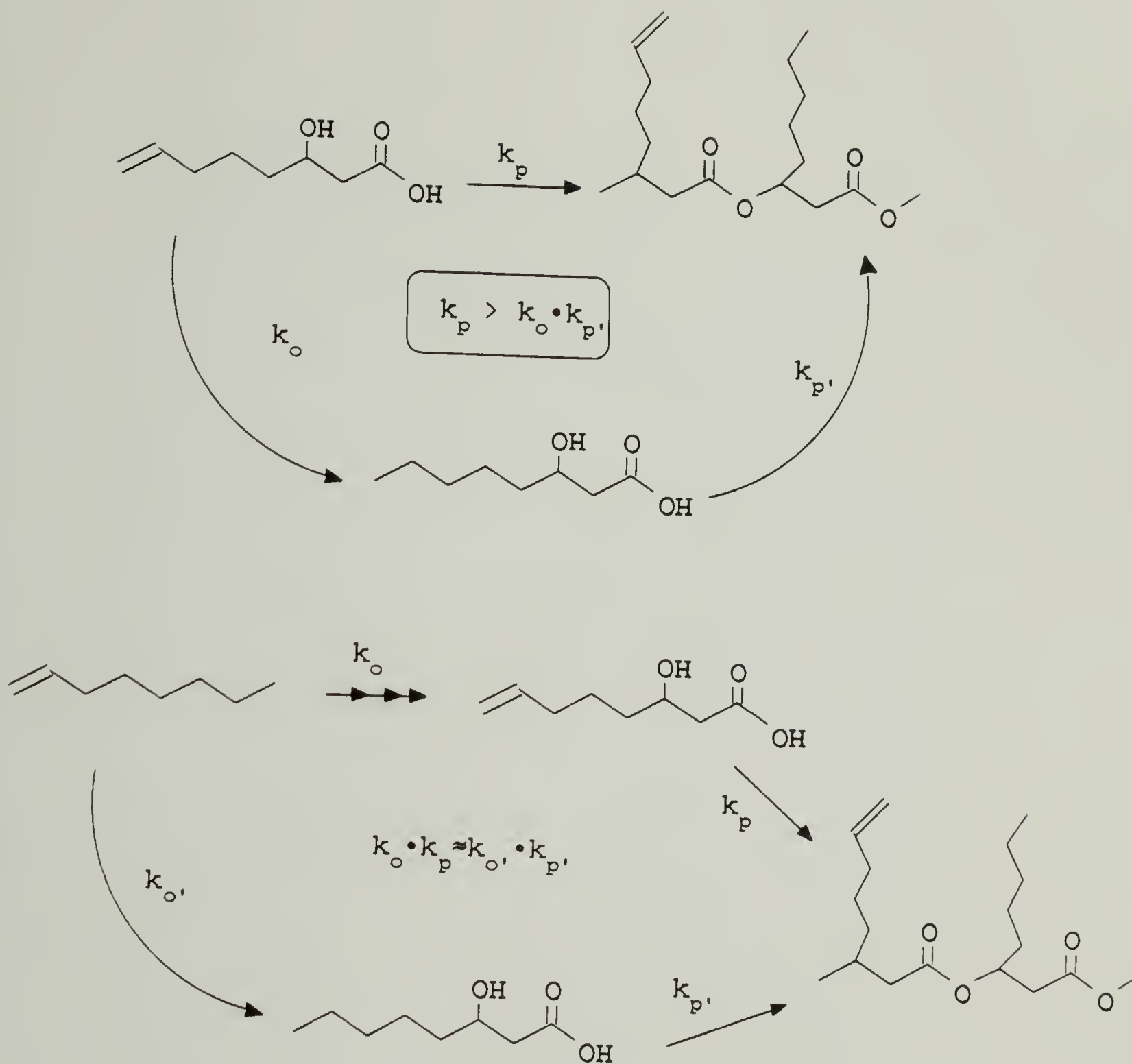


Figure 45. The pathways leading to PHAs from 3-hydroxyalkenoic acids and alkenes.

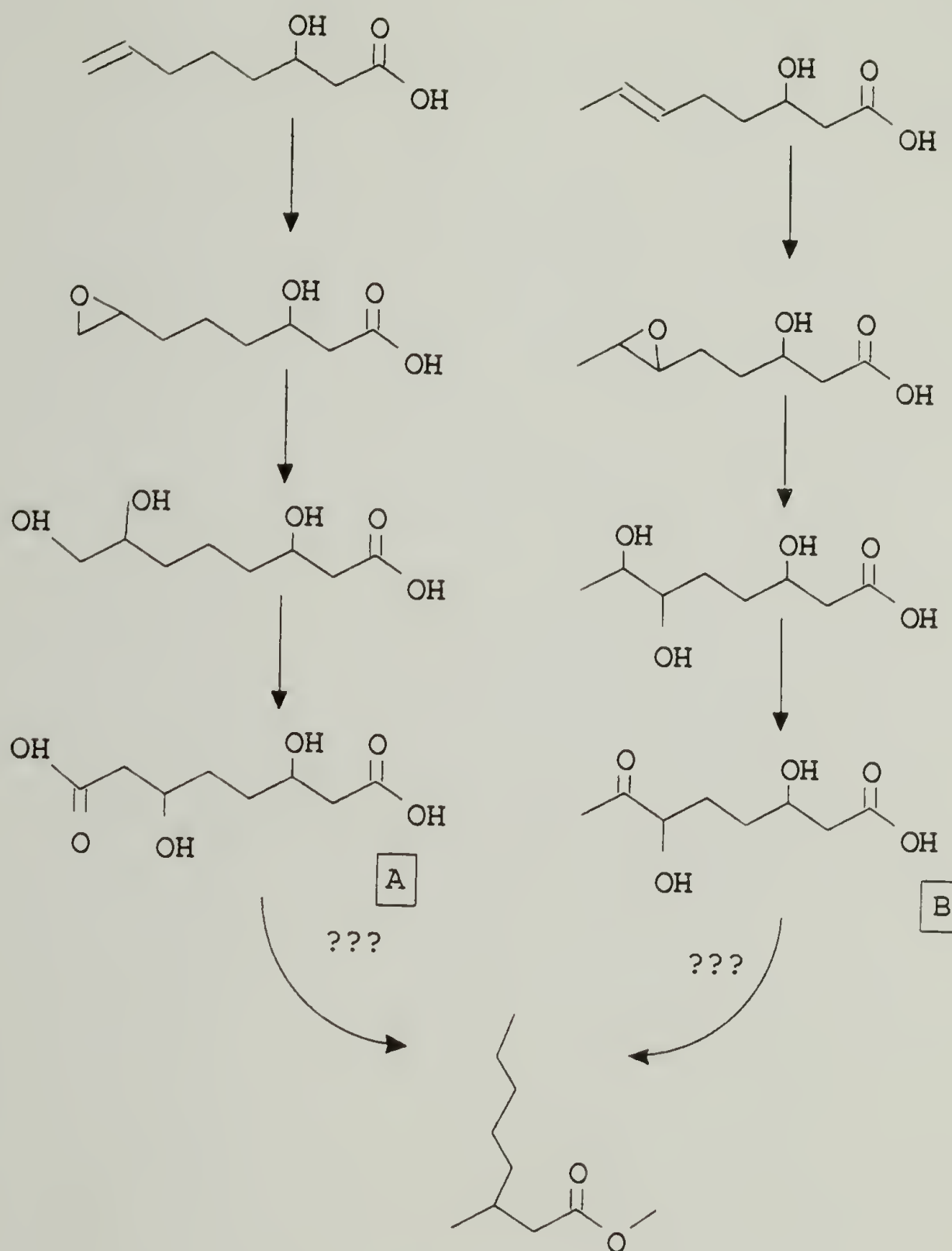


Figure 46. The products of oxidation of 3-hydroxyalkenoic acids following the mechanism in Figure 44.

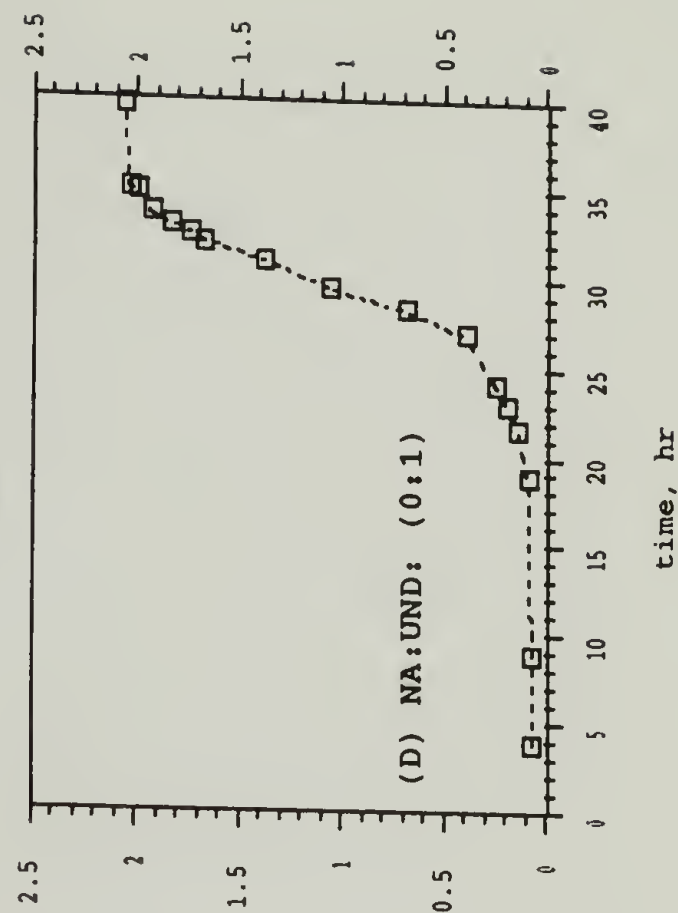
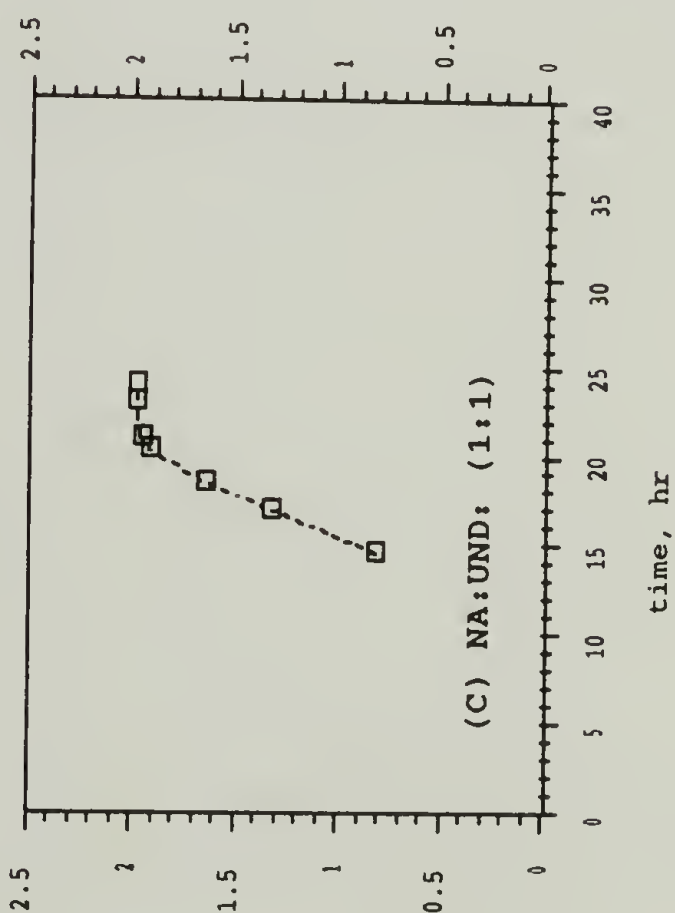
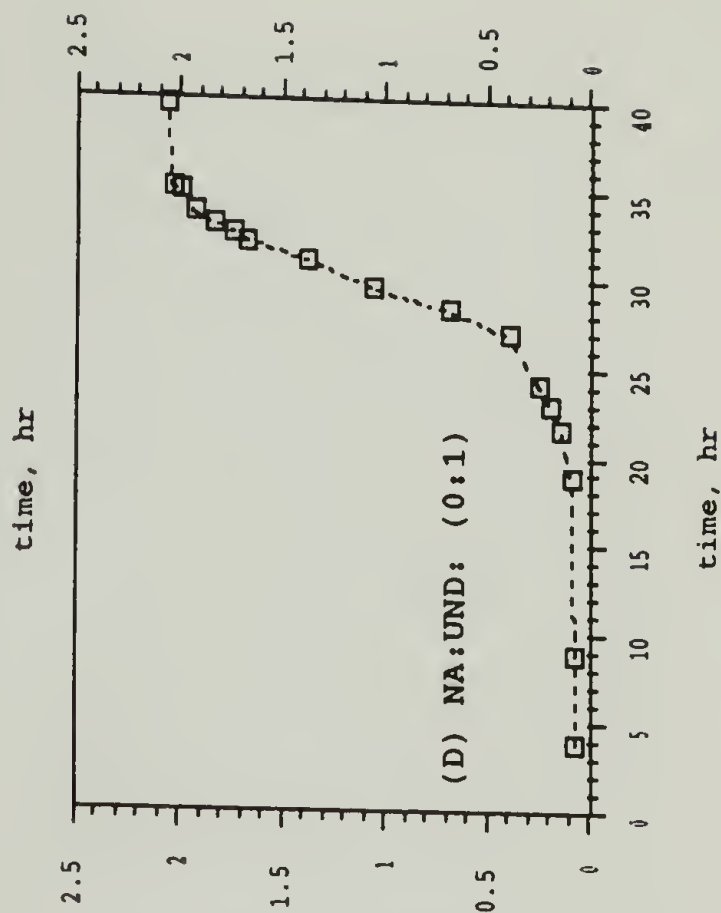
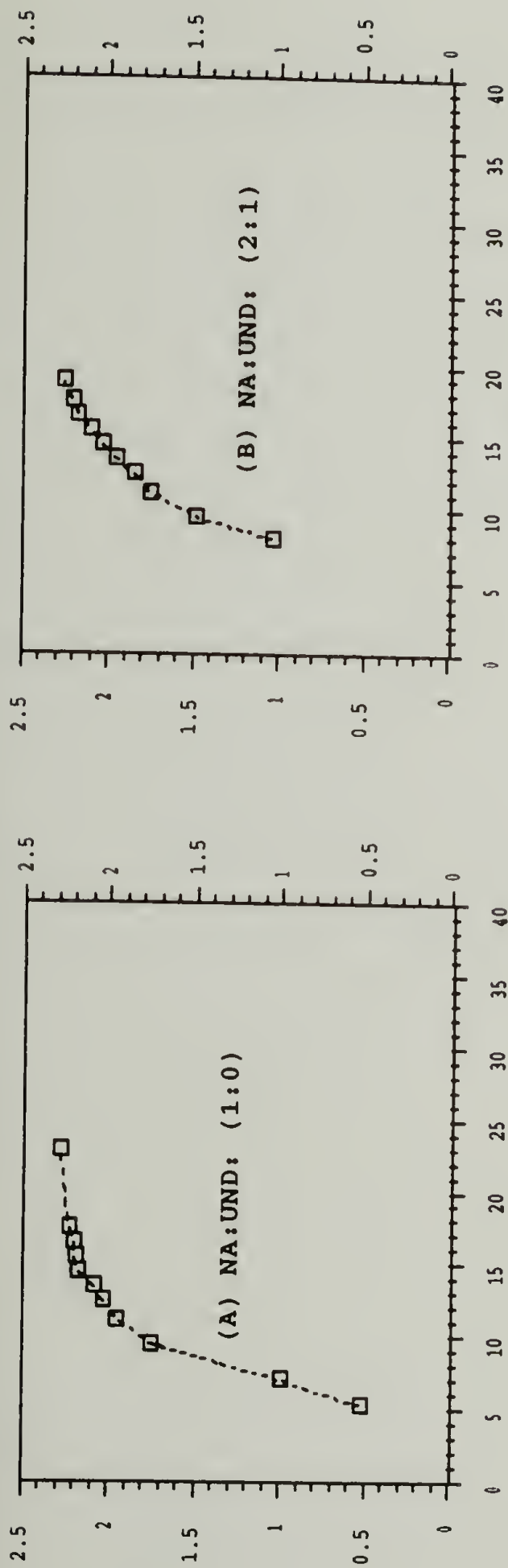


Figure 47. Growth curves of *P. oleovorans* with various mixtures of nonanoic acid, NA, and 10-undecenoic acid, UND:.



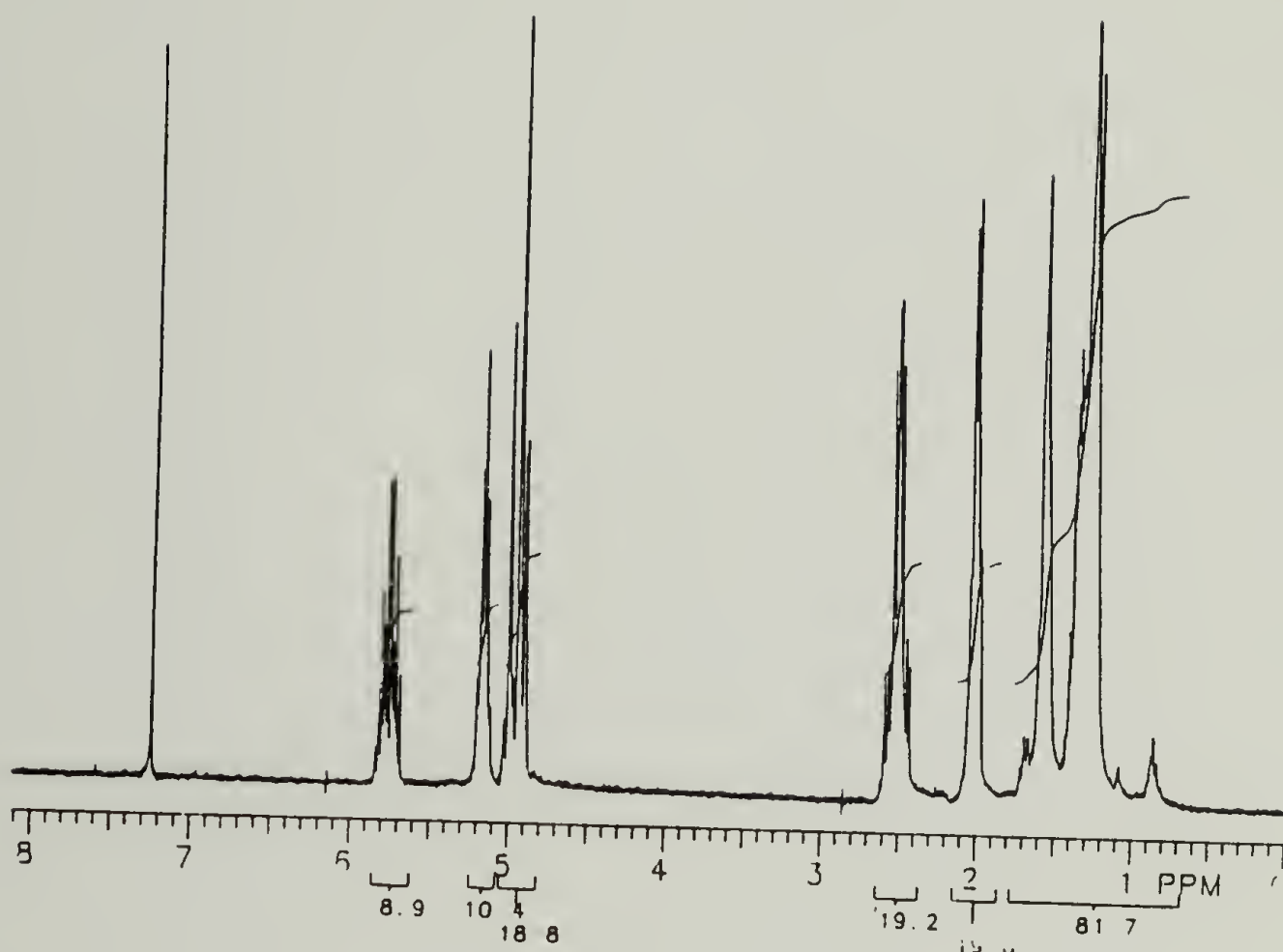


Figure 48.  $^1\text{H}$  NMR spectrum of the PHA produced from 10-undecenoic acid.

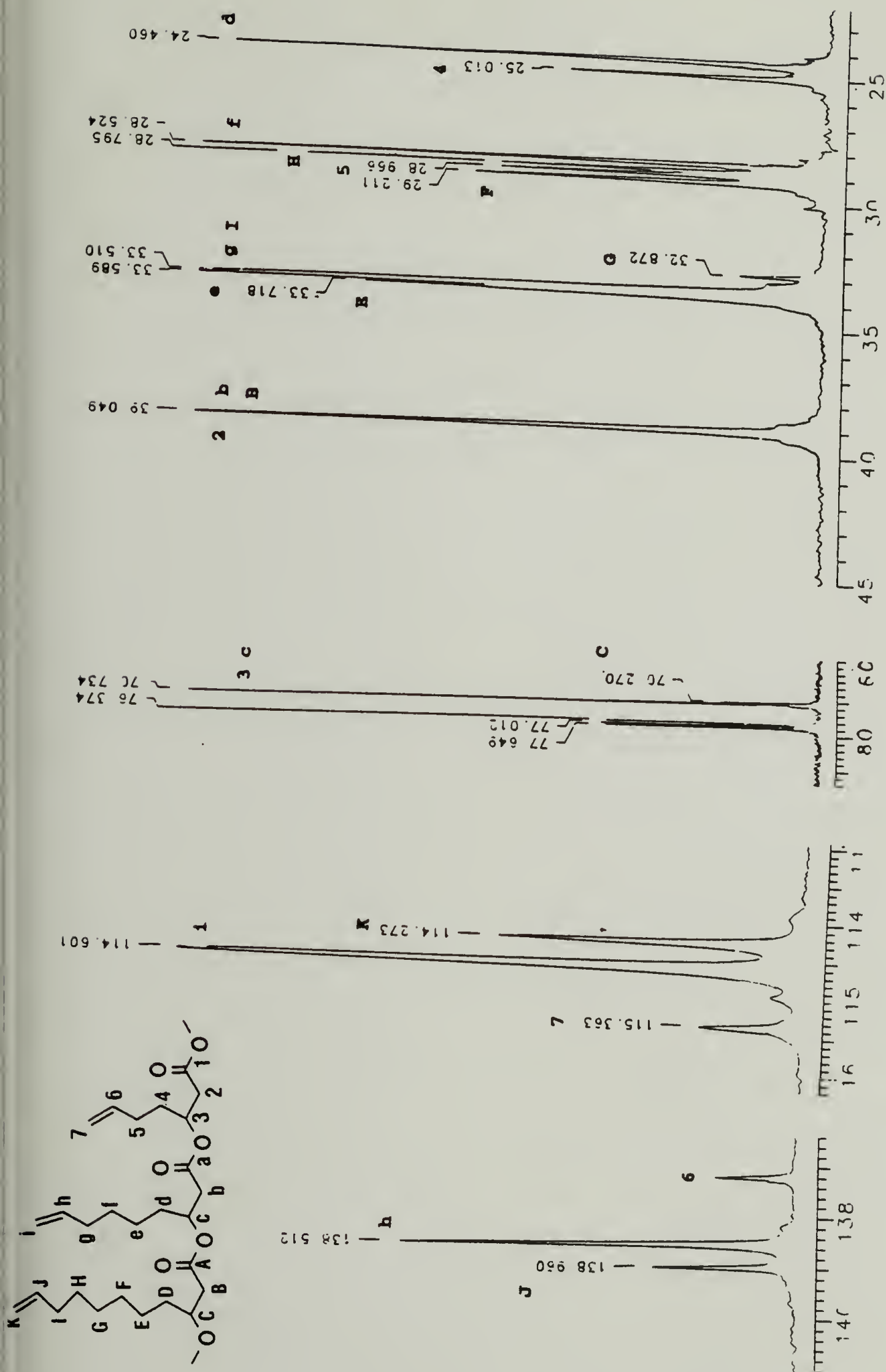


Figure 49.  $^{13}\text{C}$  NMR spectrum of the PHA produced from 10-undecenoic acid.

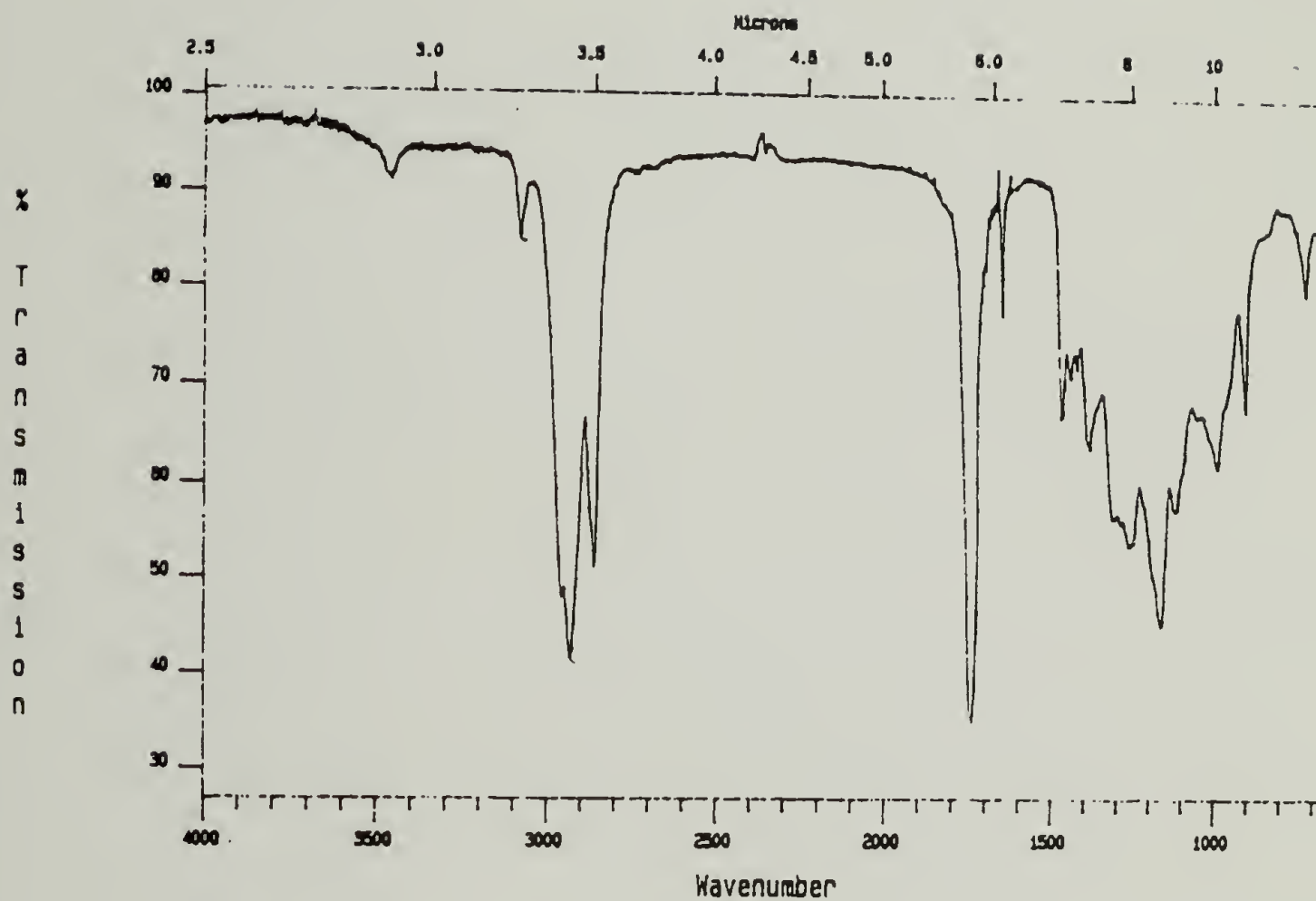


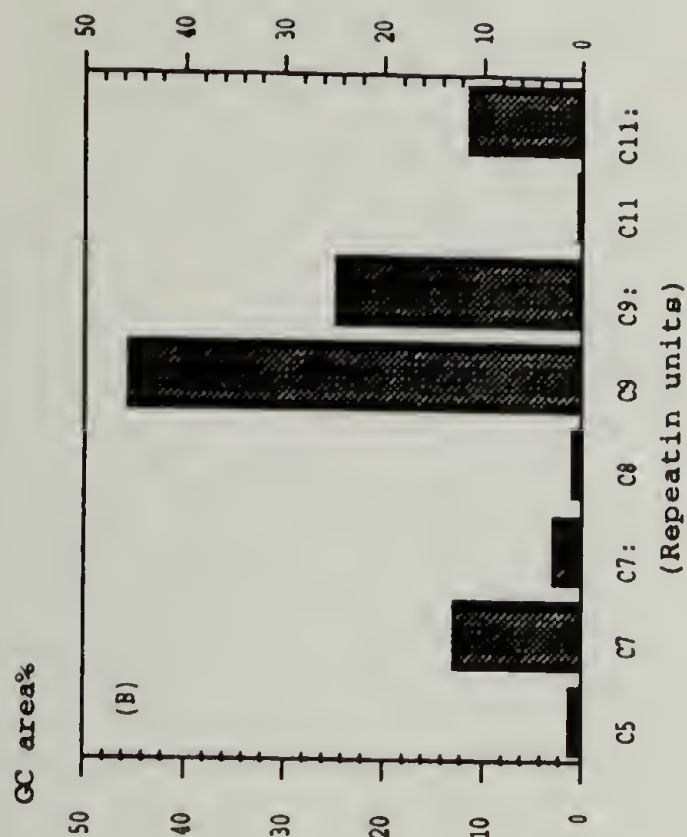
Figure 50. IR spectrum of the PHA produced from 10-undecenoic acid.



Table 17. Biomass yield, PHA yield, and PHA content in the biomass as a function of the ratio of nonanoic acid, NA, to 10-undecenoic acid, UND:.

Carbon Source (NA:UND:) <sup>1</sup>	Biomass (g/l)	PHA (g/l)	PHA Content (%)
1:0	1.10	0.43	39.1
2:1	0.80	0.27	33.8
1:1	0.62	0.20	33.3
0:1	0.60	<0.2	33.3

1, mole:mole



- (A) NA:UND: (1:1)  
 (B) NA:UND: (2:1)  
 (C) OA:UND: (2:1)

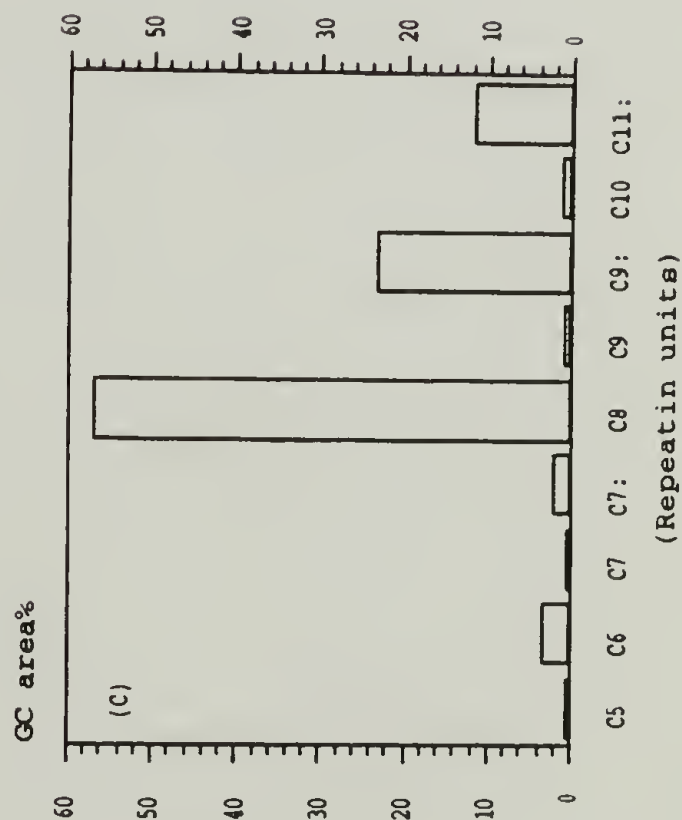
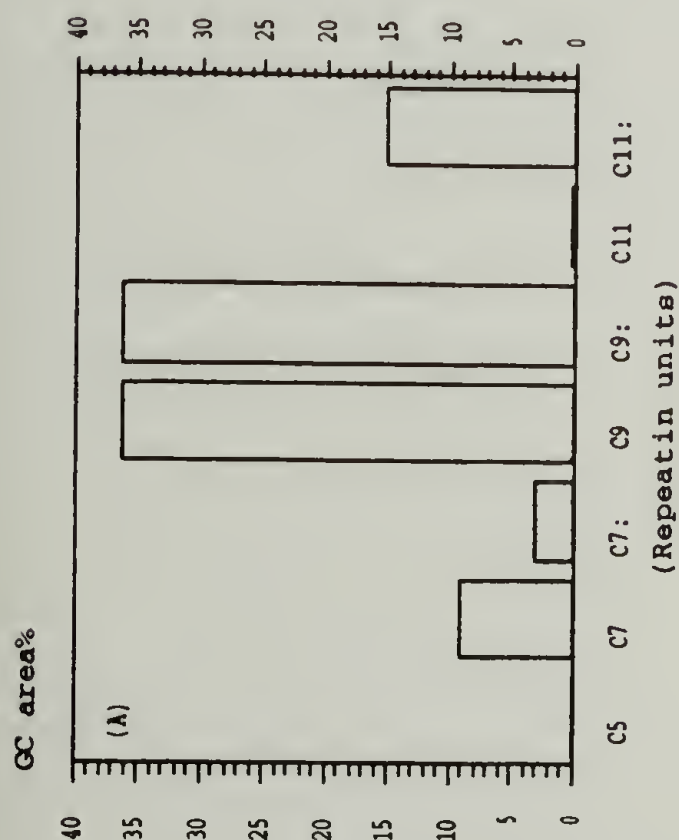


Figure 51. Repeating unit compositions of PHAs produced from various mixtures of 10-undecenoic acid, UND:, and either nonanoic acid, NA, or octanoic acid, OA.

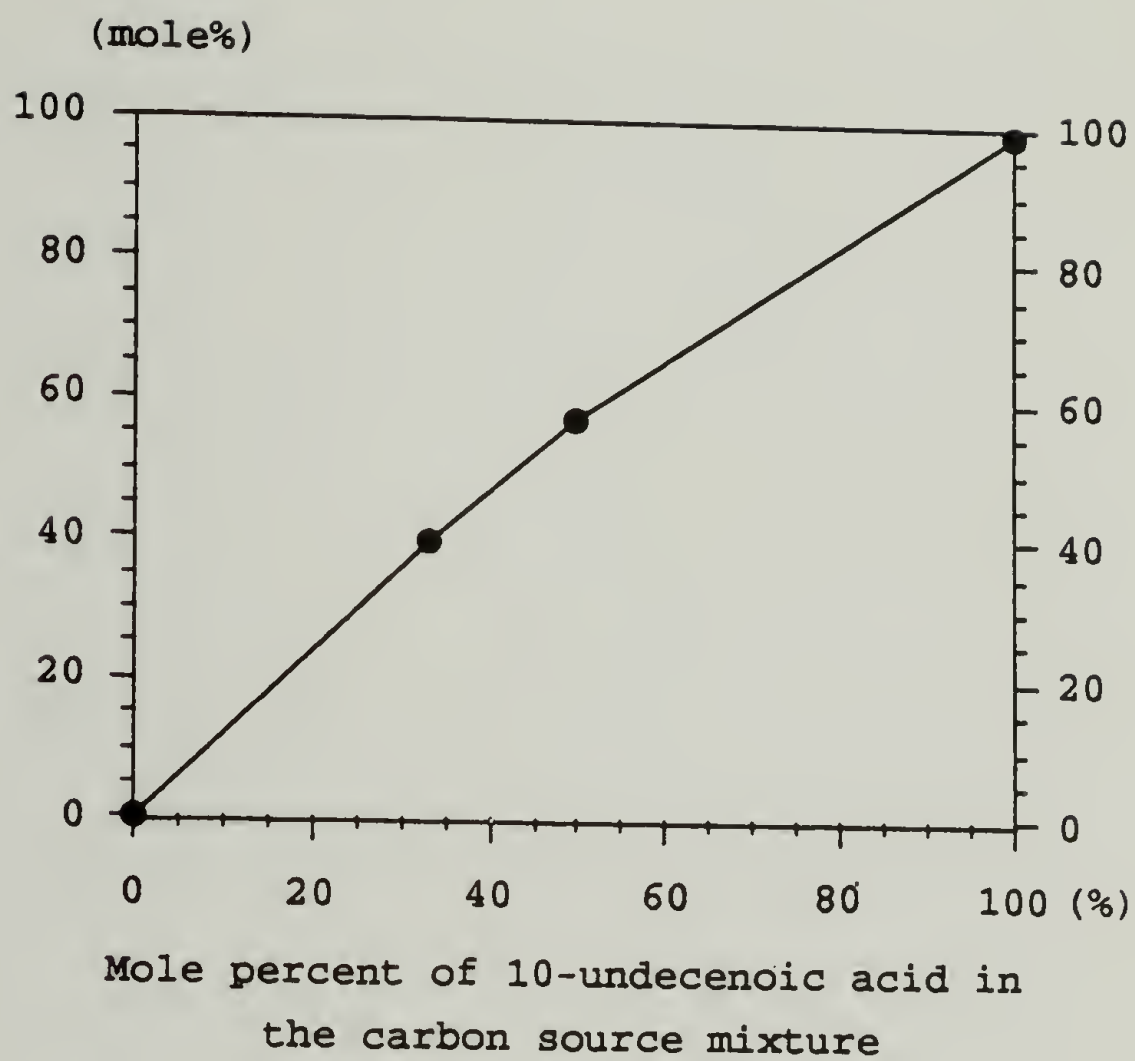


Figure 52. Mole percent of repeating units containing unsaturated units vs. mole percent of 10-undecenoic acid in the carbon source.



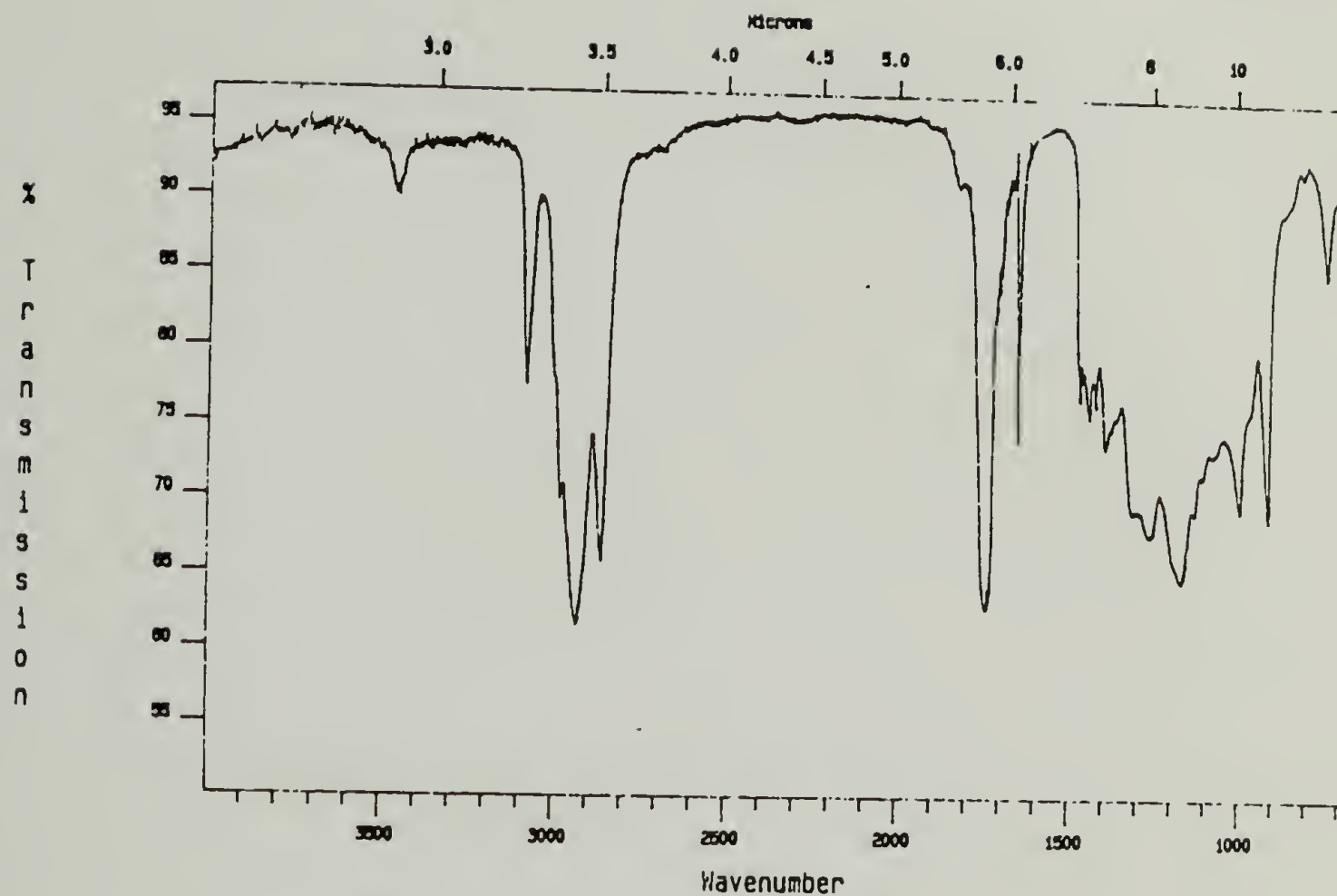


Figure 53. IR spectrum of the PHA produced from a 2:1 mixture of nonanoic acid and 10-undecenoic acid.

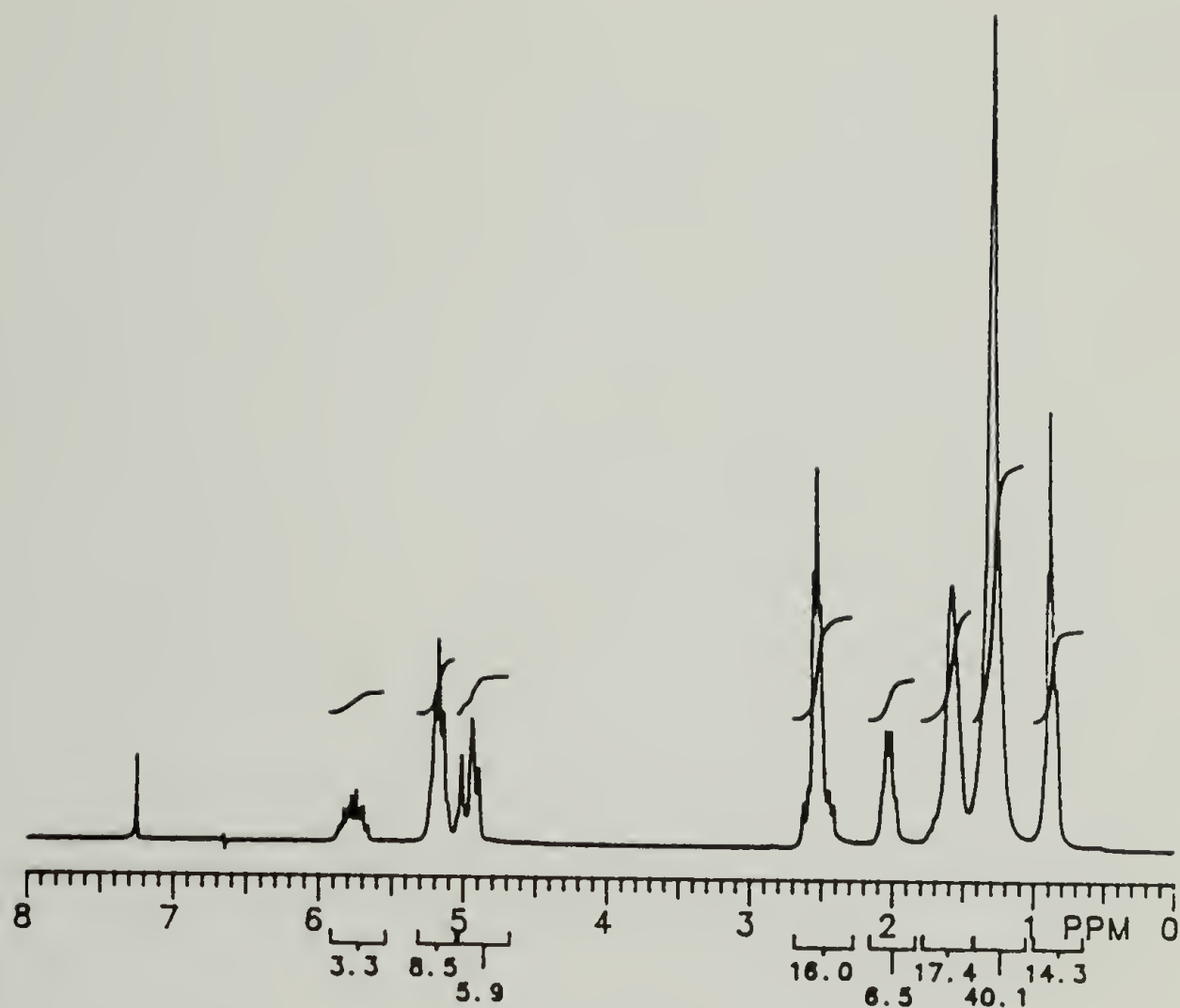


Figure 54. Proton NMR spectrum of the PHA produced from a 2:1 mixture of nonanoic acid and 10-undecenoic acid.

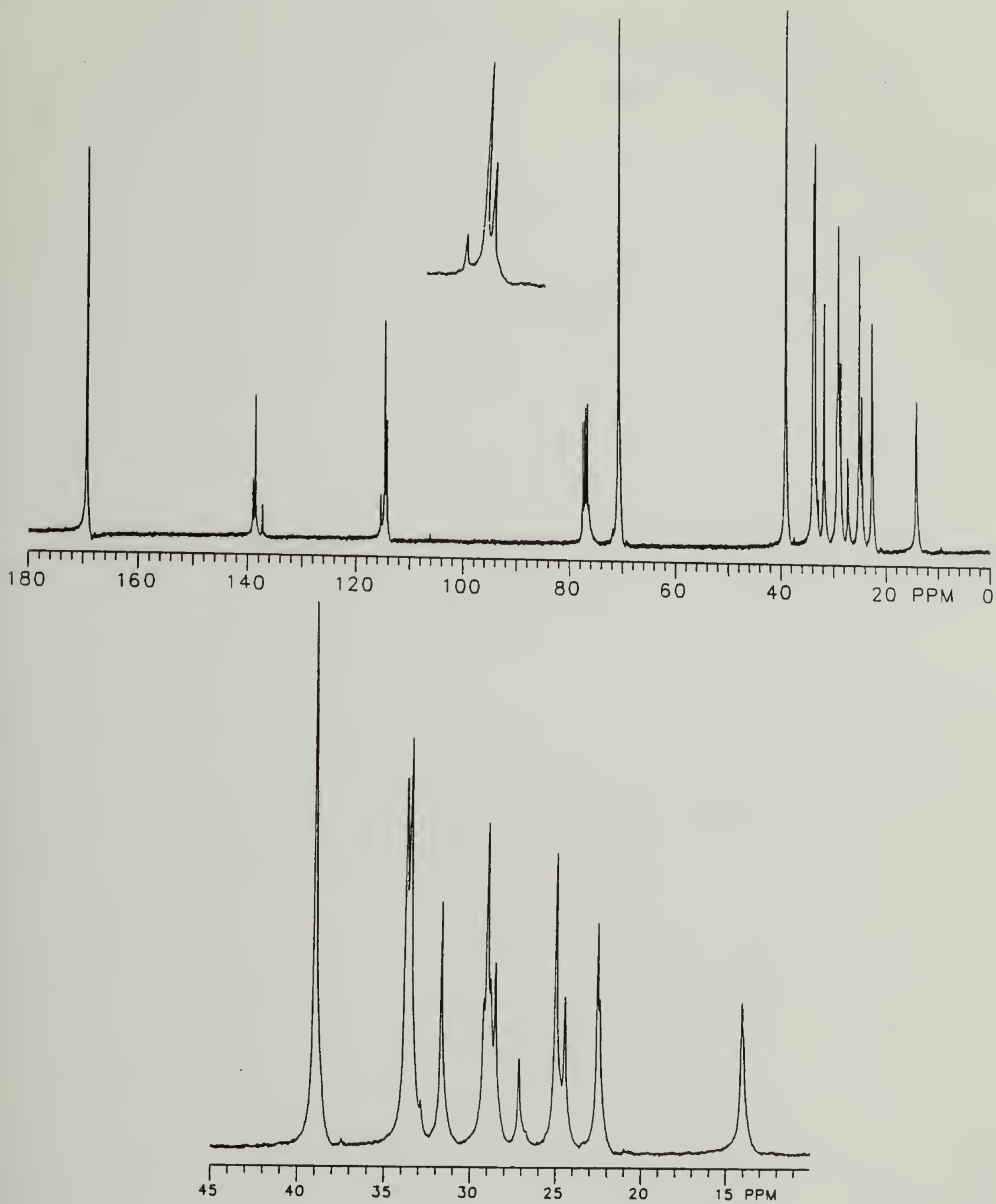


Figure 55.  $^{13}\text{C}$  NMR spectrum of the PHA produced from a 2:1 mixture of nonanoic acid and 10-undecenoic acid.





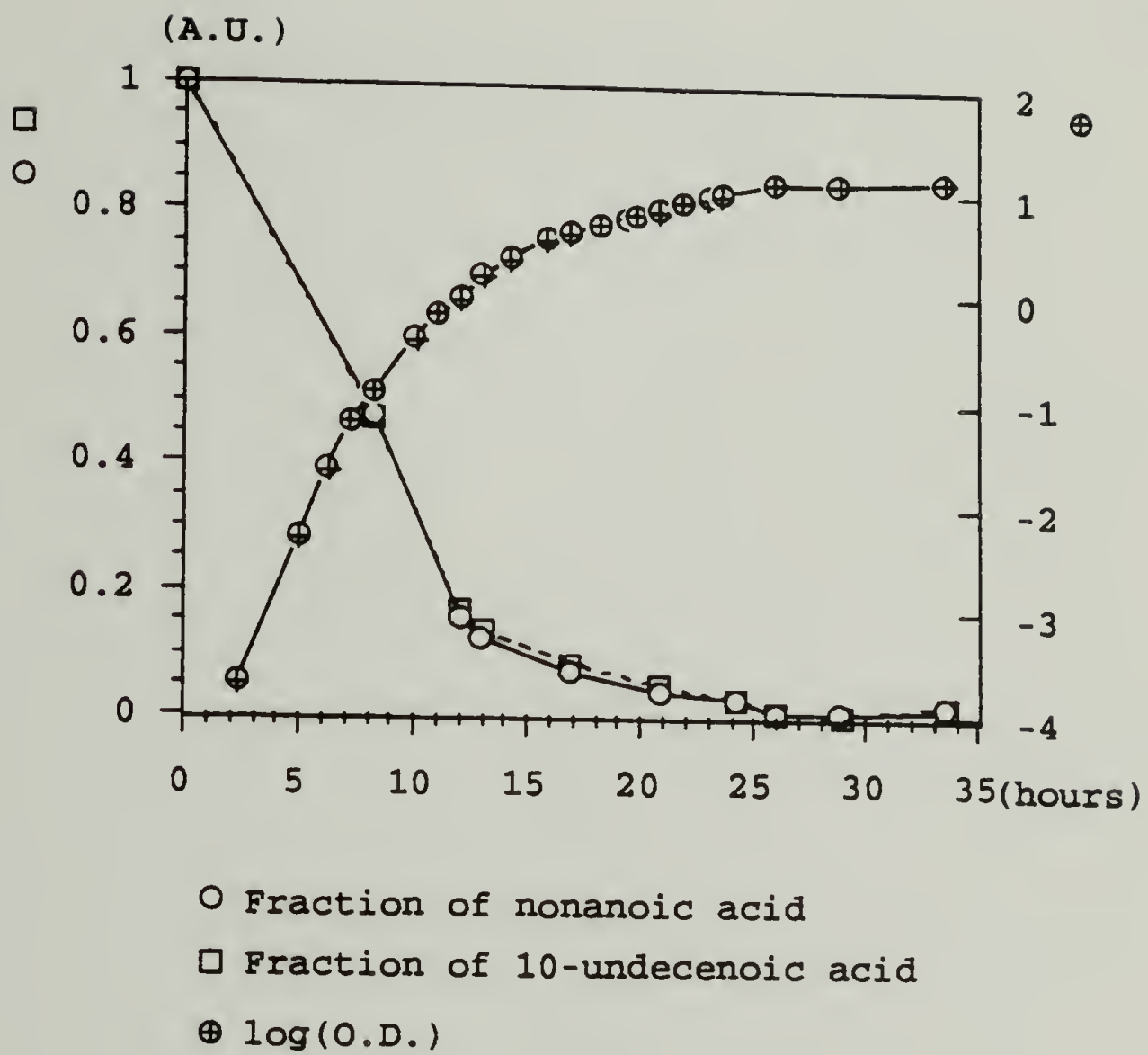


Figure 57. Log(O.D.), fraction of nonanoic acid and 10-undecenoic acid remaining in the medium vs. growth time.

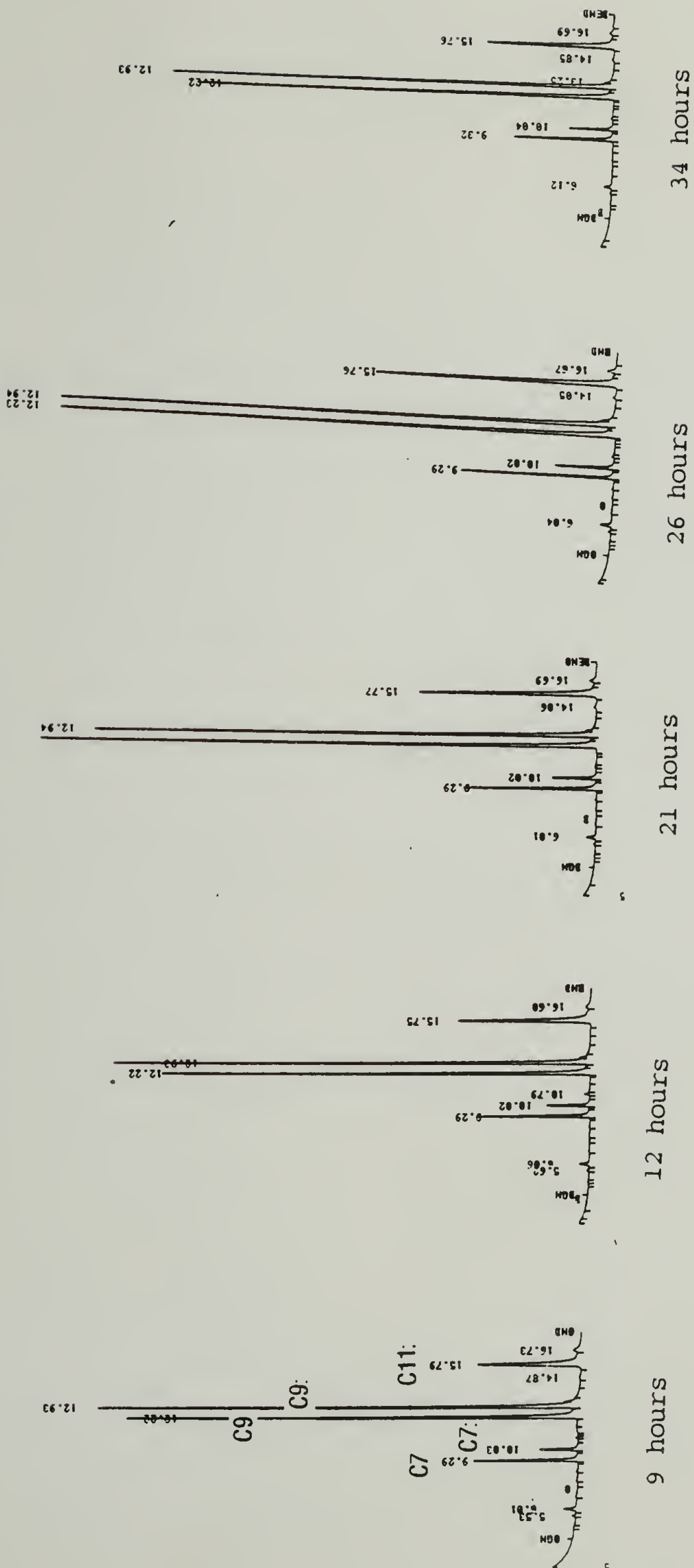




Table 18. Variation in compositions of PHAs isolated from cells grown with an equimolar mixture of nonanoic acid and 10-undecenoic acid with respect to time.

Repeating	Growth time, hr									
	unit	8.2	12.0	13.0	16.8	20.8	24.2	26.0	28.8	33.5
C5		1.30	1.34	1.05	0.96	0.88	0.63	0.84	1.11	0.90
C7		7.85	8.58	8.26	7.92	8.65	7.99	7.92	8.06	8.32
C7:		2.74	3.08	3.22	2.97	2.77	3.19	3.00	2.78	3.42
C8	-I	0.21	0.39	-	-	-	-	-	-	-
C9		34.87	34.58	34.48	34.49	36.12	34.99	34.99	35.15	34.82
C9:		34.81	33.79	34.24	34.15	32.29	33.87	33.81	33.87	35.04
C11	-	-	-	-	-	0.34	-	0.32	0.45	0.02
C11:		18.43	18.42	18.35	19.51	18.96	19.32	19.12	18.58	17.47

I, not measurable amount

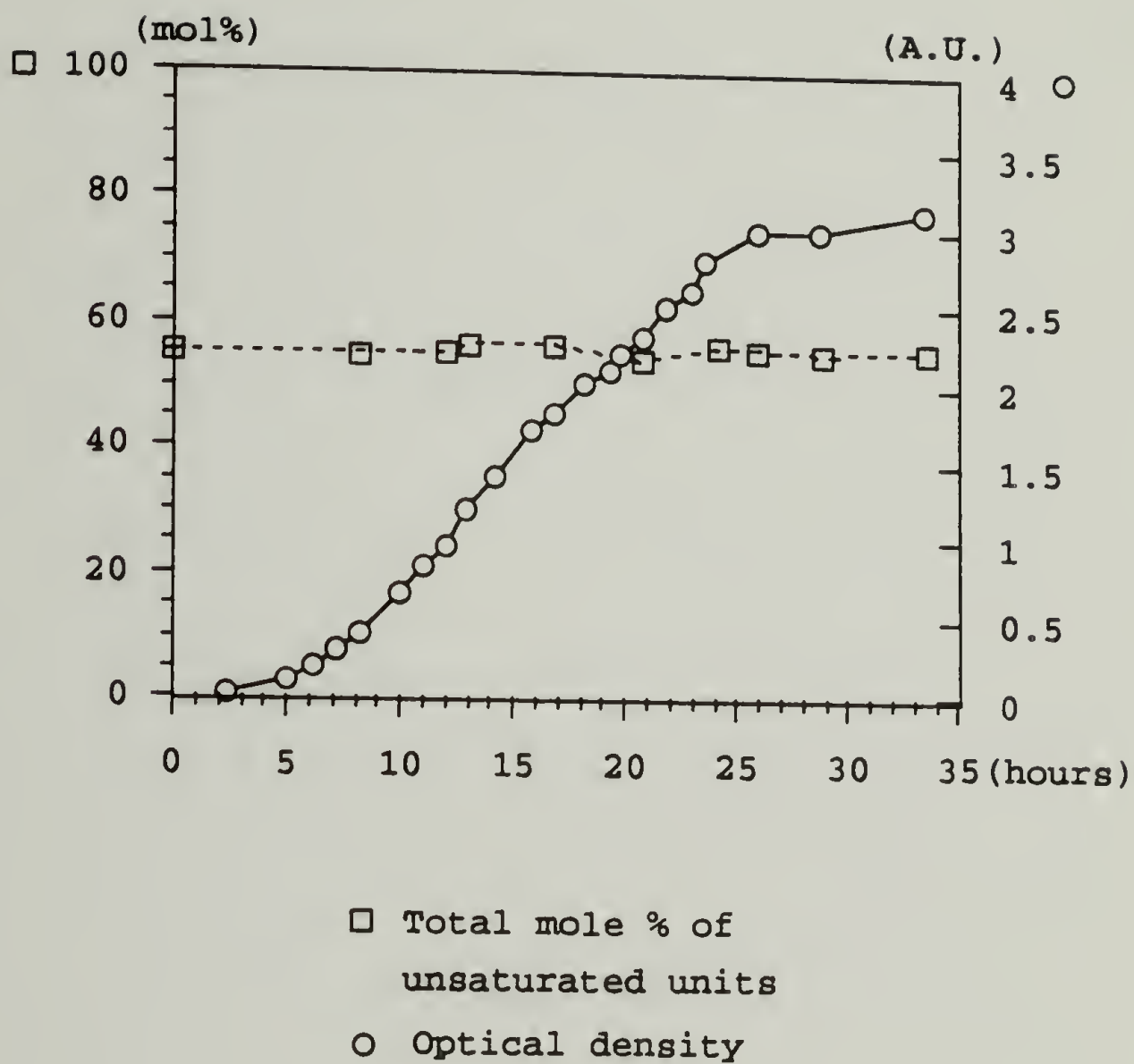
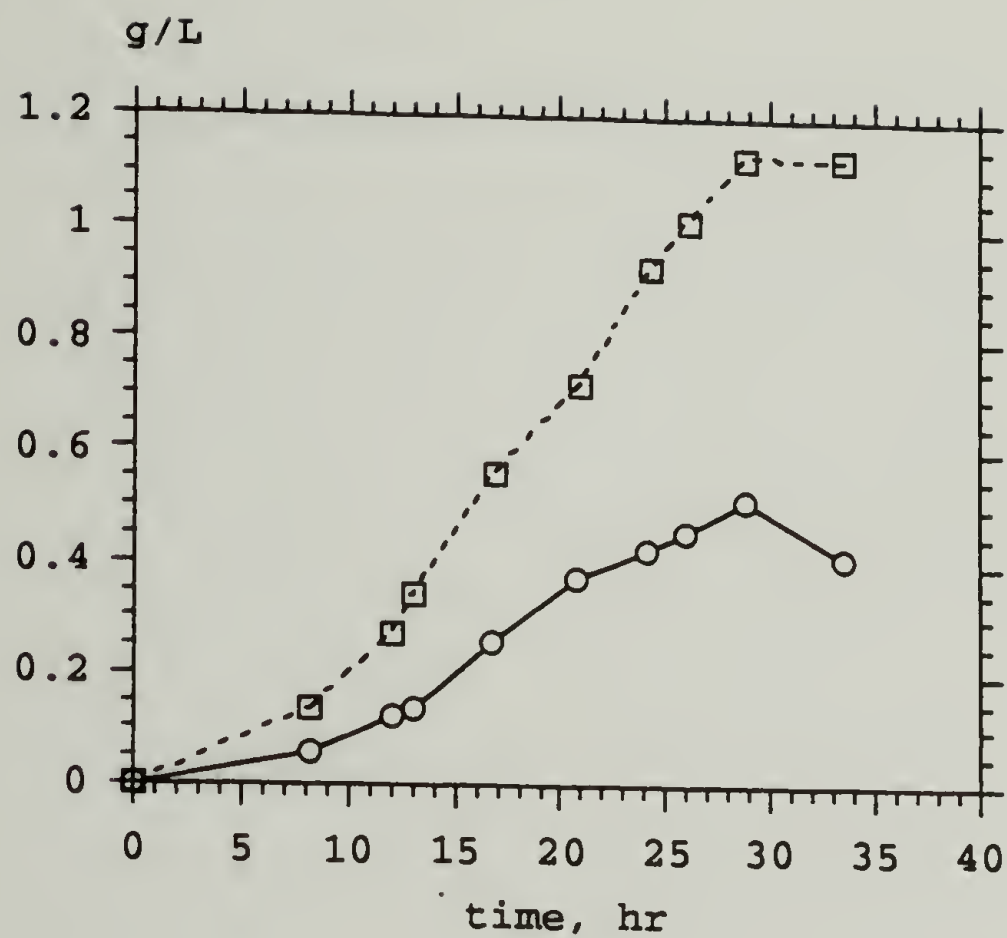


Figure 59. Growth curve of *P. oleovorans* grown with an equimolar mixture of nonanoic acid and 10-undecenoic acid, and total mole percent of unsaturated units in the PHA.



--□--biomass (g/L)  
 —○—PHA (g/L)

Figure 60. Biomass yield and PHA yield produced by *P. oleovorans* grown with an equimolar mixture of nonanoic acid and 10-undecenoic acid vs. growth time.



Table 19. Relative amount of repeating units in PHAs obtained with nonanoic acid and 10-undecenoic acid and isolated from cells grown for different period of time.

Repeating unit <sup>I</sup>	Growth time, hr									
	8.2	12.0	13.0	16.8	20.8	24.2	26.0	28.8	33.5	
C5	2.96	3.02	2.41	2.20	1.94	1.45	1.91	2.51	2.05	
C7	17.83	19.28	18.87	18.27	18.94	18.33	18.11	18.19	18.90	
C9	79.20	77.70	78.73	79.53	79.12	80.22	79.98	79.31	79.05	
C7:	4.90	5.57	5.78	5.25	5.12	5.66	5.37	5.03	6.12	
C9:	62.18	61.11	61.35	60.30	59.78	60.07	60.45	61.33	62.65	
C11:	32.92	33.32	32.87	34.45	35.10	34.27	34.19	33.64	31.23	

I, Cn represents 3-hydroxyalkanoate unit containing n carbon atoms; Cn: represents 3-hydroxyalkenoate unit containing n carbon atoms.

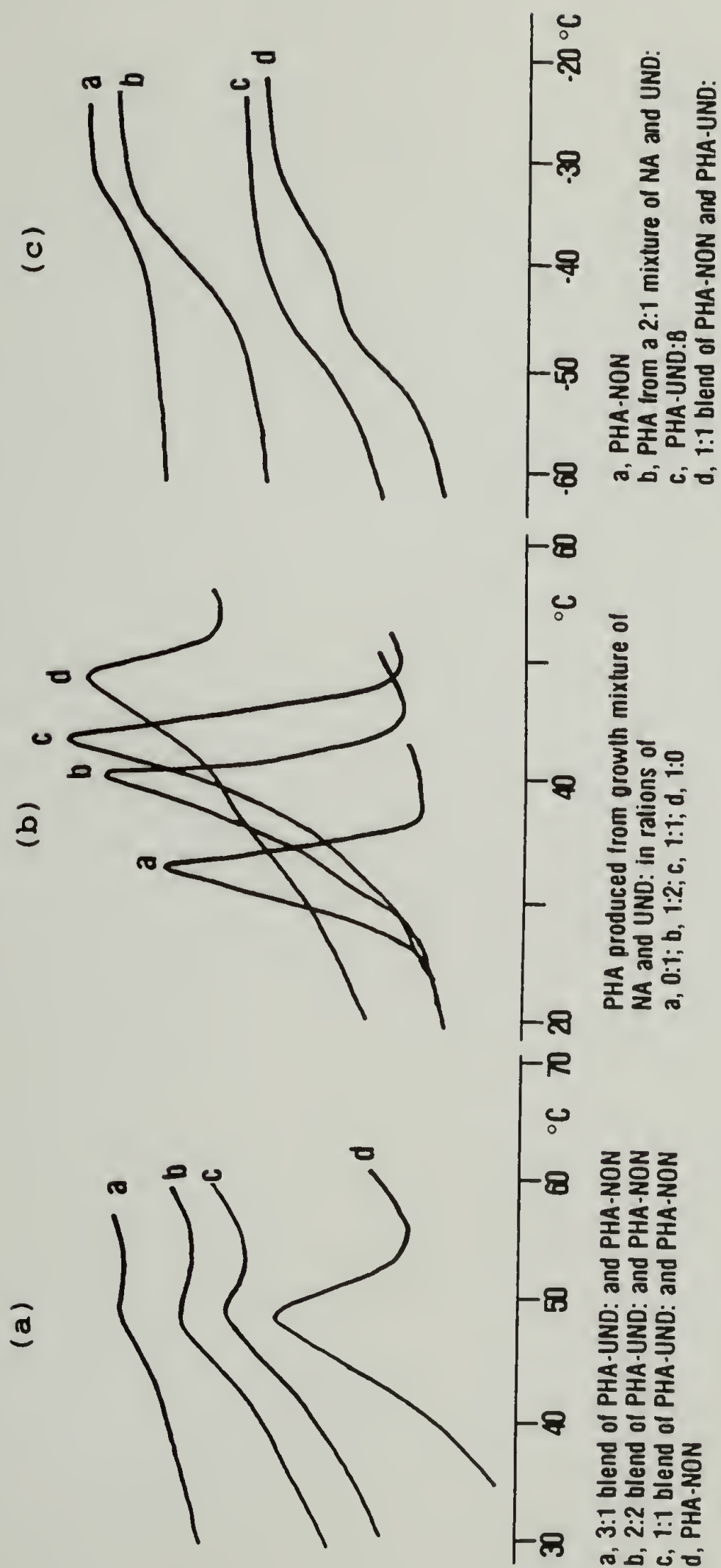


Figure 61. DSC thermograms of PHA-NON and PHA-UND: and their blends and PHAs produced from growth mixtures of nonanoic acid, NA, and 10-undecenoic acid, UND:.

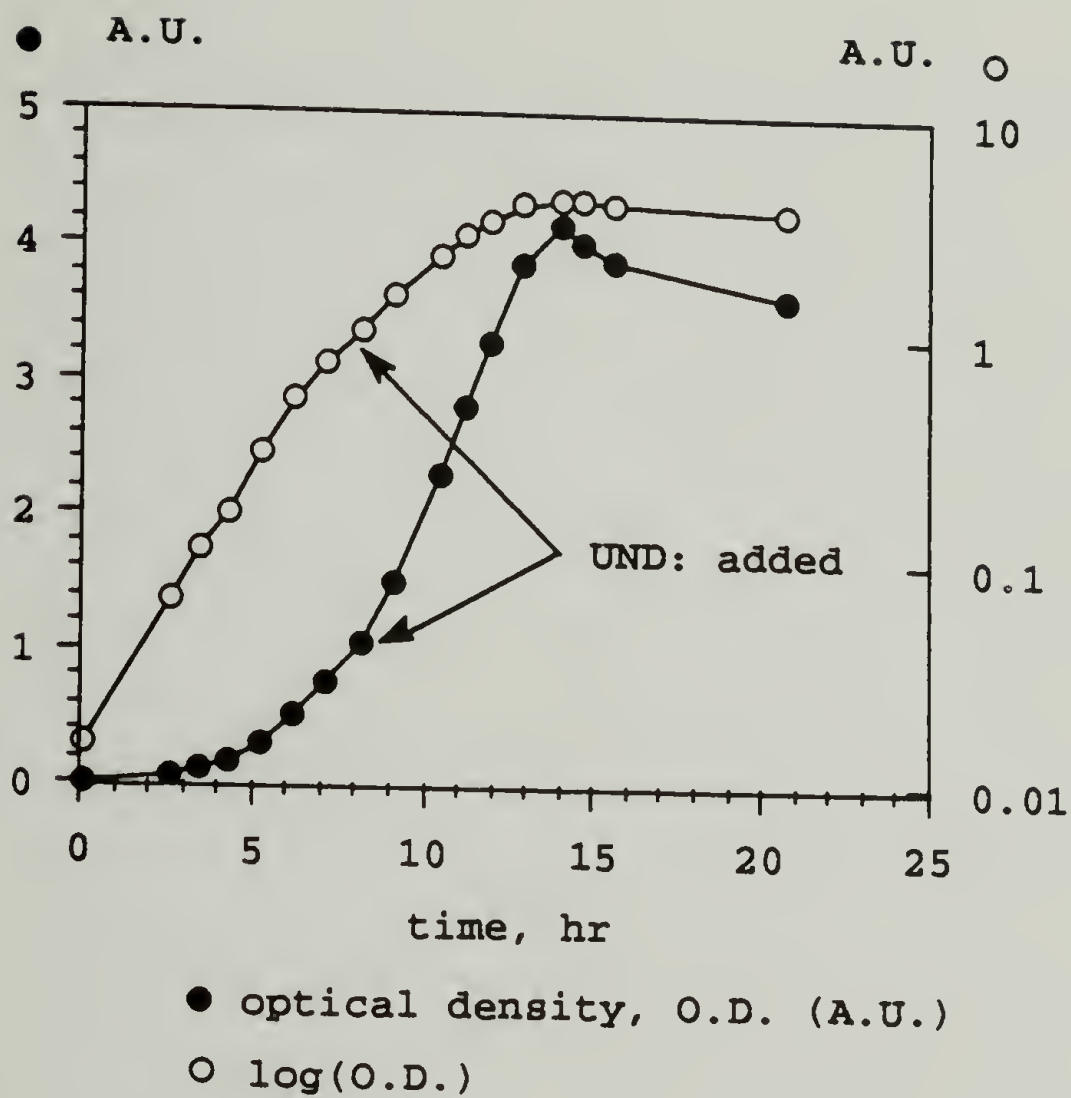
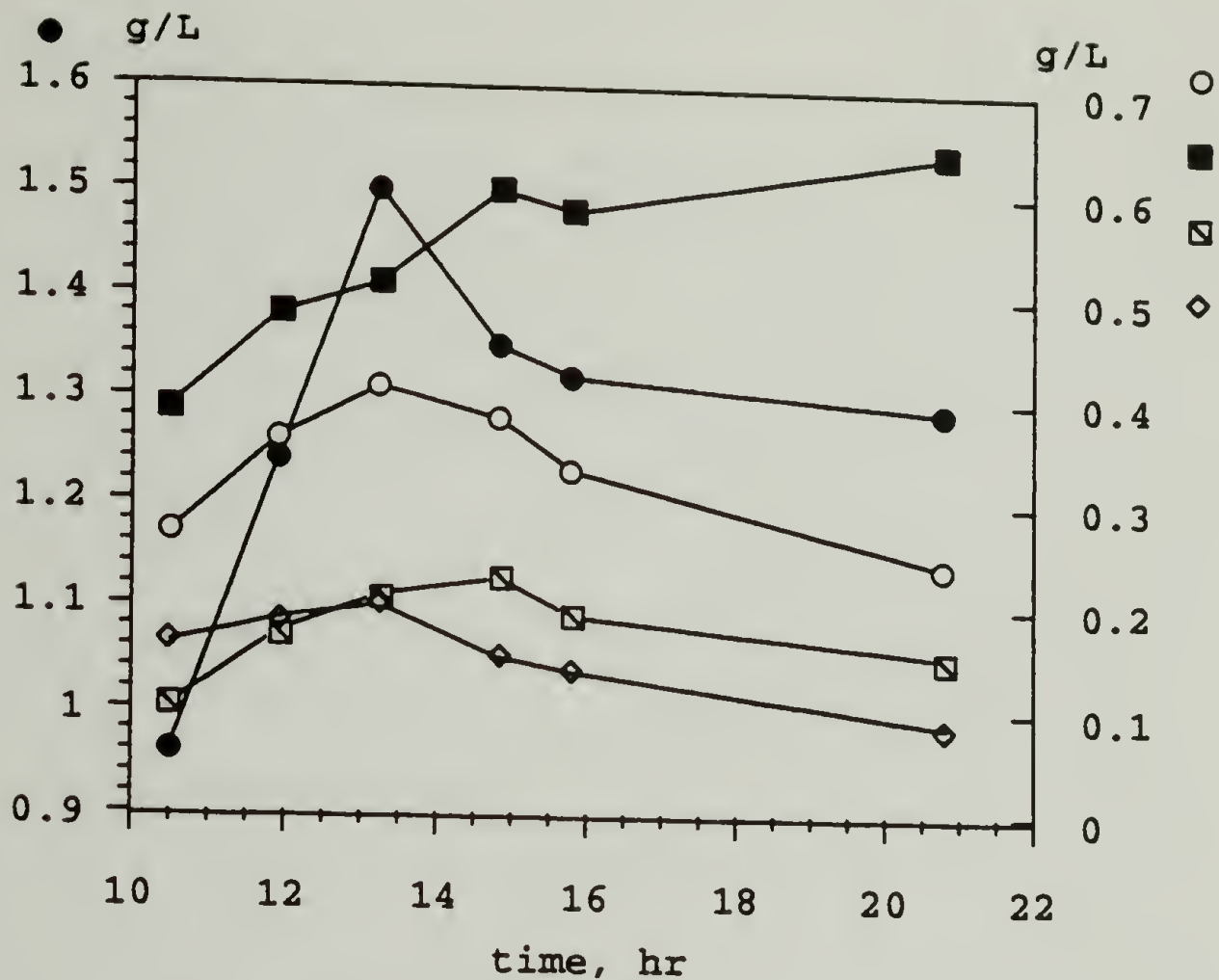


Figure 62. The growth curves obtained by feeding *P. oleovorans* with nonanoic acid and 10-undecenoic acid in sequence.





- biomass yield (g/L)
- PHA yield (g/L), (A)
- fraction of unsaturated units, (B)
- relative amount of PHA-UND:, (A)•(B)
- ◇ relative amount of PHA-NON, (A)-(A)•(B)

Figure 63. The biomass yield, PHA yield, fractions of PHA-NON, and PHA-UND: from a culture fed with nonanoic acid and 10-undecenoic acid in sequence vs. growth time.

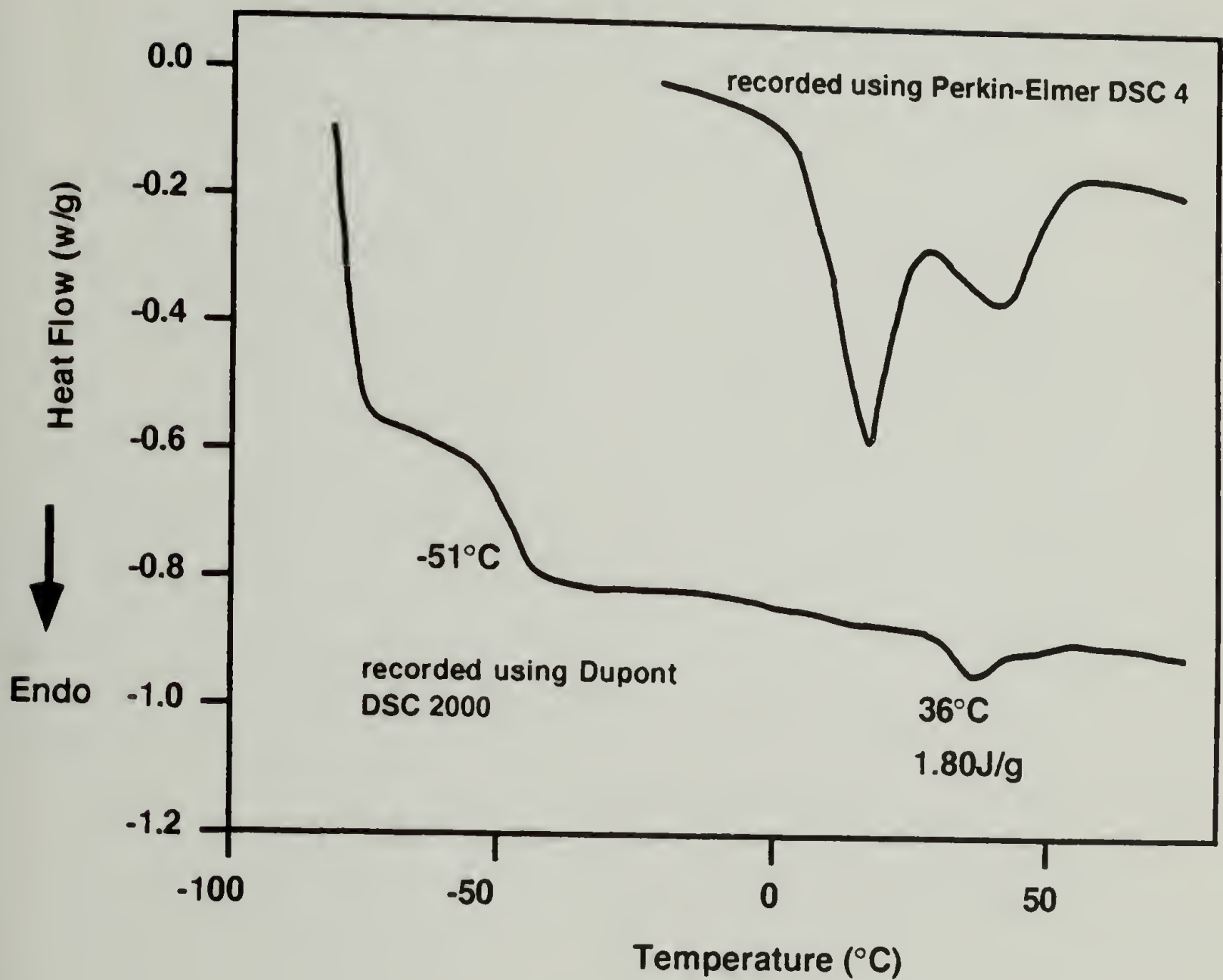


Figure 64. DSC thermogram of PHA produced by *P. oleovorans* fed with nonanoic acid and 10-undecenoic acid in sequence.

Table 20. Weight loss of crosslinked samples with diborane after extraction with chloroform.

Sample	Weight loss after extraction with chloroform (wt%)
Blend of PHA-NON and PHA-UND: (wt:wt)	
1:1	38.6
2:1	35.6
1:2	27.6
PHA from sequential feeding	4.6



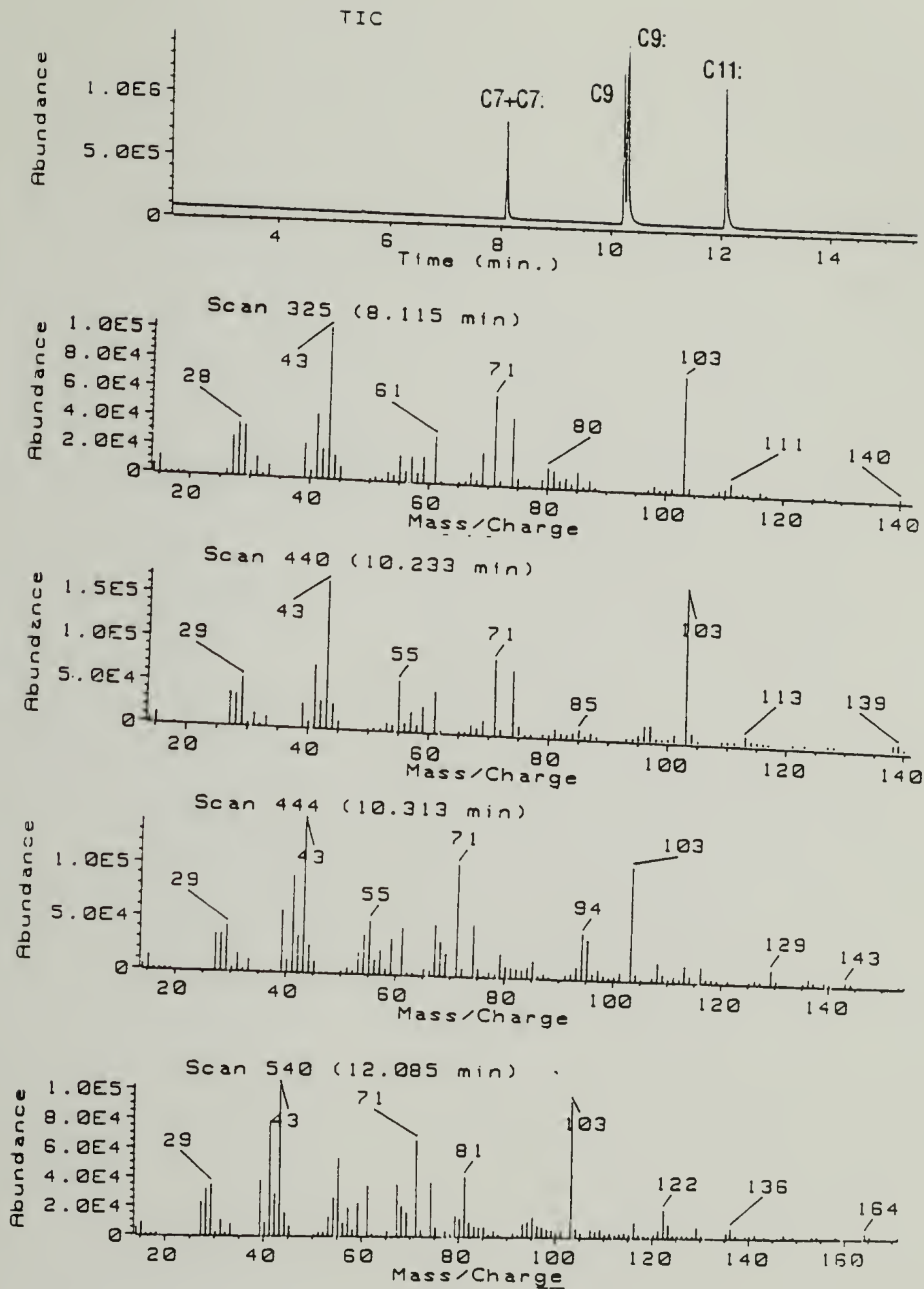


Figure 65. TIC and mass spectra of repeating units in the PHA produced by feeding nonanoic acid and 10-undecenoic acid in sequence.

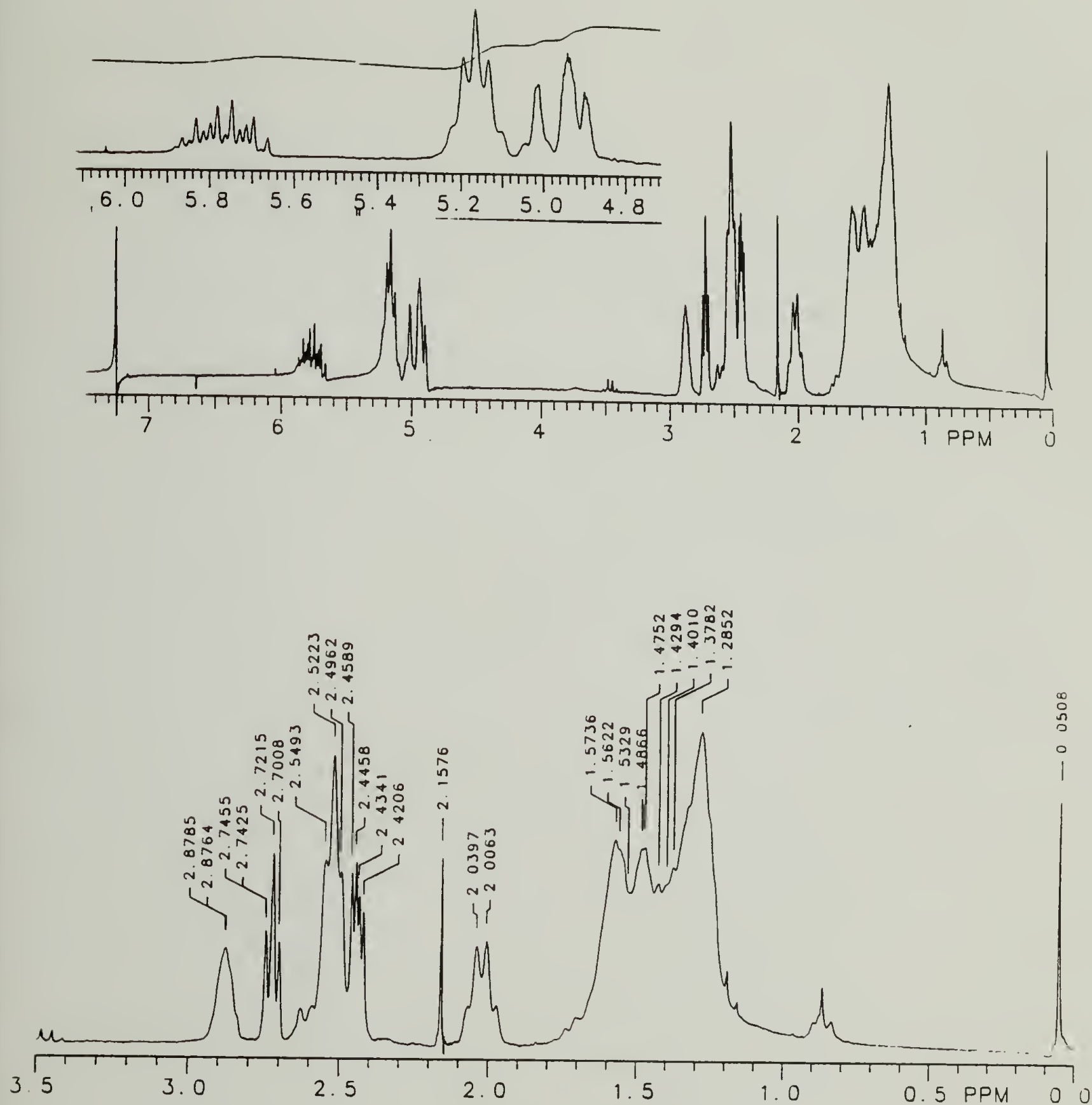


Figure 66. NMR spectrum of the epoxidation product of PHA-UND:.

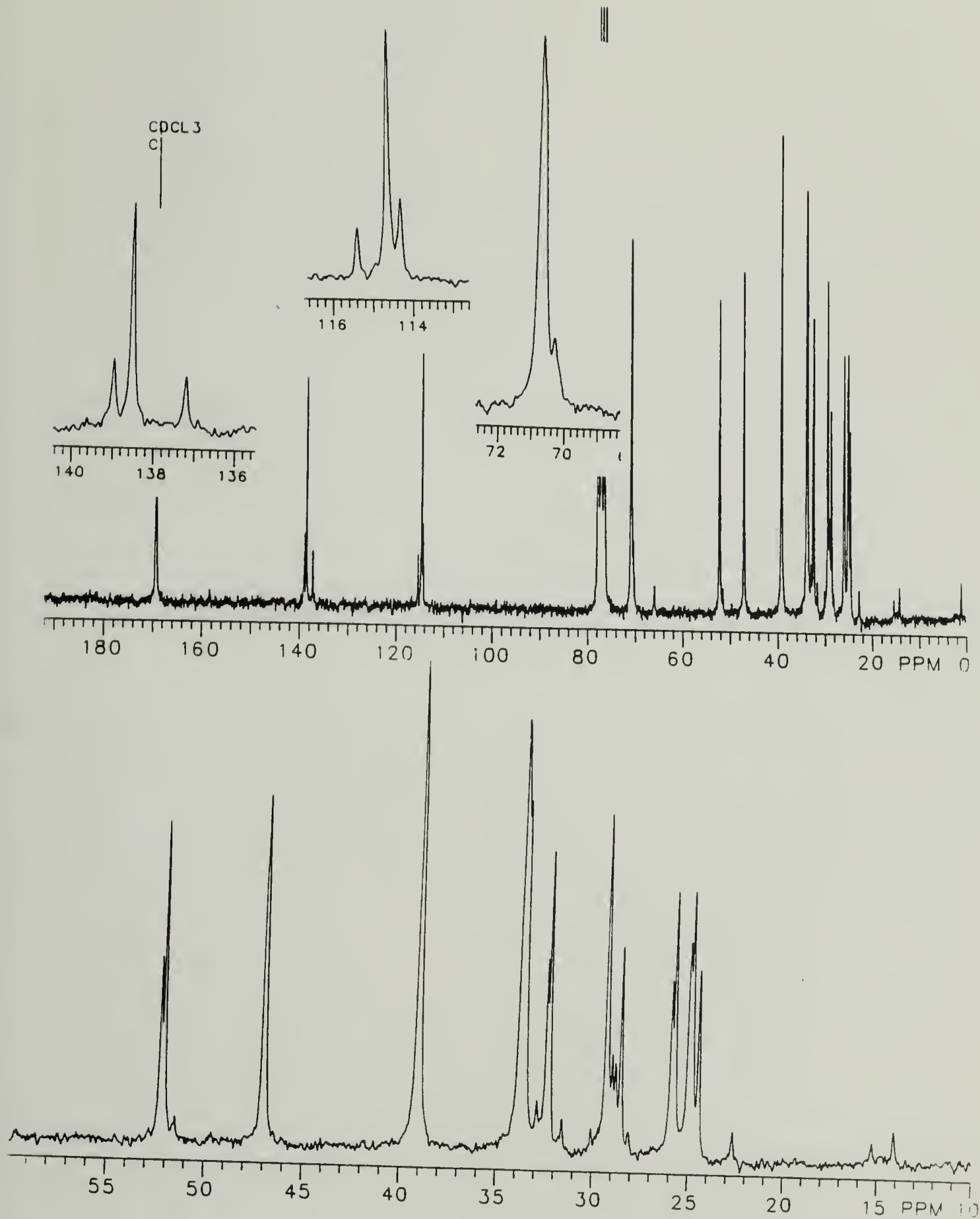


Figure 67.  $^{13}\text{C}$  NMR spectrum of the epoxidation product of PHA UND:



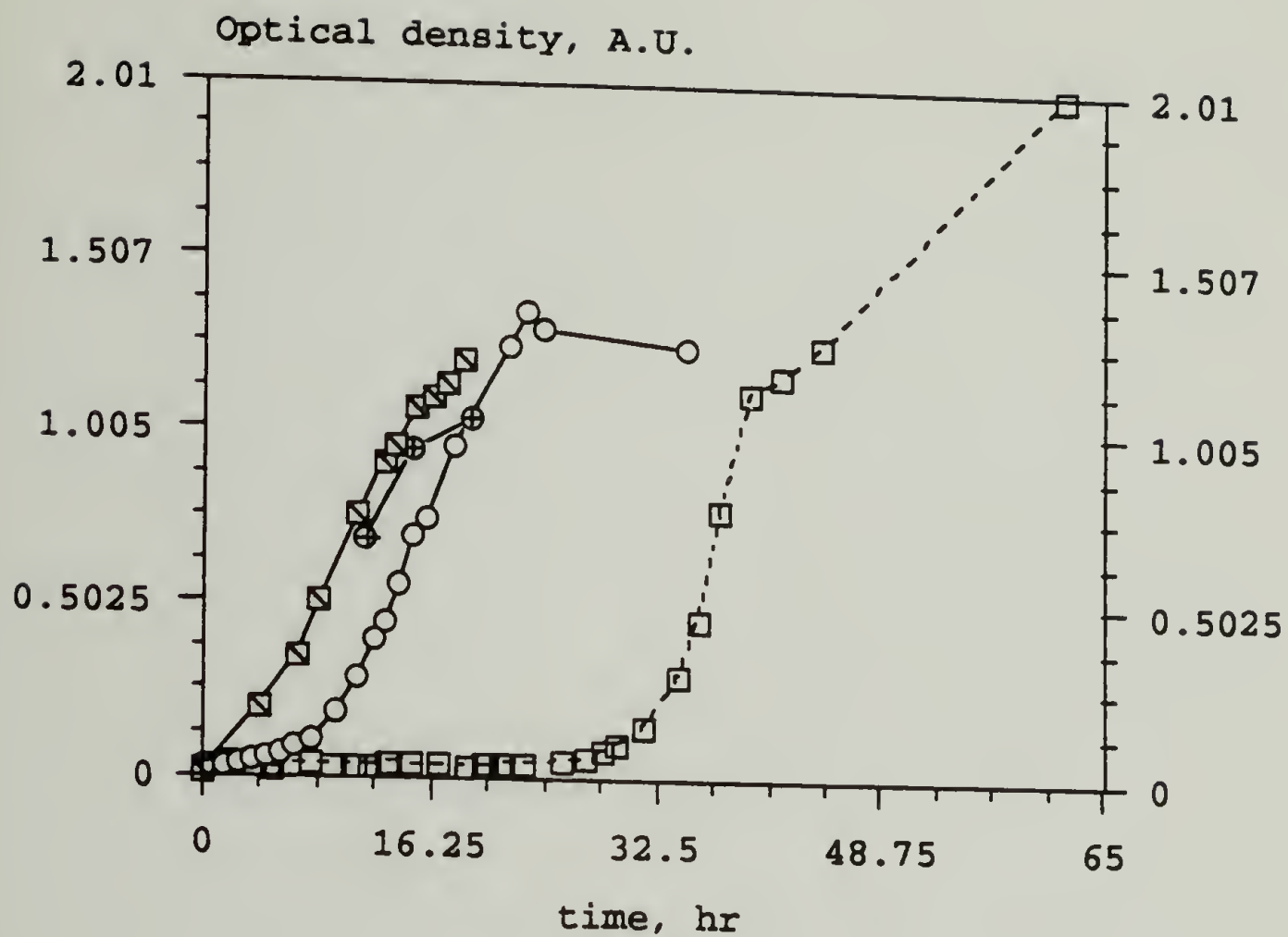


Figure 68. Growth curves of *P. oleovorans* for mixtures of nonanoic acid with either 11-bromoundecanoic acid, 8-bromooctanoic acid, or 6-bromohexanoic acid.

Table 21. Biomass yield and bromine content of the PHAs produced by *P. oleovorans* grown with various mixtures of alkanolic acids and bromoalkanoic acids.

Carbon source (1)	Carbon Source (2)	Ratio (1):(2)	Biomass (g/l)		PHA (g/l)	Cell materials [B] - [A]	PHA content (wt%)	Functional units (mol%)	Weight % of bromine
			[A]	[B]					
NA	11BRUA	1:1	0.68	0.10	0.59	14.0	37.5	21.67	
NA <sup>2</sup>	11BRUA	1:1	0.69	0.18	0.51	26.1	3.7	ND <sup>3</sup>	
NA	8BROA	1:1	0.63	0.08	0.55	13.0	25.0	10.35	
NA	8BROA	2:1	0.55	0.21	0.34	38.2	4.2	ND	
NA	6BRHA	1:1	0.42	0.02	0.40	4.5	25.0	15.30	
OA	8BROA	1:1	0.31	0.11	0.20	35.5	2.0	0.98	
OA	8BROA	1:2	0.37	0.08	0.29	21.6	3.0	1.90	

1, Abbreviations for carbon sources: NA is nonanoic acid, 11BRUA is 11-bromoundecanoic acid, 8BROA is 8-bromooctanoic acid, 6BRHA is 6-bromohexanoic acid, OA is octanoic acid. 2, Carbon sources were fed consecutively. 3, Not determined.

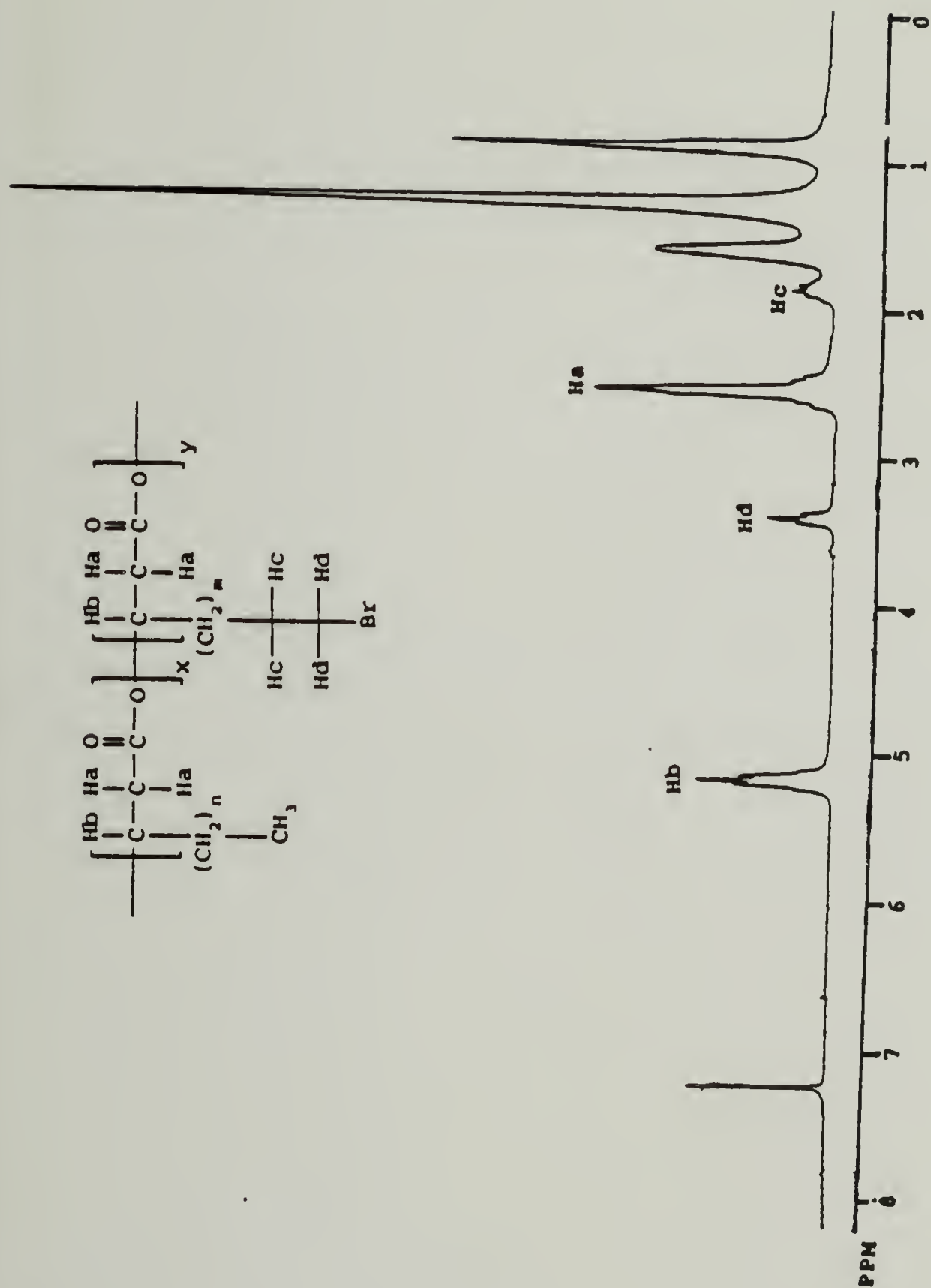


Figure 69. NMR spectrum of a PHA obtained from cells grown with an equimolar mixture of nonanoic acid and 6-bromohexanoic acid.



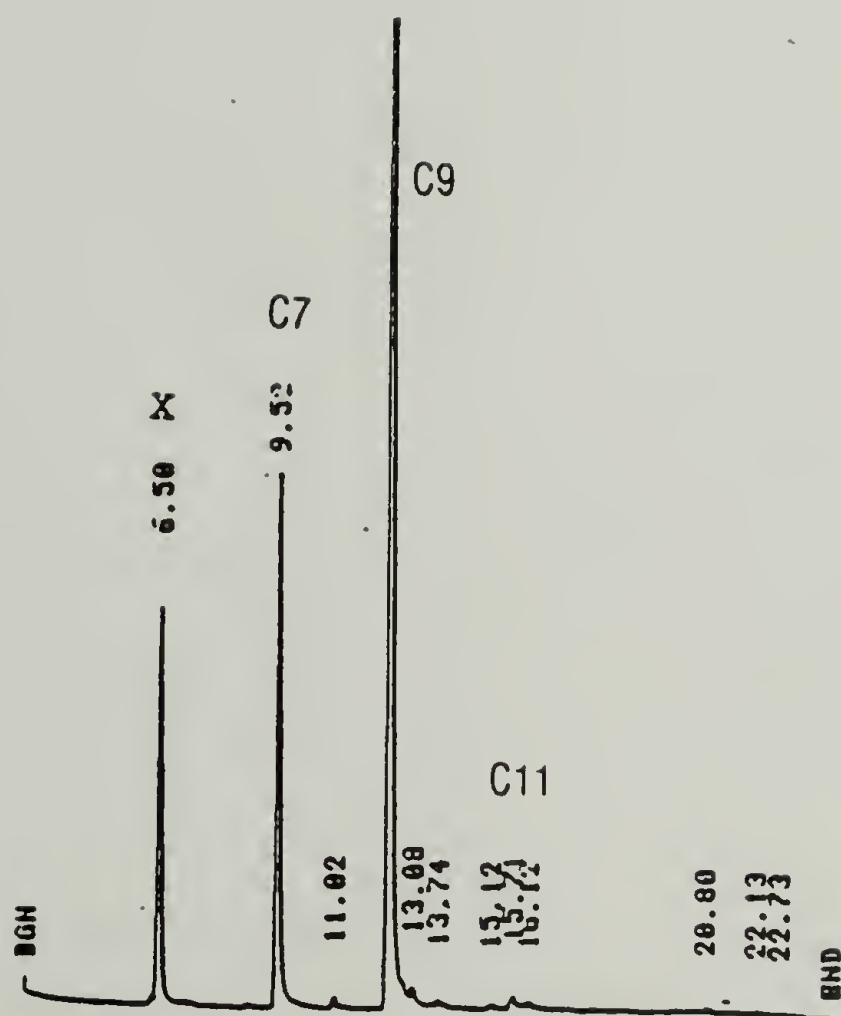


Figure 70. Gas chromatogram of a methanolized sample of a PHA produced from an equimolar mixture of nonanoic acid and 6-bromohexanoic acid.

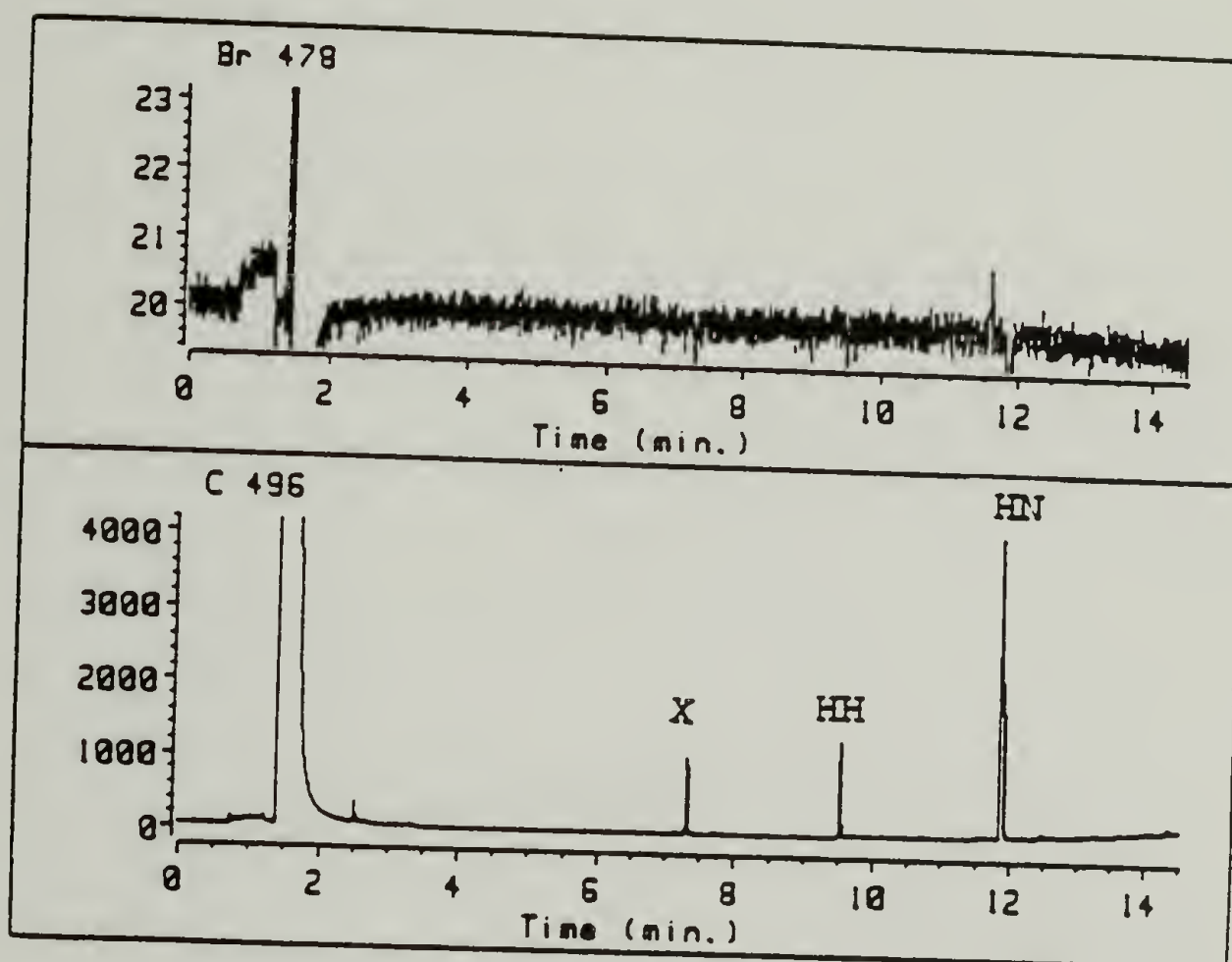


Figure 71. GC/AED chromatogram of the methanolized sample of a PHA produced from an equimolar mixture of nonanoic acid and 6-bromohexanoic acid.

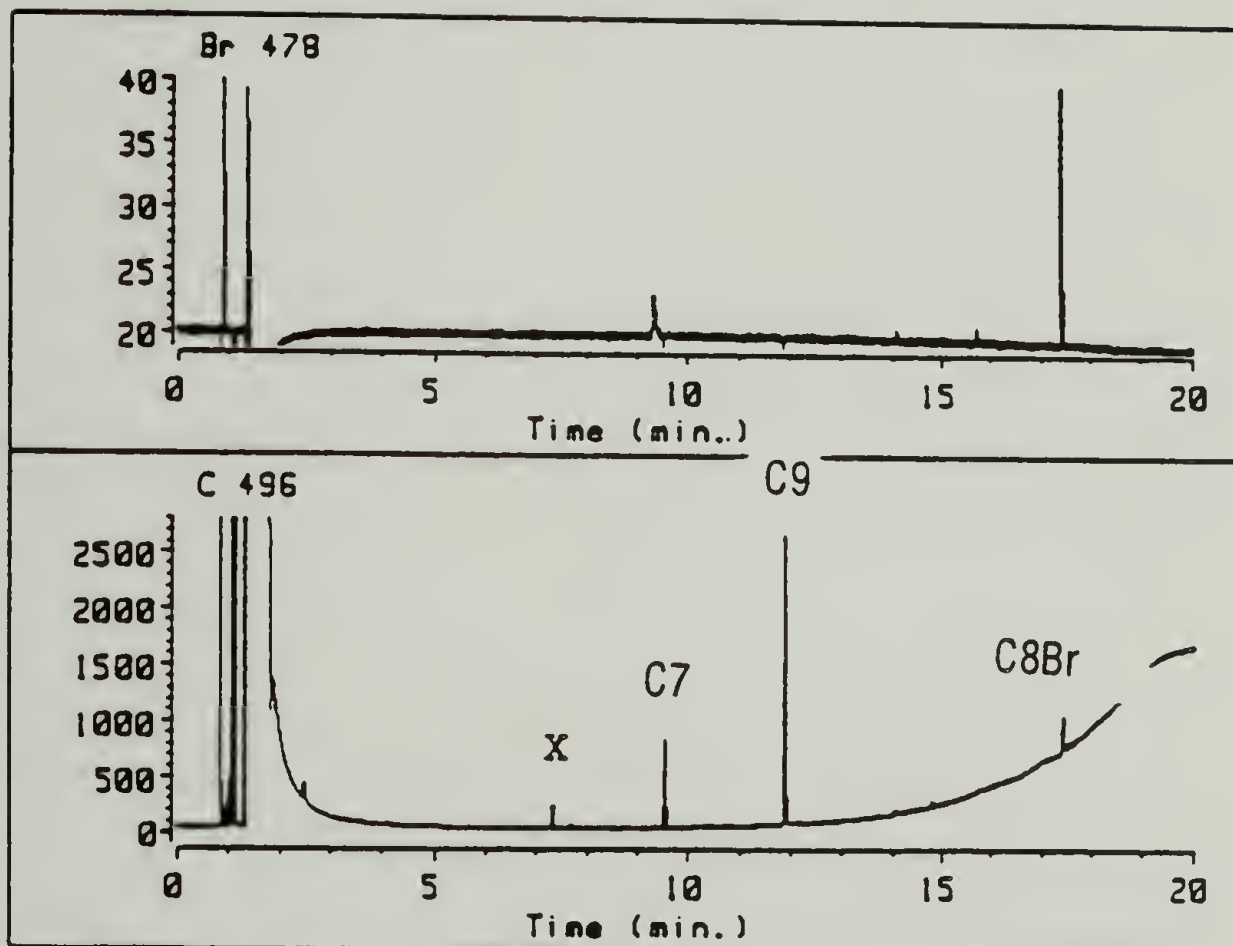


Figure 72. GC/AED chromatogram of the methanolized sample of a PHA produced from an equimolar mixture of nonanoic acid and 8-bromooctanoic acid.



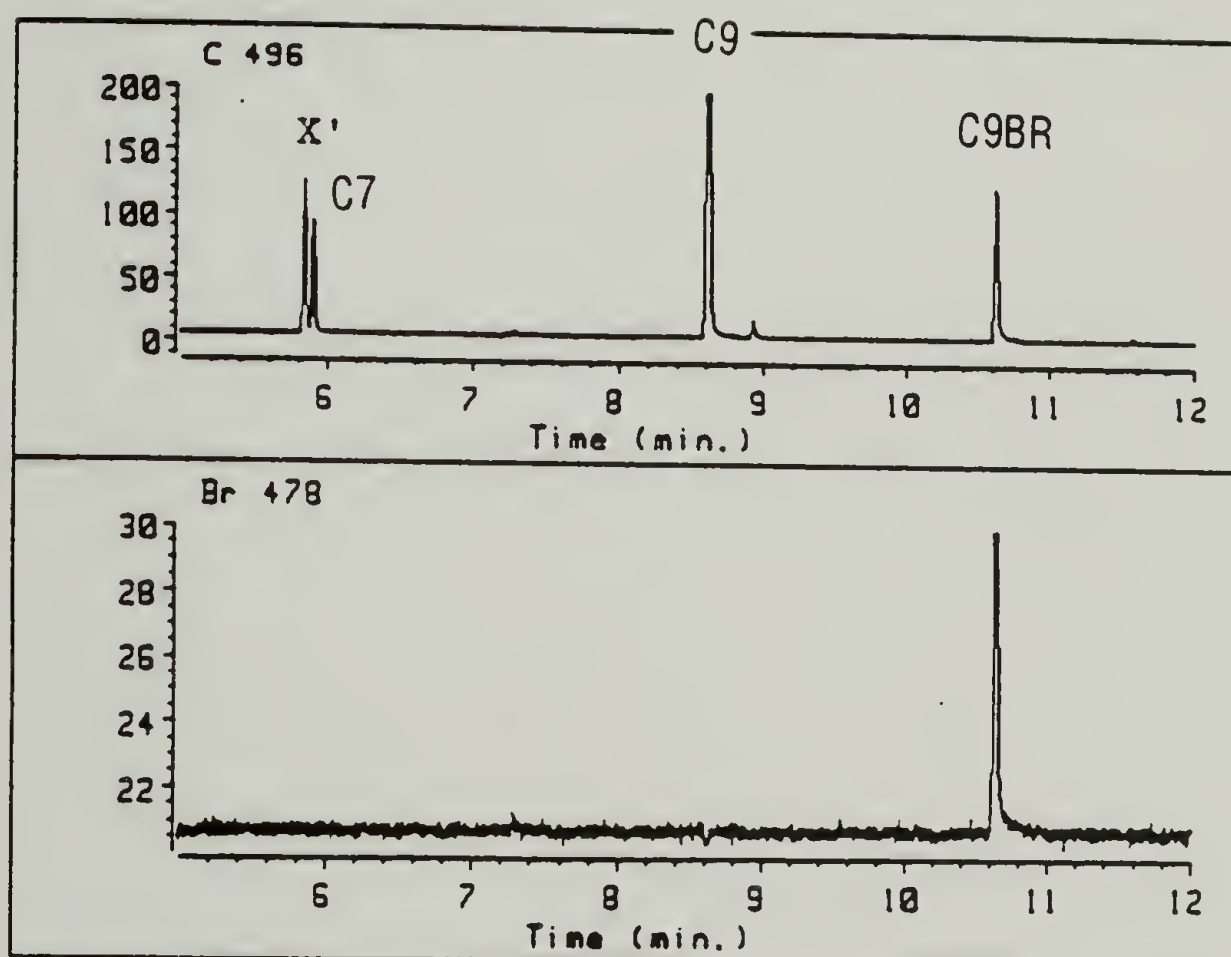


Figure 73. GC/AED chromatogram of the methanolized sample of a PHA obtained from cells grown with an equimolar mixture of nonanoic acid and 11-bromoundecanoic acid.

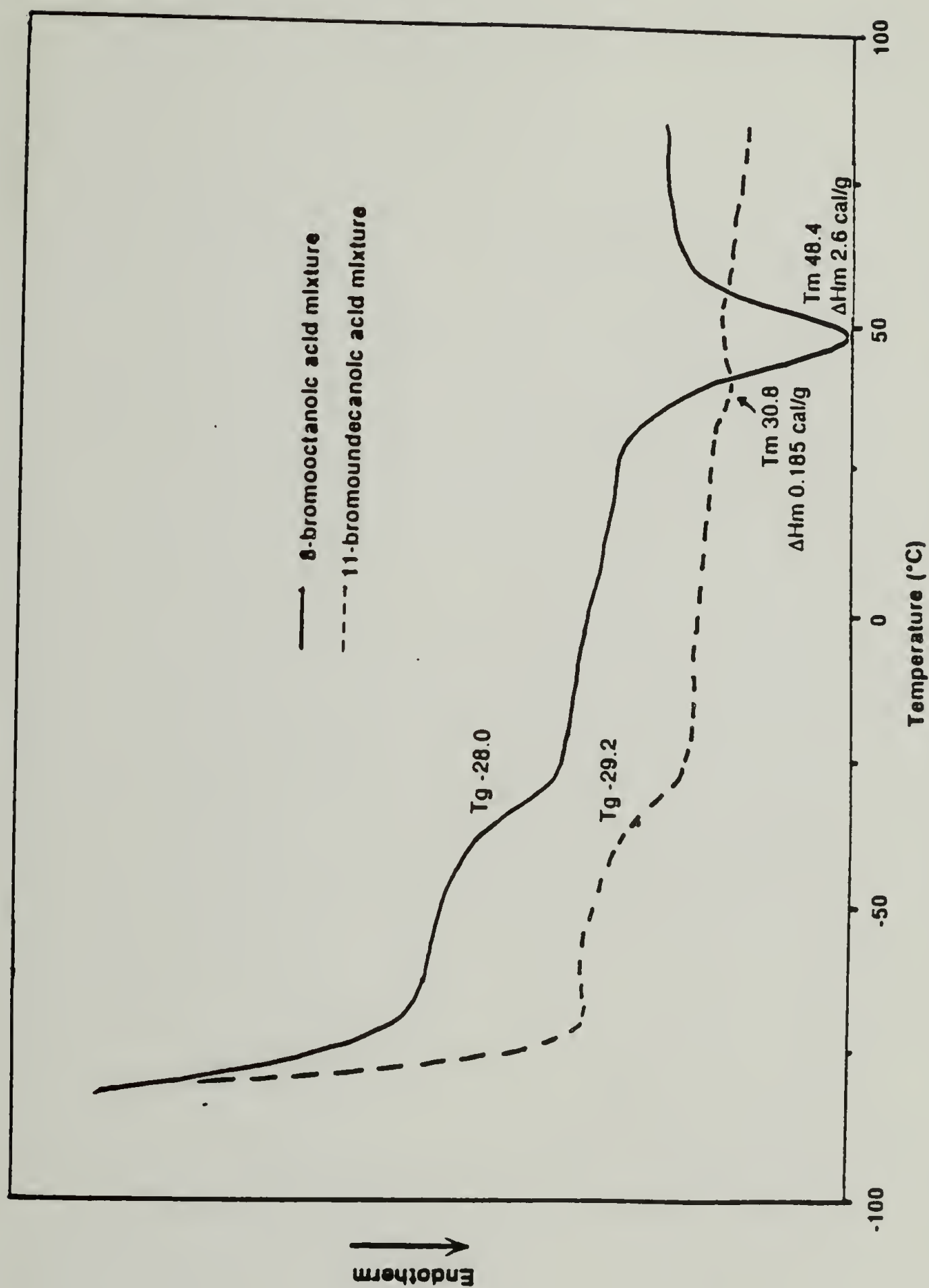


Figure 74. DSC thermogram of PHAs produced from equimolar mixtures of nonanoic acid and either 8-bromooctanoic acid or 11-bromoundecanoic acid.

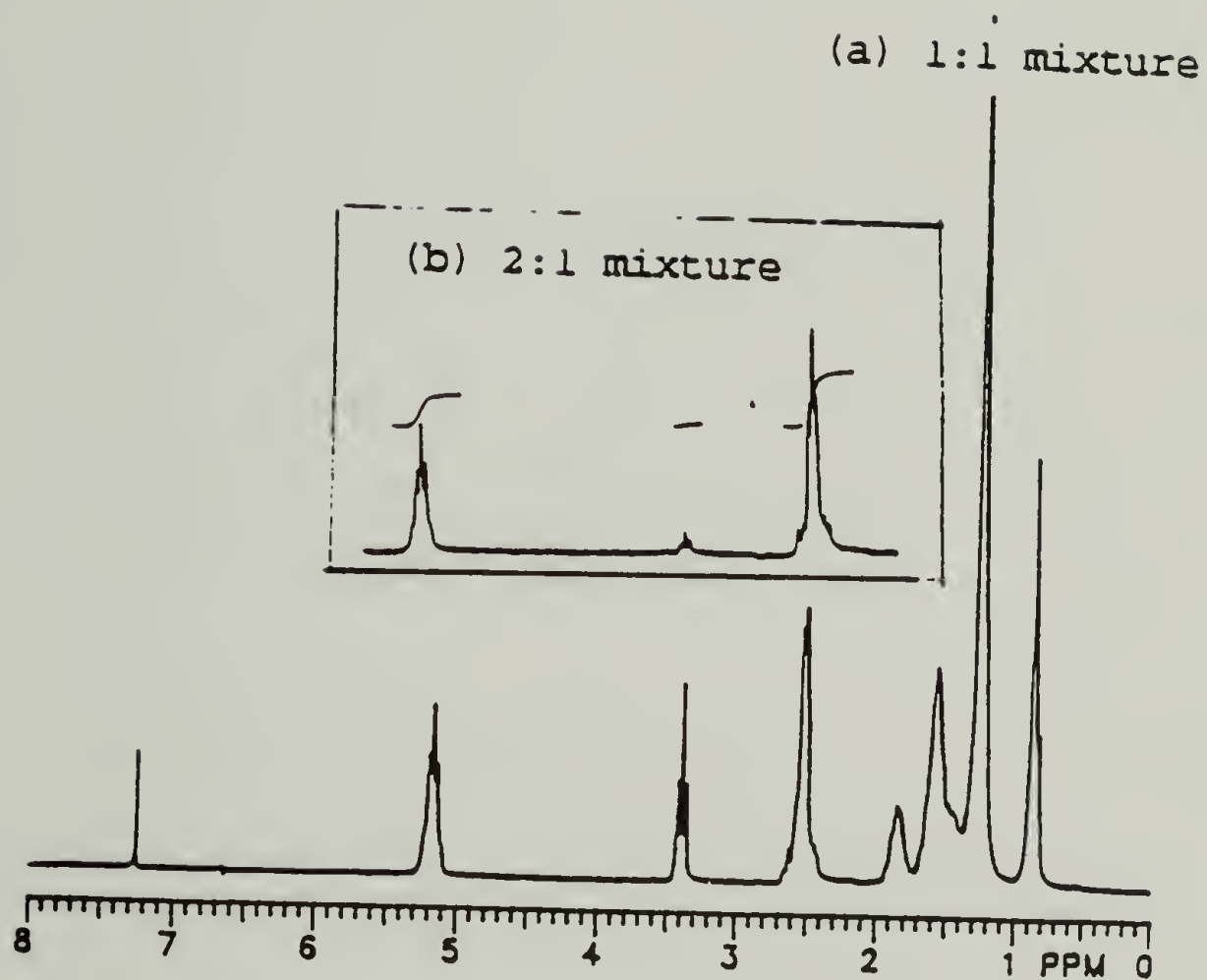


Figure 75. NMR spectra of PHAs produced from 1:1 and 2:1 mixtures of nonanoic acid and 8-bromooctanoic acid.



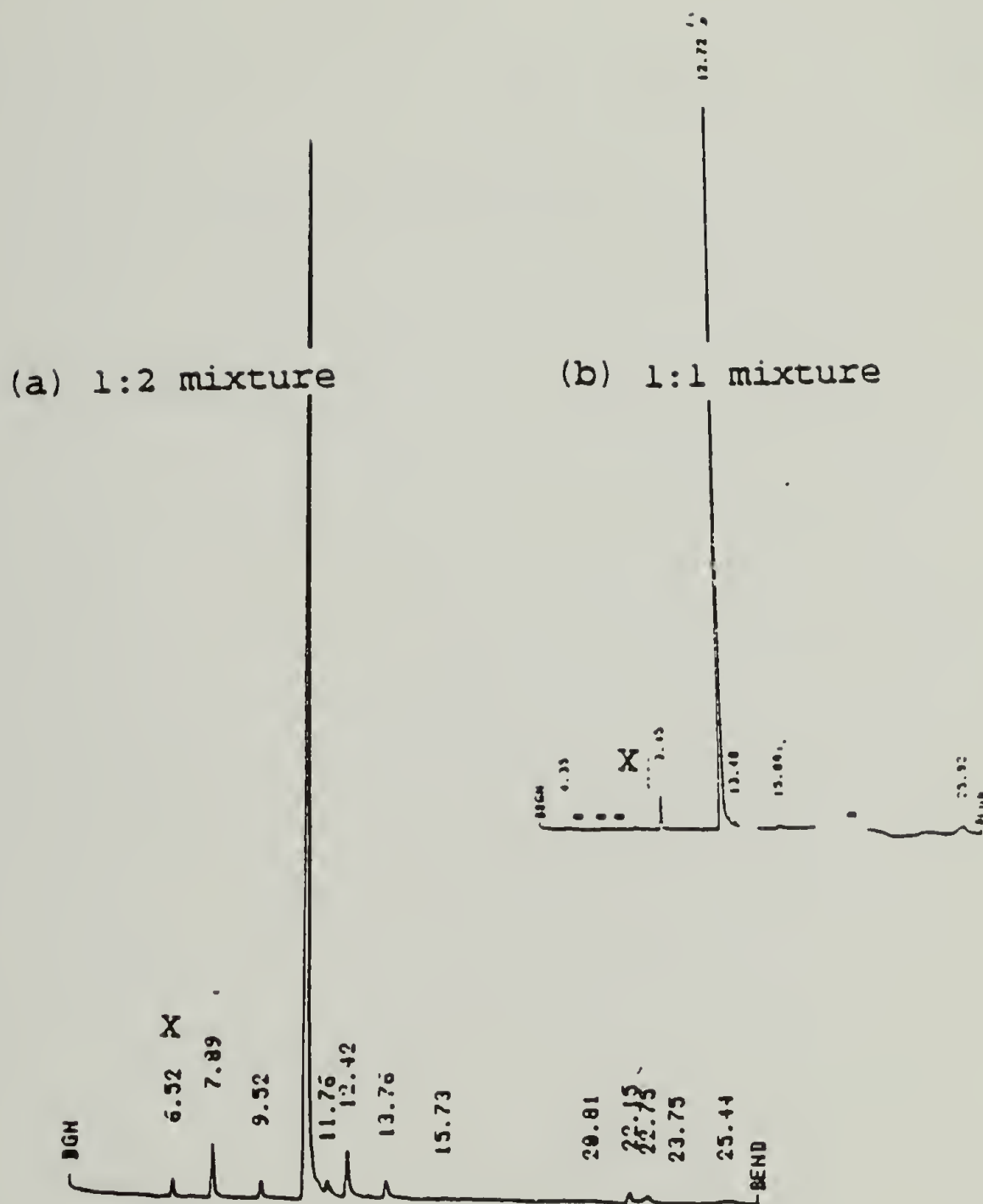


Figure 76. Gas chromatograms of methanolized samples of PHAs produced from 1:1 and 1:2 mixtures of octanoic acid and 8-bromooctanoic acid.

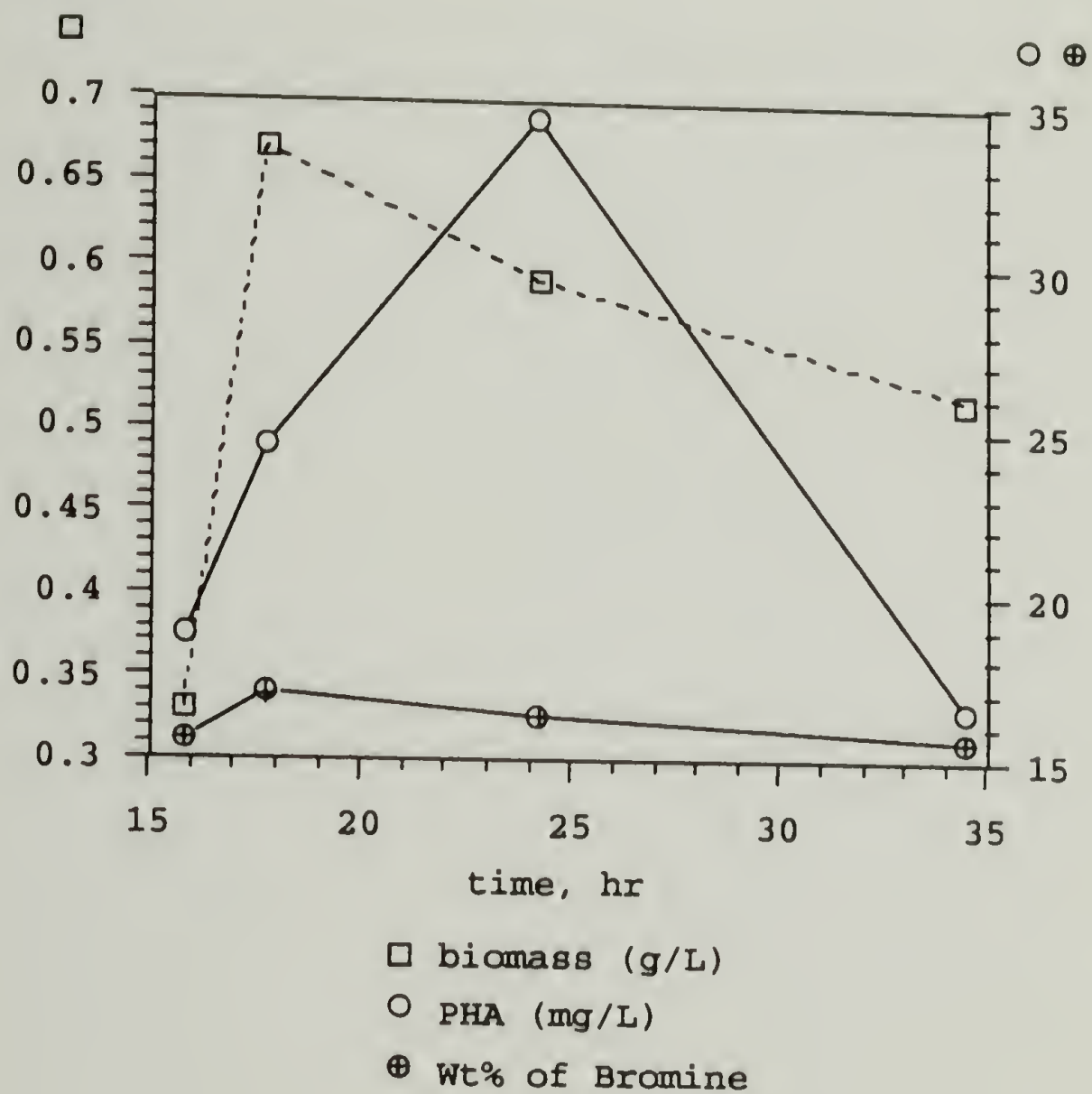
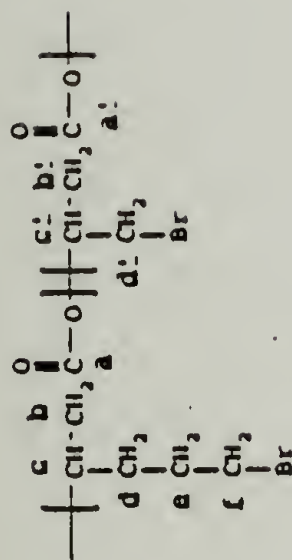


Figure 77. Weight percent of bromine in the PHA, biomass yield, and PHA yield vs. growth time determined from a culture grown with an equimolar mixture of nonanoic acid and 8-bromooctanoic acid.



Peaks marked with Arabic number are from repeating units derived from nonanoic acid, see  $^{13}\text{C}$  NMR of PHA-NON shown in Section III.E.

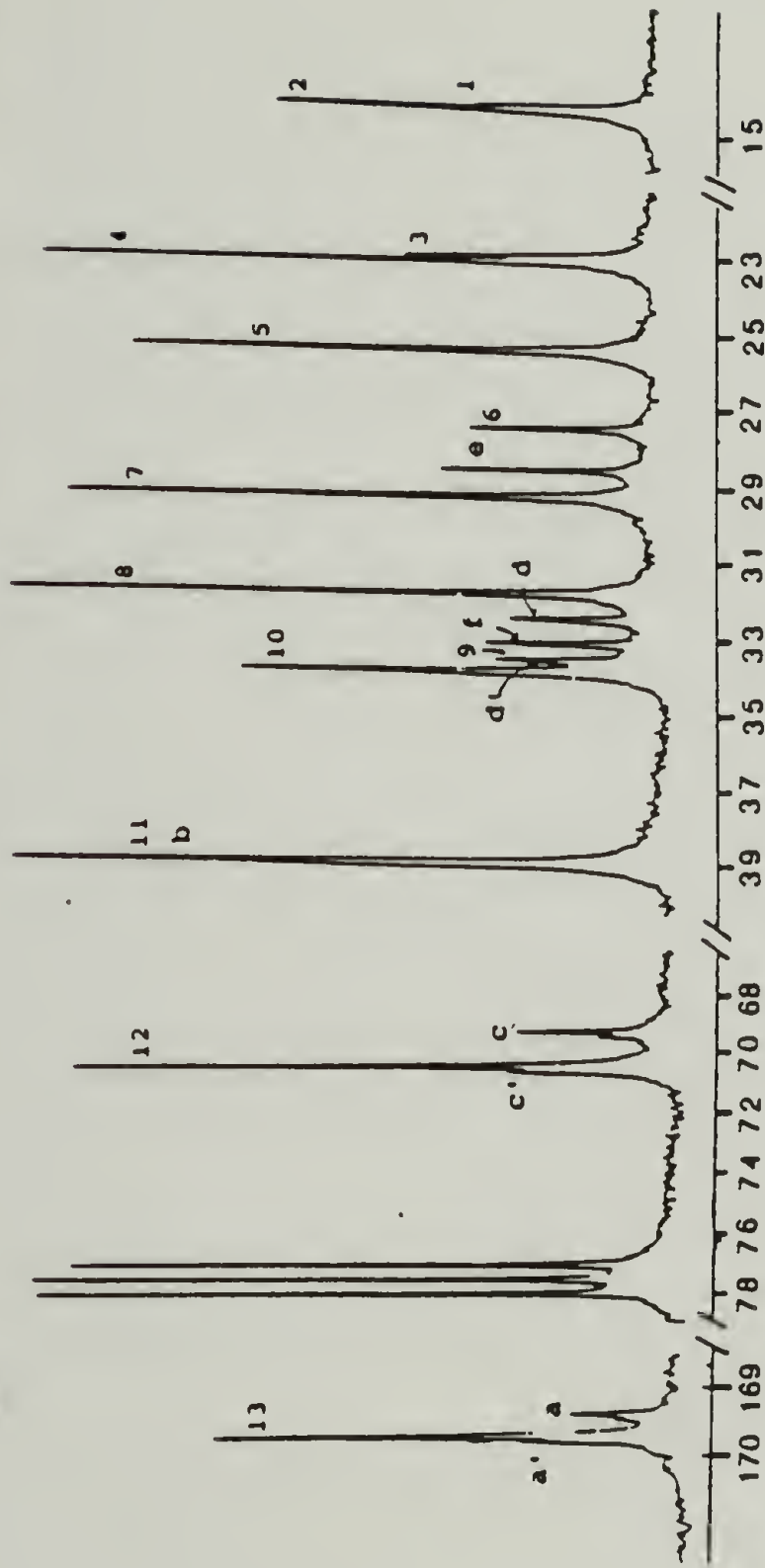


Figure 78.  $^{13}\text{C}$  NMR spectrum of a PHA produced from an equimolar mixture of nonanoic acid and 6-bromohexanoic acid.



Peaks marked with 'N' are from repeating units derived from nonanoic acid, see <sup>13</sup>C NMR of PHA-NON shown in Section III.E.

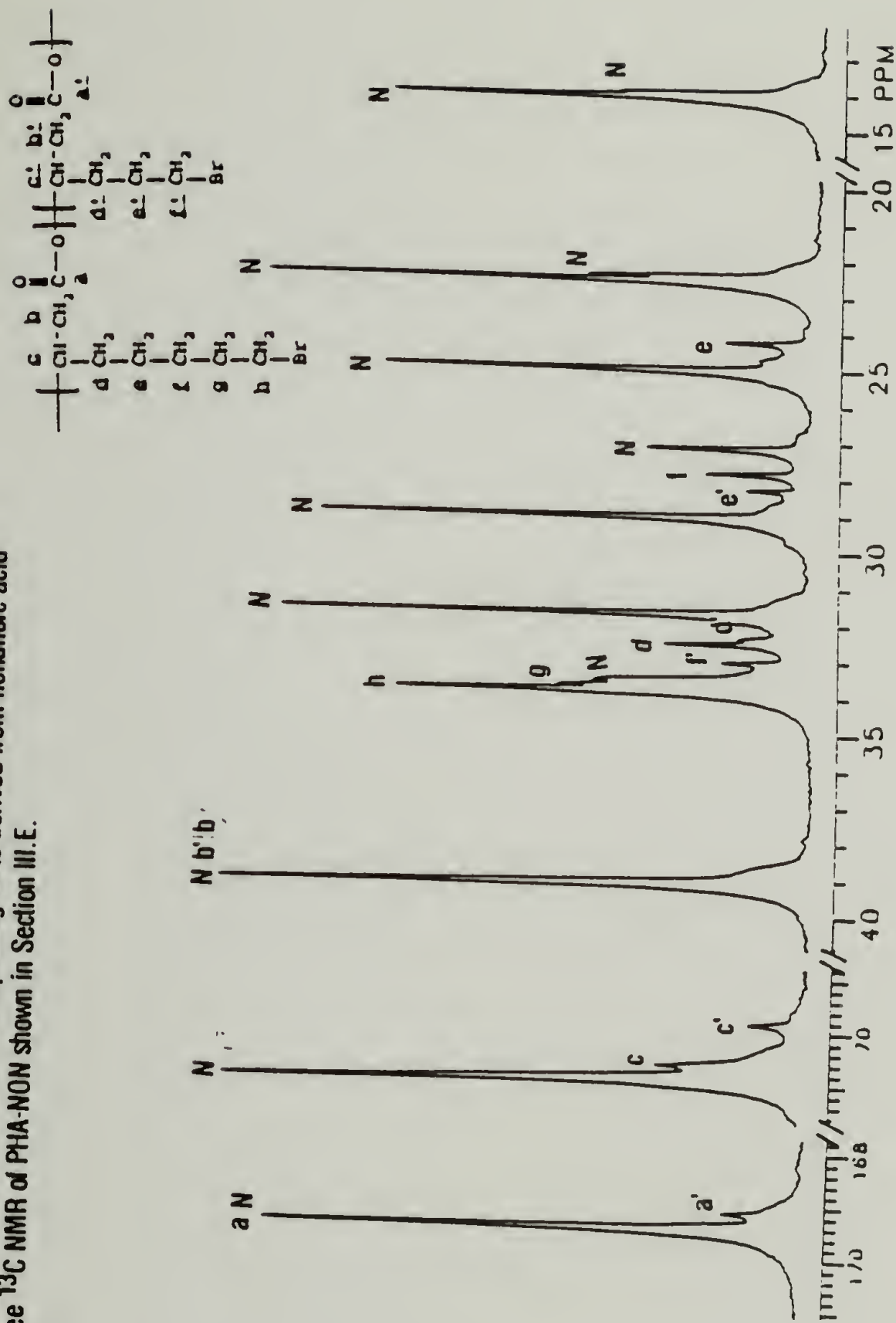


Figure 79. <sup>13</sup>C NMR spectrum of a PHA produced from an equimolar mixture of nonanoic acid and 8-bromooctanoic acid.

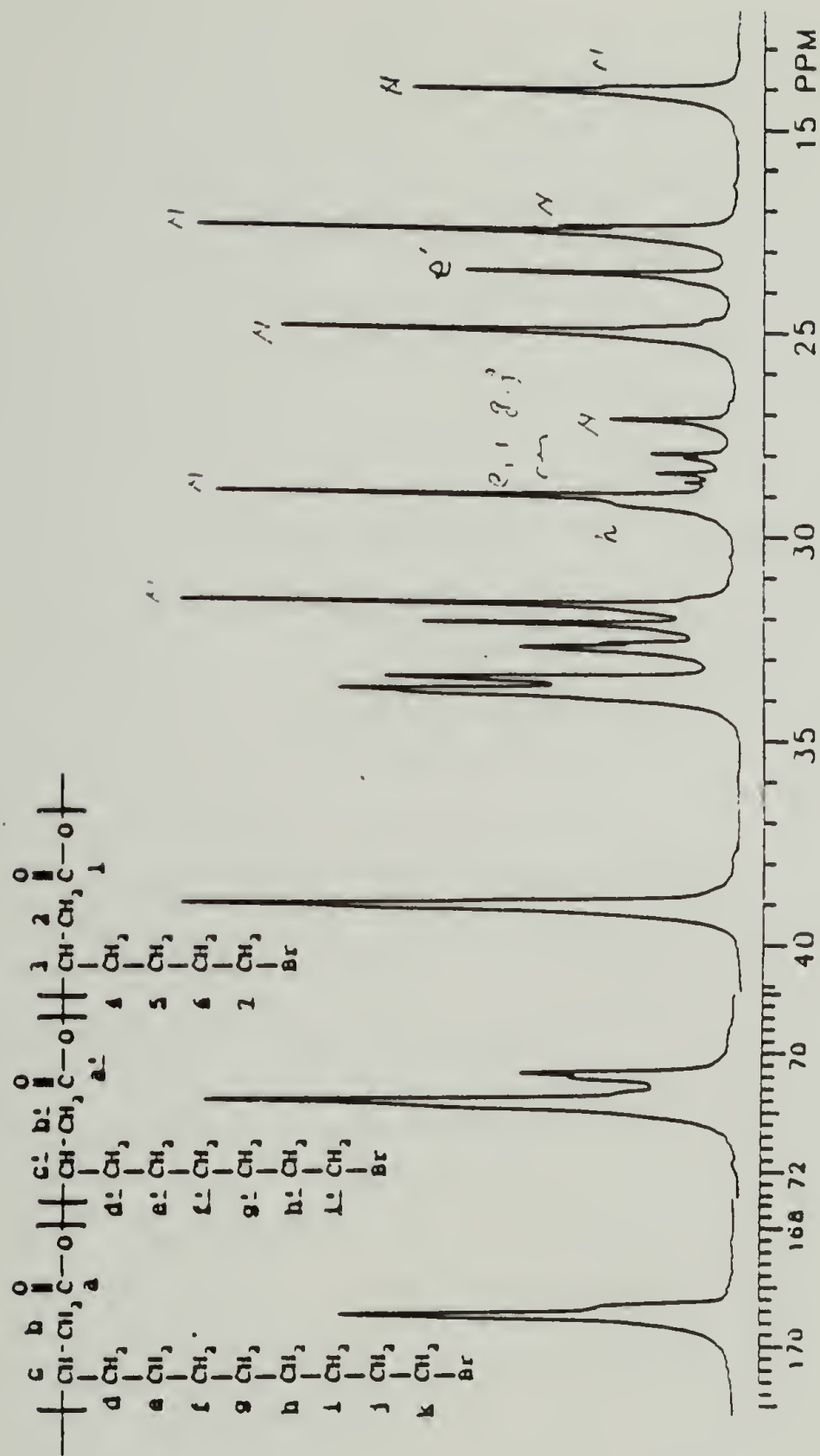


Figure 80.  $^{13}\text{C}$  NMR spectrum of a PHA produced from an equimolar mixture of nonanoic acid and 11-bromoundecanoic acid.

Table 22. Fermentation results from various mixtures of 5-phenylvaleric acid, PVA, and nonanoic acid, NA, or octanoic acid, OA.

Carbon source (mol:mol)	Growth time (hours)	O.D. at harvest	Biomass yield (g/l)	PHA yield (g/l)	PHA content <sup>1</sup> (wt%)	Phenyl group (mole%)
NA:PA (1:0)	18	2.3	1.10	0.43	39.1	0.0
NA:PA (2:1)	15.5	2.0	0.78	0.29	37.2	12.6
NA:PA (1:1)	17.5	2.0	0.77	0.29	37.7	25.7
NA:PA (1:2)	15.0	1.7	0.50	0.16	31.6	40.6
NA:PA (2:1) <sup>2</sup>	12.0	1.2	0.83	0.13	15.1	14.9
OA:PA (1:0)	18.8	2.2	1.01	0.36	36.0	0.0
OA:PA (1:1)	13.5	1.5	0.51	0.20	39.2	24.3

- 1, weight percent of polymer based on dry weight of biomass
- 2, aeration rate and agitation rate were 21/min and 100 r.p.m. for first 10 hours and then increased to 61/min and 200 r.p.m. for two hours before harvesting.



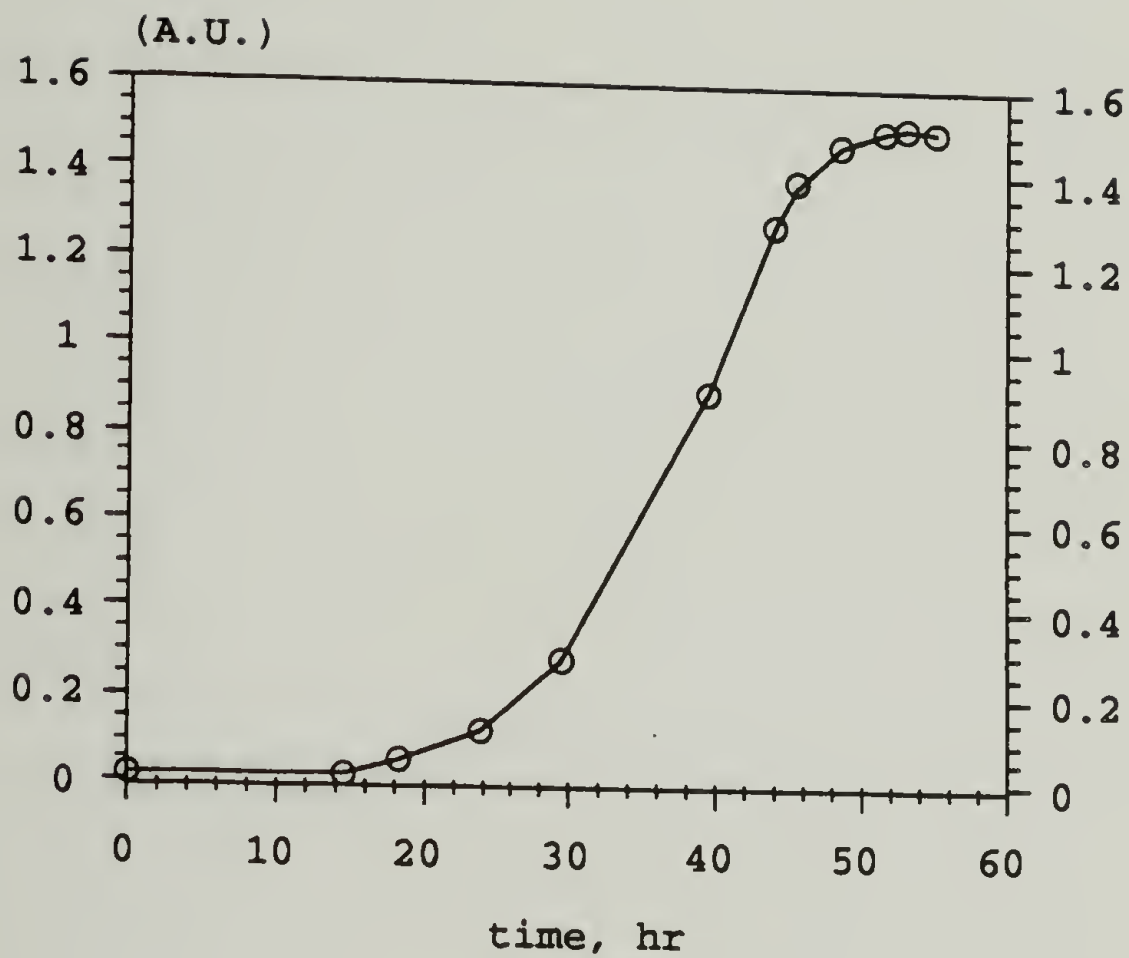


Figure 81. Growth curve of *P. oleovorans* growth with 10 mM 5-phenylvaleric acid.

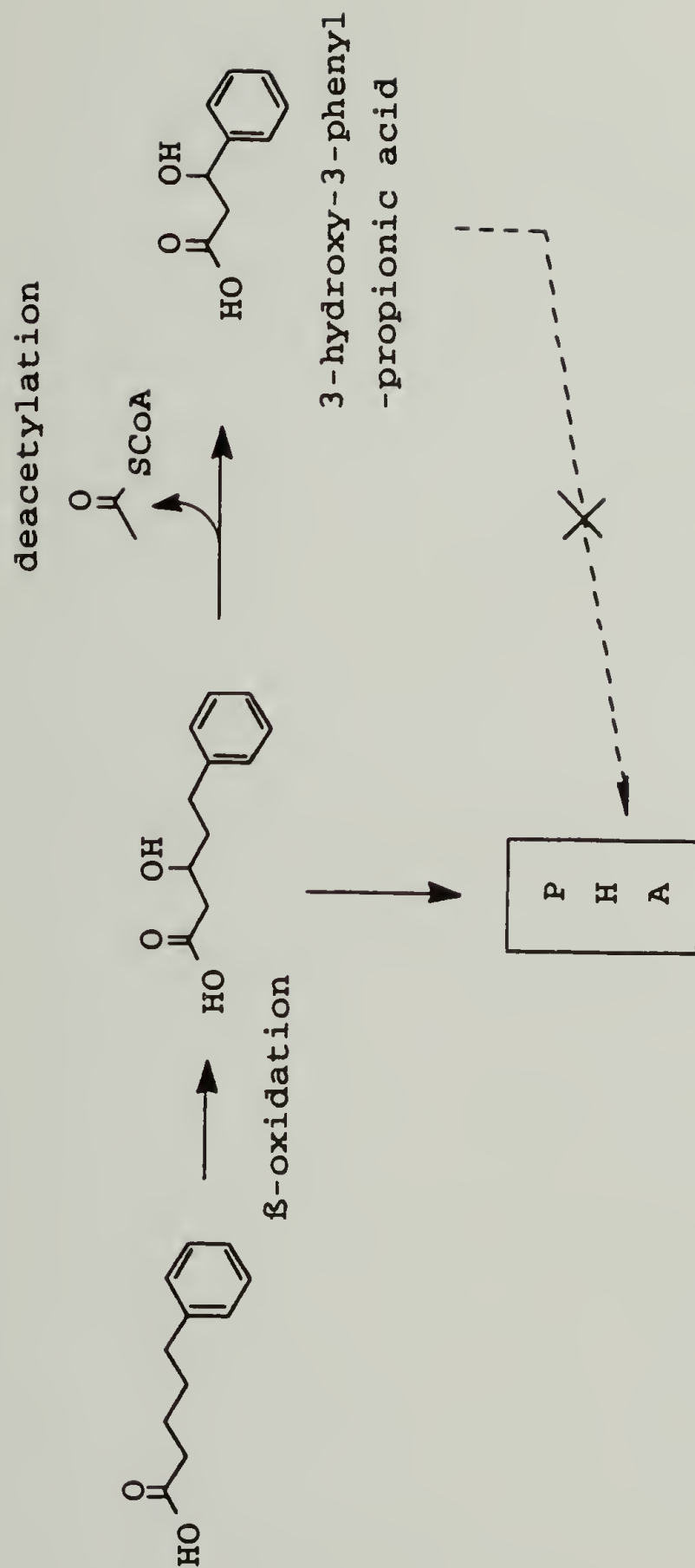


Figure 82. Possible production pathway of 3-hydroxy-3-phenylpropionic acid from 5-phenylvaleric acid.

Table 23. Repeating unit compositions of PHAs obtained using various carbon source mixtures.

Carbon Source	Repeating unit in PHAs obtained, Cn <sup>a</sup> (mole%)							
	C5	C6	C7	C8	C9	C10	C11	HPV <sup>b</sup>
NA:PVA (1:0)	1.7	- <sup>c</sup>	25.3	-	70.1	-	2.9	-
NA:PVA (2:1)	1.7	-	21.7	-	60.7	-	3.3	12.6
NA:PVA (1:1)	1.3	-	17.3	-	54.2	-	1.5	25.7
NA:PVA (1:2)	0.6	-	16.0	-	41.1	-	1.7	40.6
NA:PVA (2:1) <sup>d</sup>	1.5	-	21.3	-	60.3	-	2.0	14.9
OA:PVA (1:0)	-	10.7	-	83.3	-	6.1	-	-
OA:PVA (1:1)	-	7.3	-	64.5	-	3.9	-	24.3

a, Cn represents a 3-hydroxyalkanoate containing n carbon atoms.

b, 3-hydroxy-5-phenylvalerate unit

c, not measurable amounts

d, aeration rate and agitation rate were 21/min and 100 r.p.m. for first 10 hours and then increased to 61/min and 200 r.p.m. for two hours before harvesting.



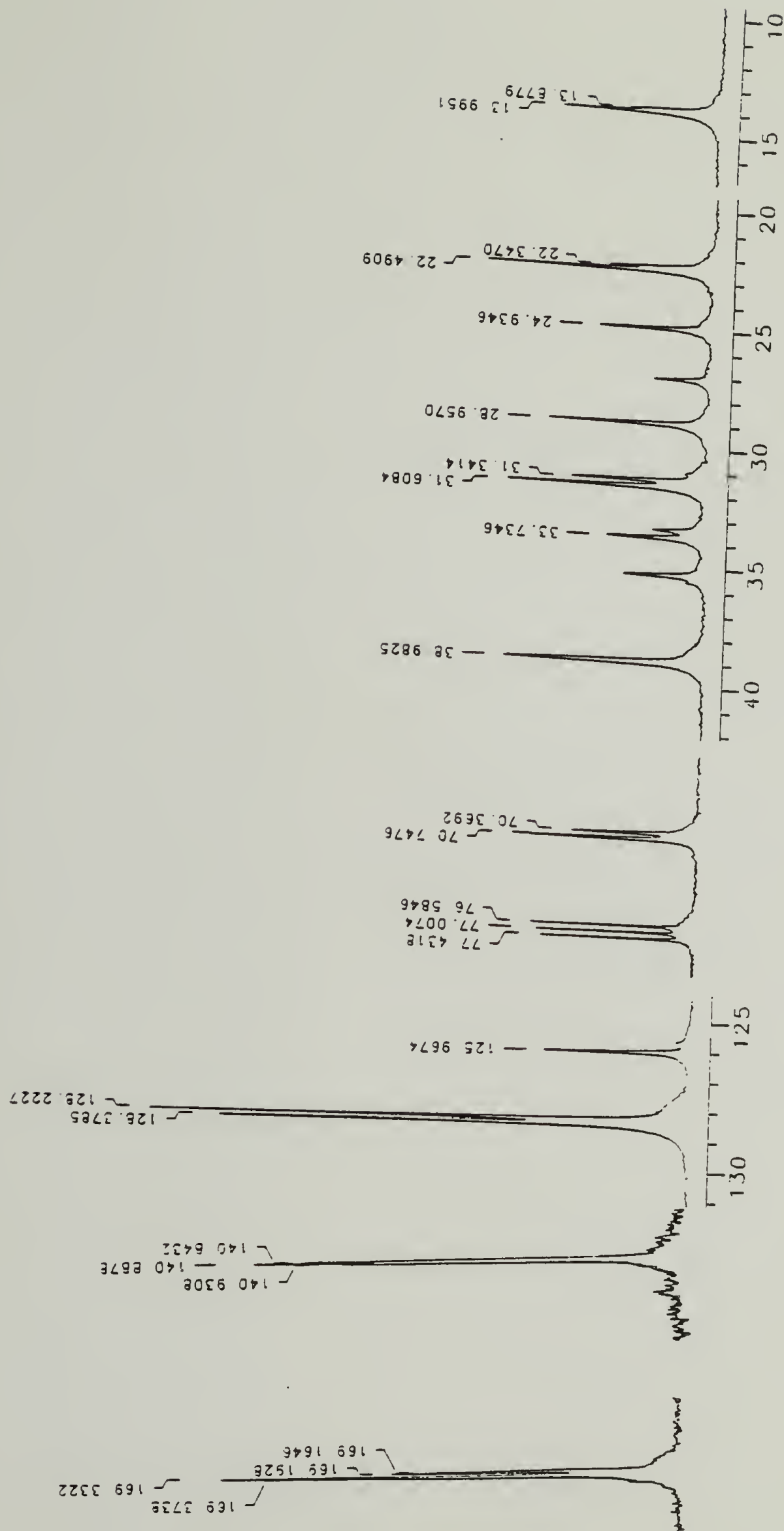


Figure 83.  $^{13}\text{C}$  NMR spectrum of a PHA produced from a 2:1 mixture of nonanoic acid and 5-phenylvaleric acid.

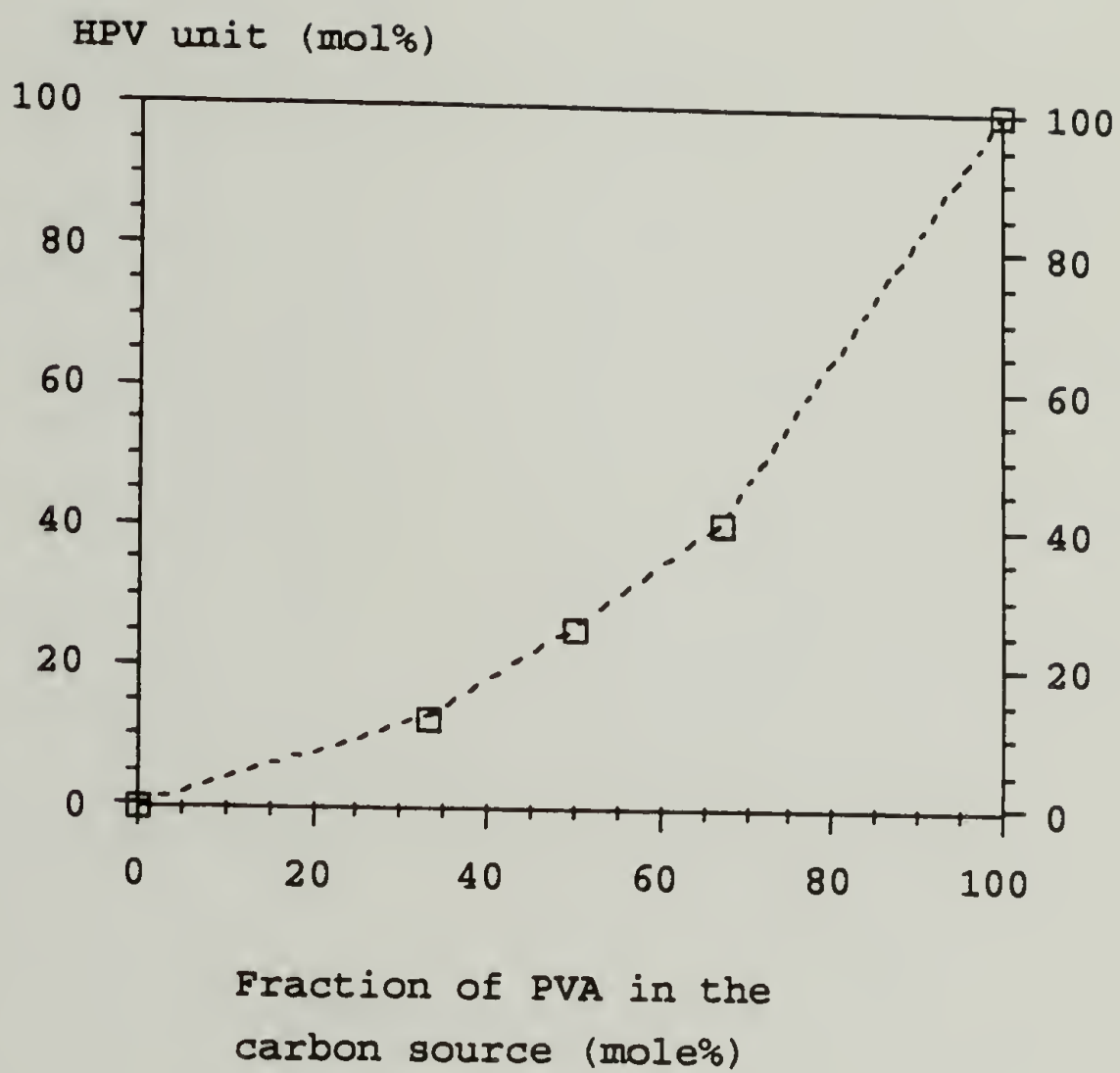


Figure 84. Mole percent of HPV units in the PHA vs. mole percent of 5-phenylvaleric acid in the source mixture.

Table 24. Relative amount of repeating units from nonanoic acid, NA, and octanoic acid, OA, in the PHAs obtained from mixtures of 5-phenylvaleric acid, PVA, and either NA or OA.

Carbon source	Repeating unit, GC area% <sup>1</sup>						
	C5	C6	C7	C8	C9	C10	C11
NA:PVA (1:0)	1.7	- <sup>2</sup>	25.3	-	70.1	-	2.9
NA:PVA (2:1)	2.0	-	24.8	-	69.5	-	3.8
NA:PVA (1:1)	1.8	-	23.3	-	73.0	-	2.0
NA:PVA (1:2)	1.0	-	26.9	-	69.2	-	2.9
NA:PVA (2:1) <sup>3</sup>	1.8	-	25.0	-	70.8	-	2.4
OA:PVA (1:0)	-	10.7	-	83.3	-	6.0	-
OA:PVA (1:1)	-	9.6	-	85.2	-	5.2	-

1, see footnotes in Table 23.

2, not measurable amount

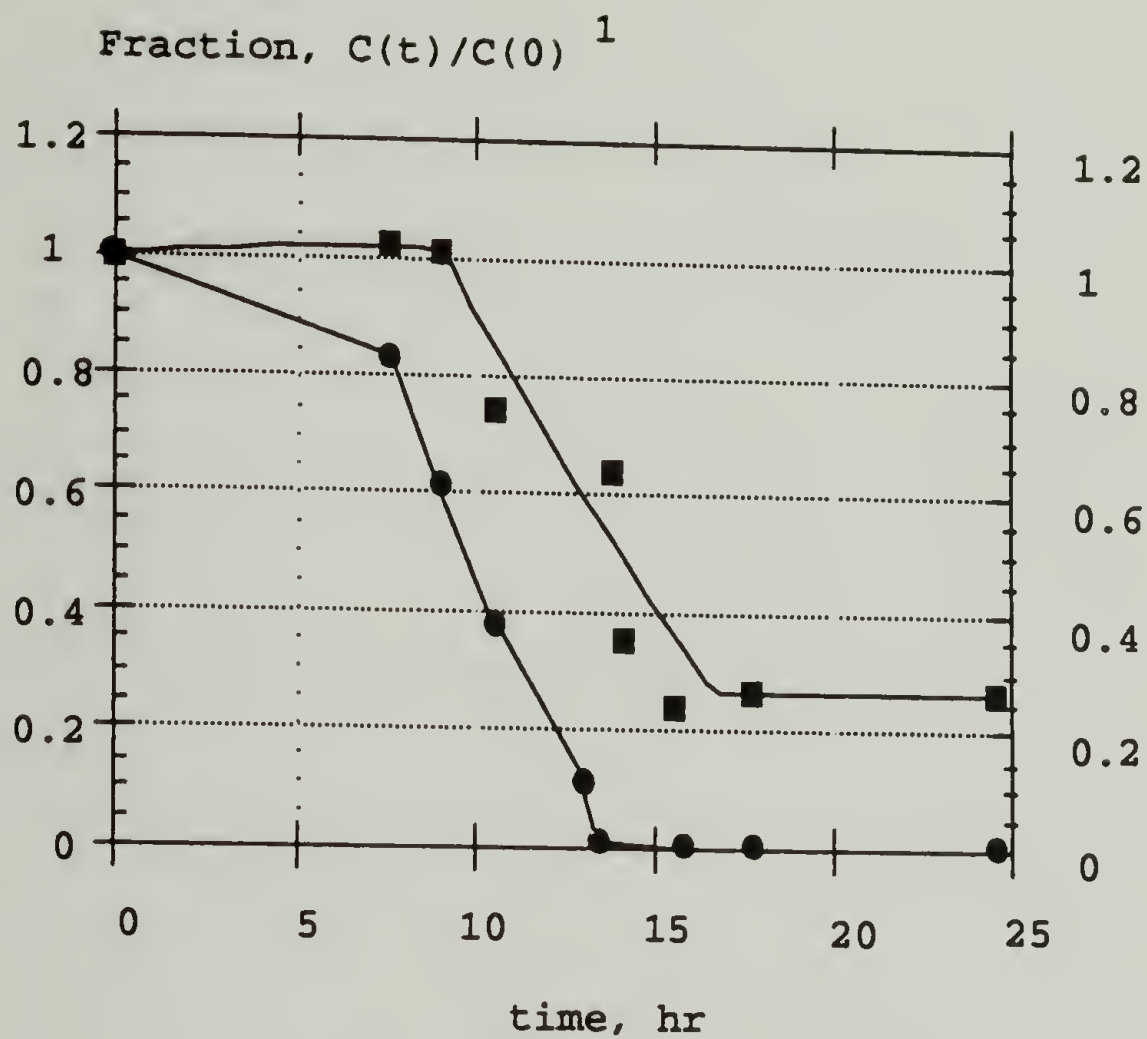
3, aeration rate and agitation rate were 21/min and 100 r.p.m. for first 10 hours and then increased to 61/min and 200 r.p.m. for two hours before harvesting.



Table 25. The relative amount of C5, C7, and C9 units in PHAs obtained from cells harvested at different times grown with an equimolar mixture of nonanoic acid, NA, and 5-phenylvaleric acid, PVA.

Harvesting time, hr	Repeating units, Cn (mole%) <sup>1</sup>		
	C5	C7	C9
6.2	1.8	29.9	68.3
9.7	1.8	29.5	68.7
13.7	1.6	25.0	73.5
15.0	1.7	25.1	73.2
16.5	2.1	25.6	72.3
18.5	1.8	23.2	75.1
24.7	1.9	24.8	73.3

1, See footnotes in Table 23.



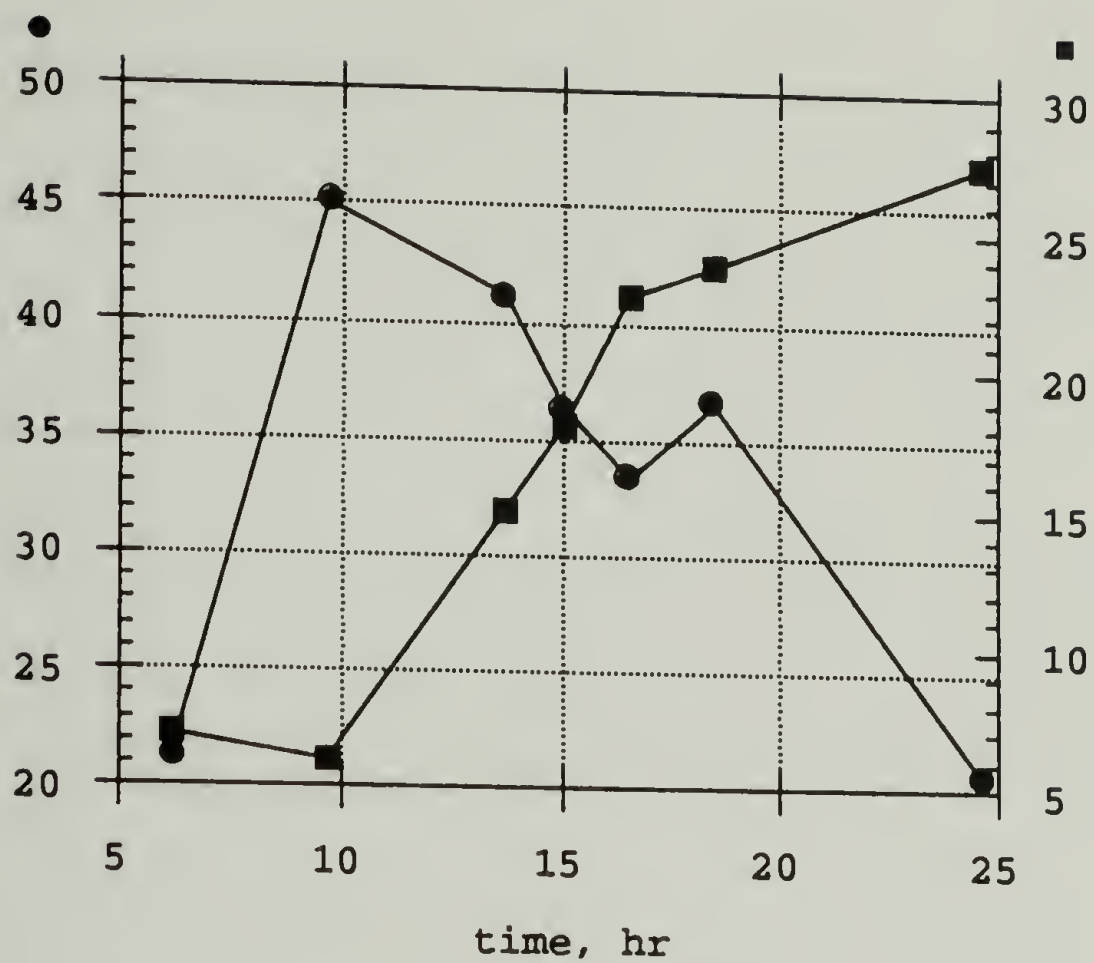
● nonanoic acid

■ 5-phenylvaleric acid

1,  $C(t)$ -concentration at time  $t$ ;

$C(0)$ -concentration at time 0

Figure 85. Fractions of nonanoic acid and 5-phenylvaleric acid remaining in the medium vs. growth time.



● PHA content (wt%)  
 ■ Mol% of HPV units

Figure 86. Fraction of HPV units and PHA contents in the biomass harvested at different growth times.



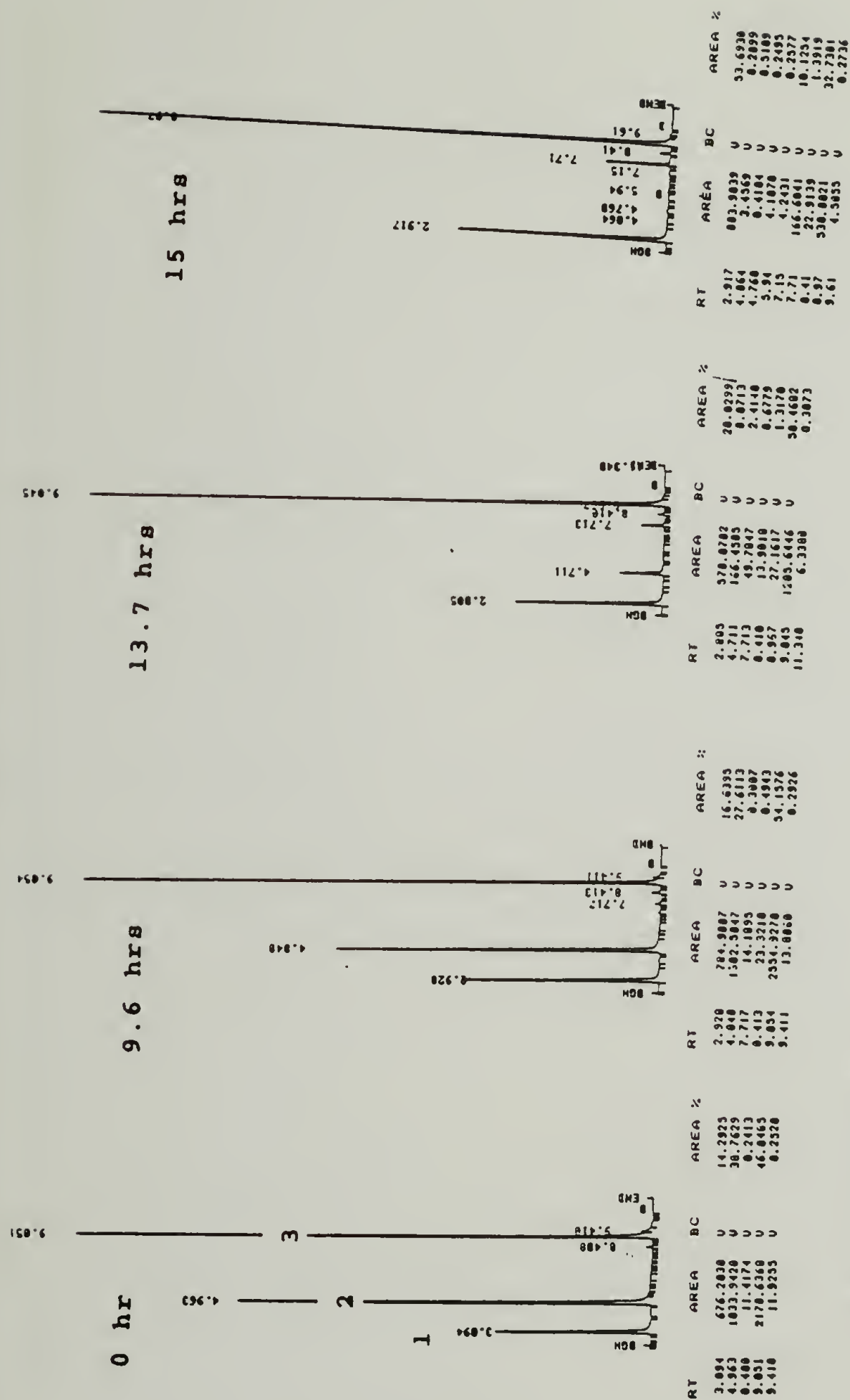


Figure 87. Gas chromatograms of growth medium for the analysis of NA and PVA remaining in the medium at different growth times.

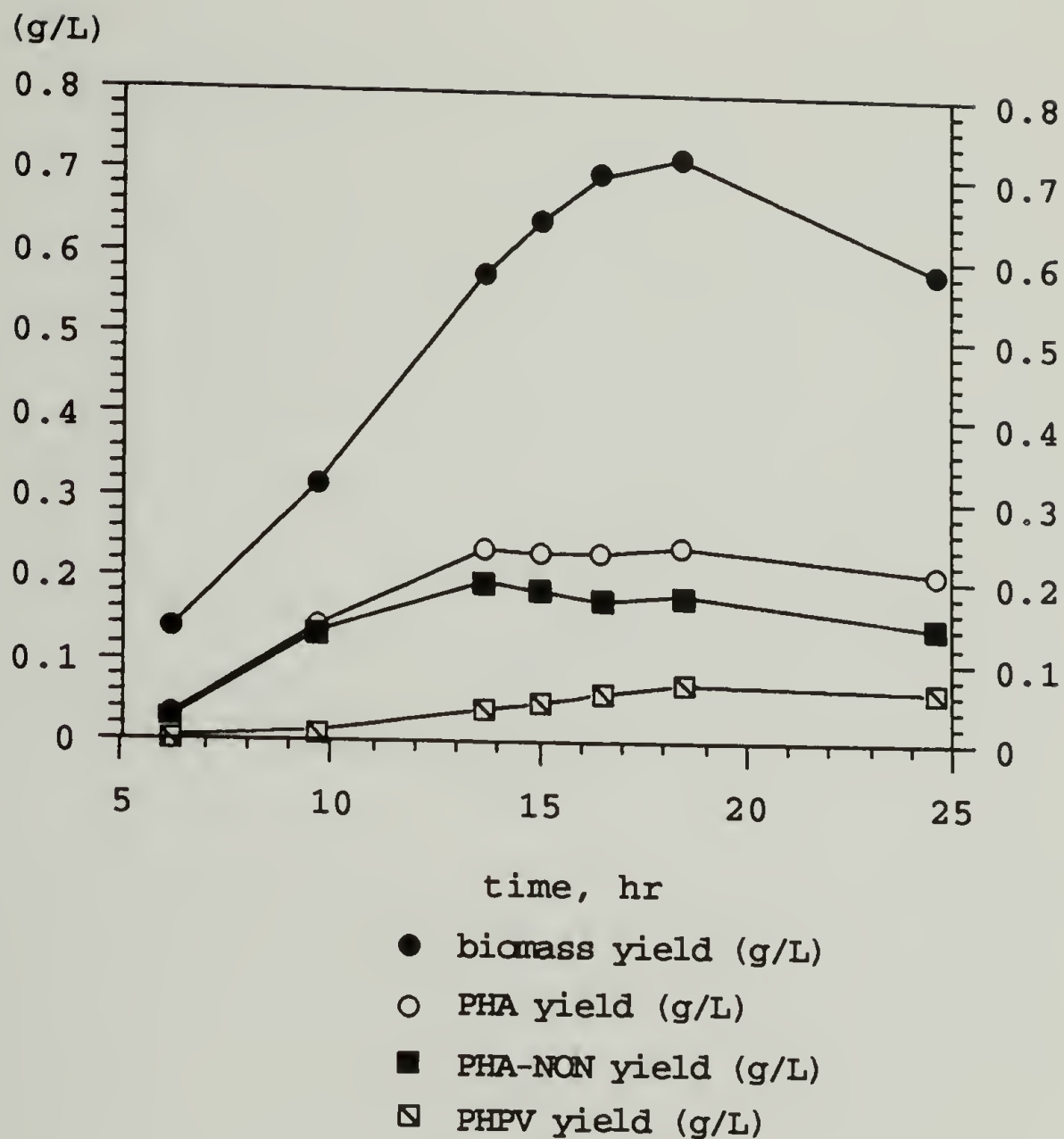


Figure 88. Biomass yield, PHA yield, PHA-NON yield, and PHPV yield from a culture grown with an equimolar mixture of NA and PVA vs. growth time.

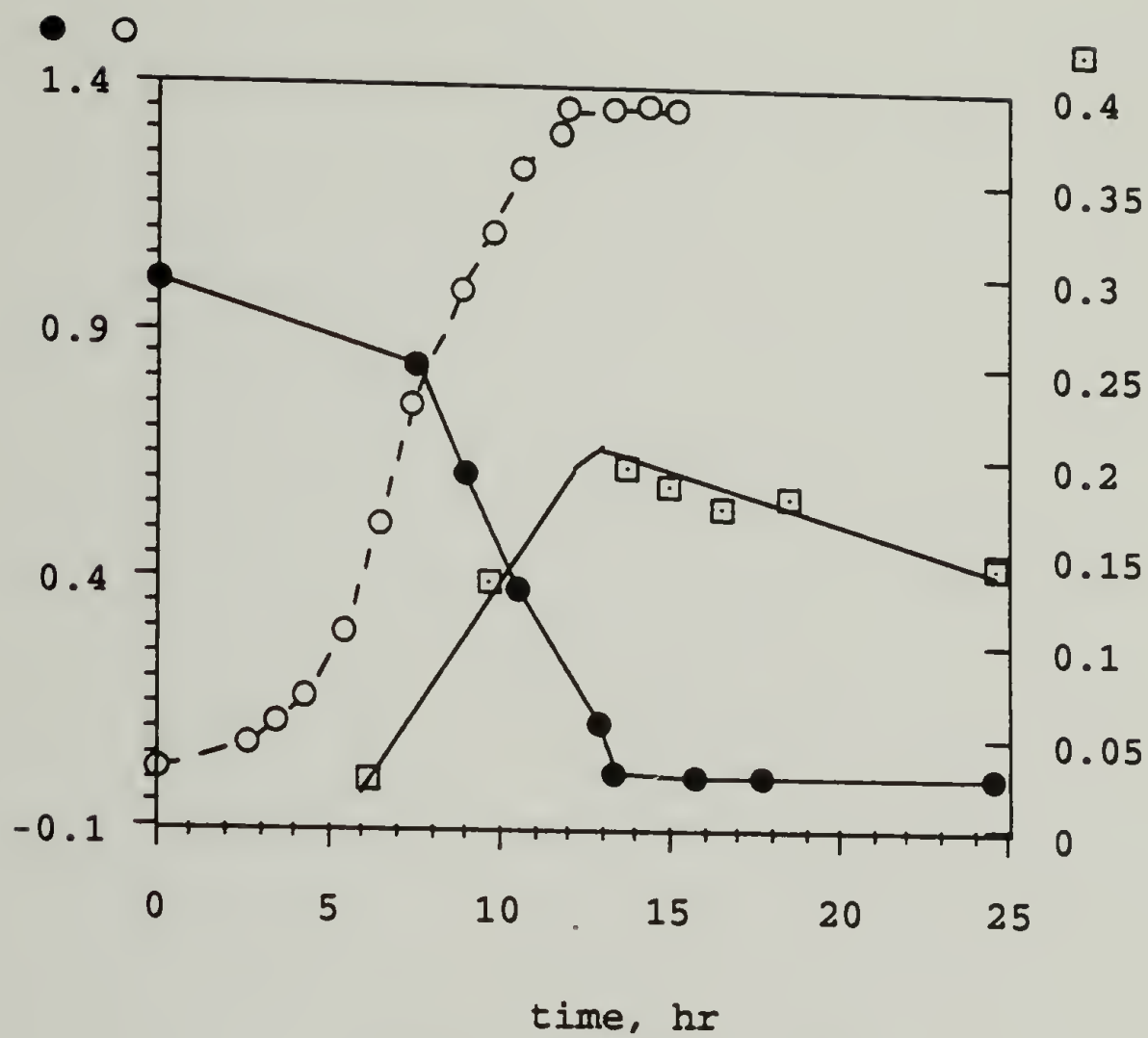


Figure 89. PHA-NON yield, fraction of nonanoic acid, NA, remaining in the medium, optical density of a culture grown with a 5 mM NA medium vs. growth time.



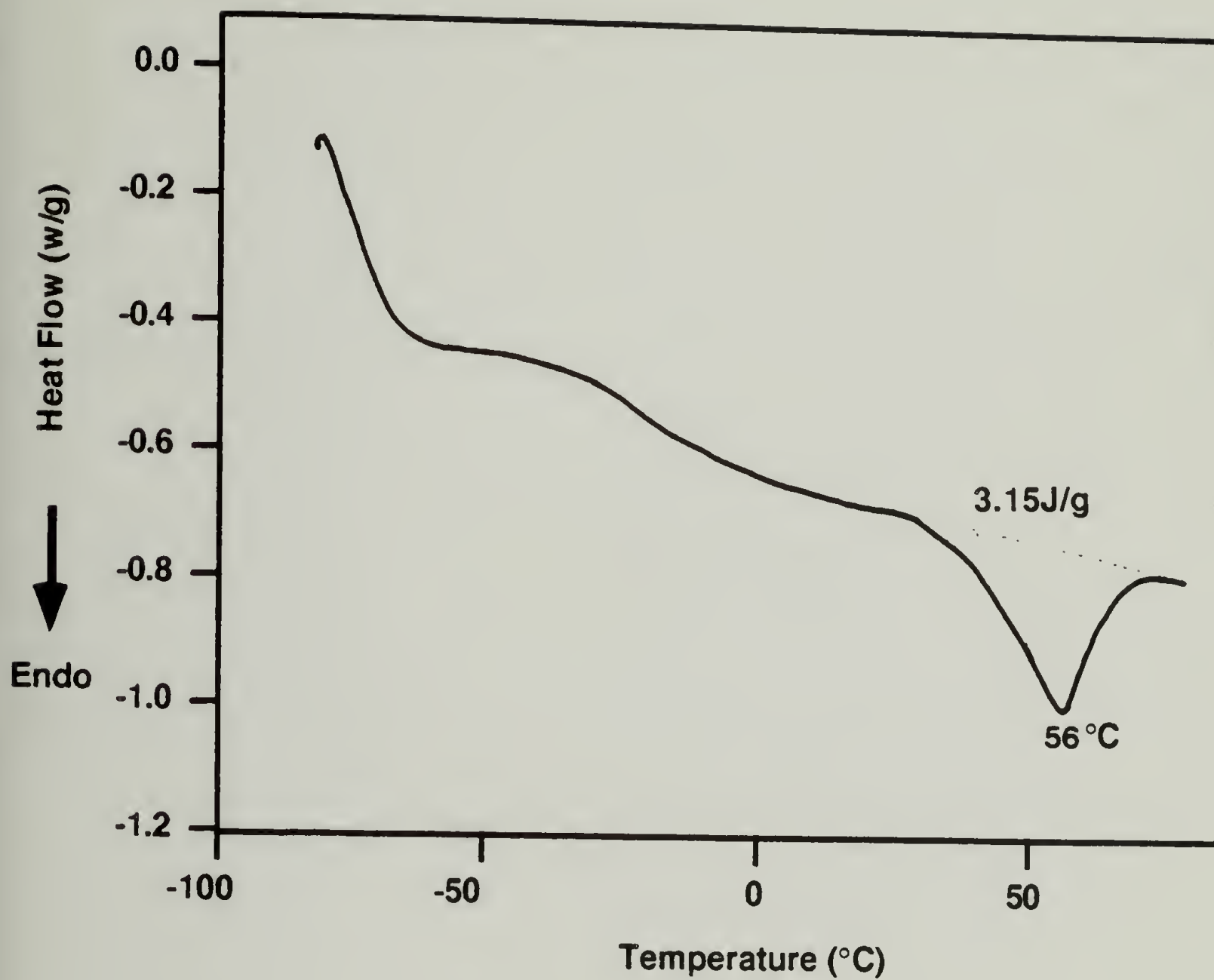


Figure 90. DSC thermogram of a PHA produced from a 2:1 mixture of OA and PVA.

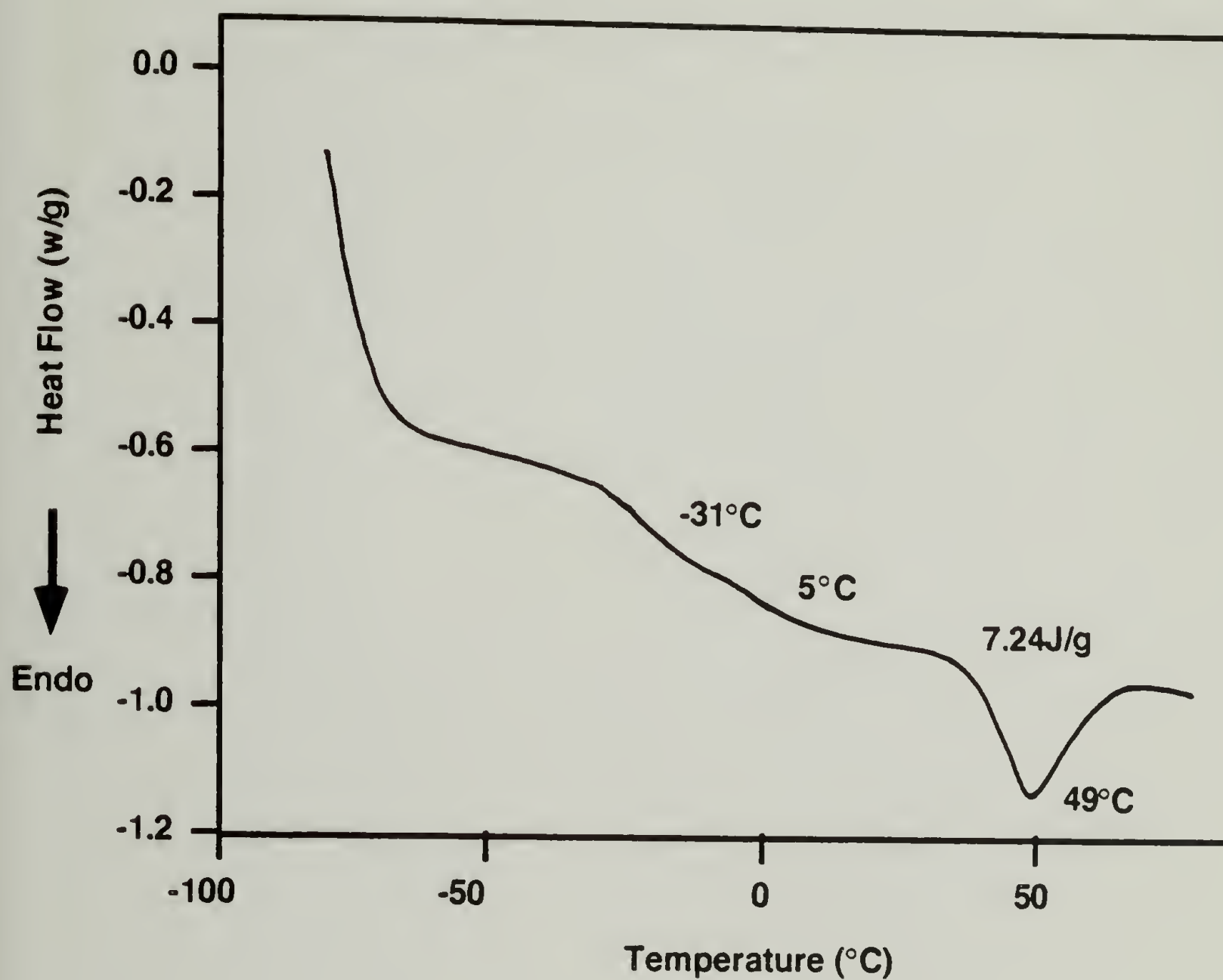


Figure 91. DSC thermogram of a PHA produced from a 1:2 mixture of NA and PVA.

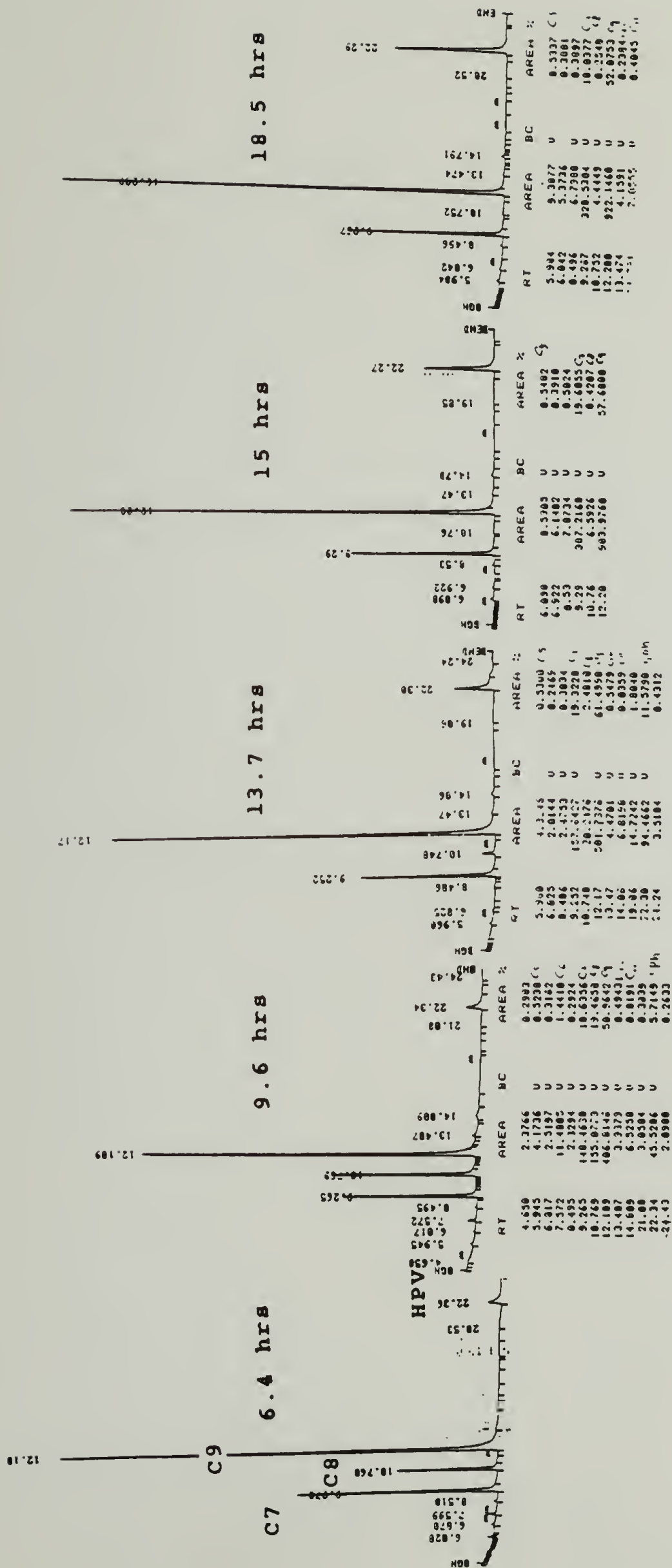


Figure 92. Gas chromatograms of methanolized samples of PHAS harvested at different growth times.



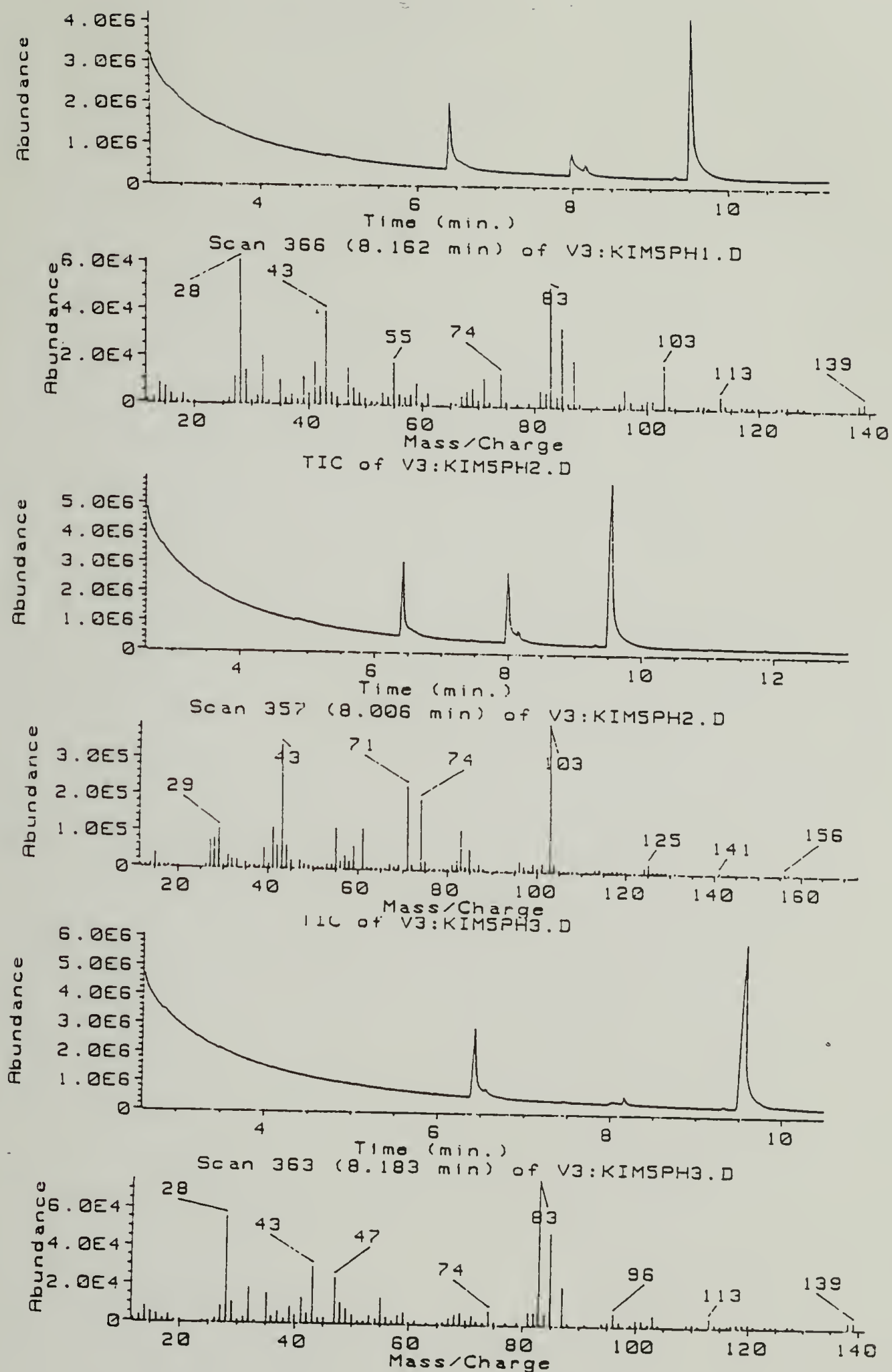


Figure 93. TIC and mass spectrum of C8 unit in PHA harvested in early growth phase.

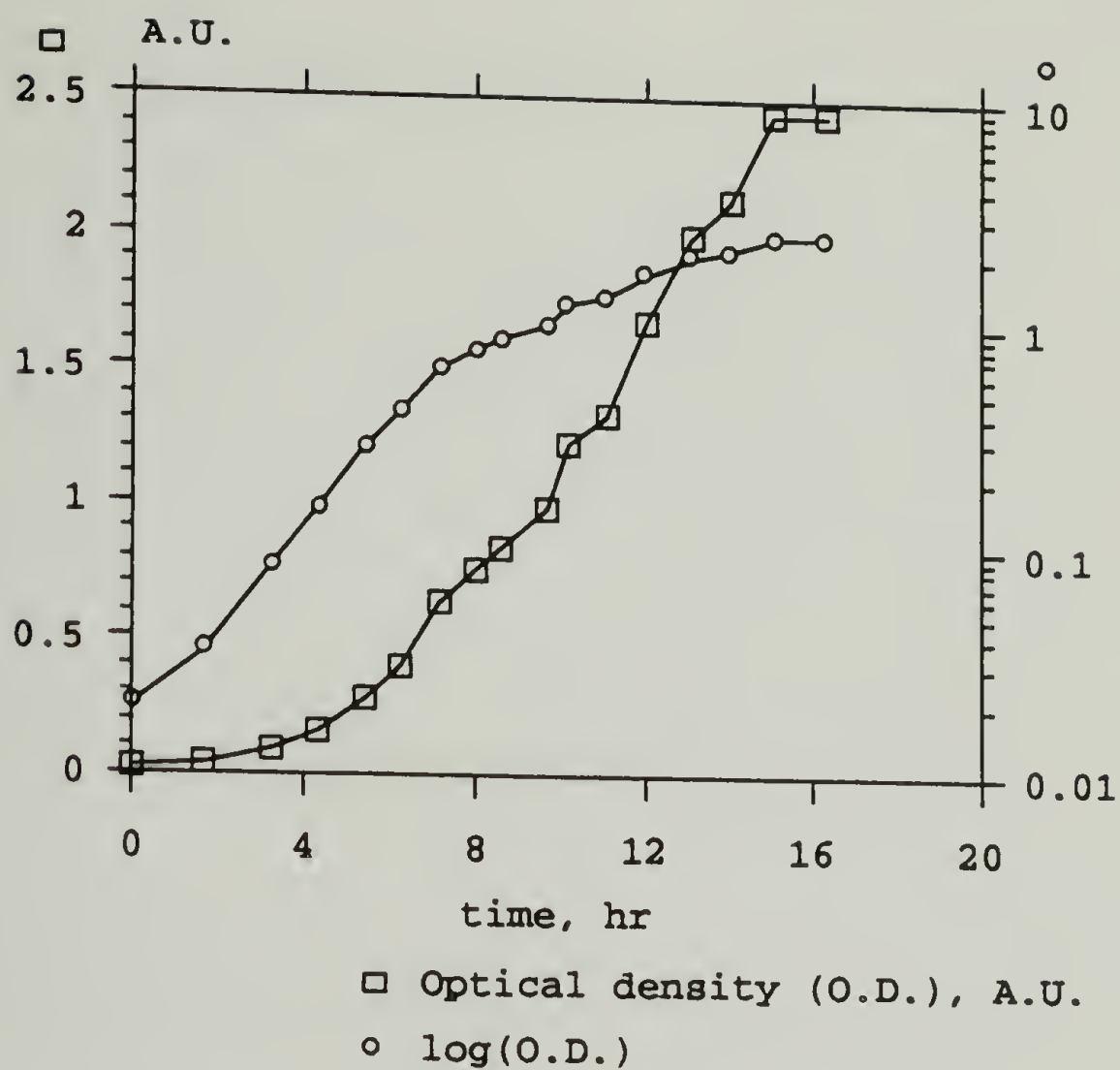
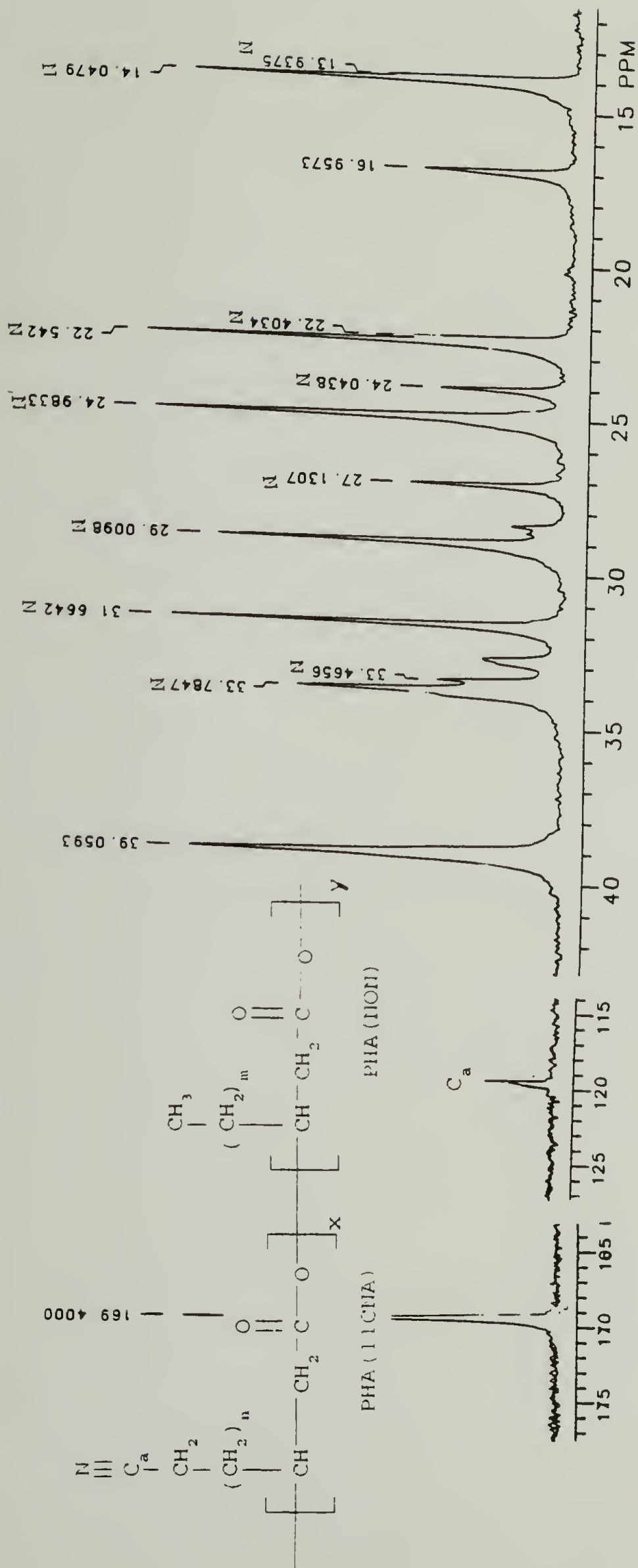


Figure 94. Growth plots for *P. oleovorans* grown with an equimolar mixture of nonanoic acid and 11-cyanoundecanoic acid.







N represents C's from PHA (NON)

Figure 96. <sup>13</sup>C NMR spectrum of the PHA produced on an equimolar mixture of nonanoic acid and 11-cyanoundecanoic acid.

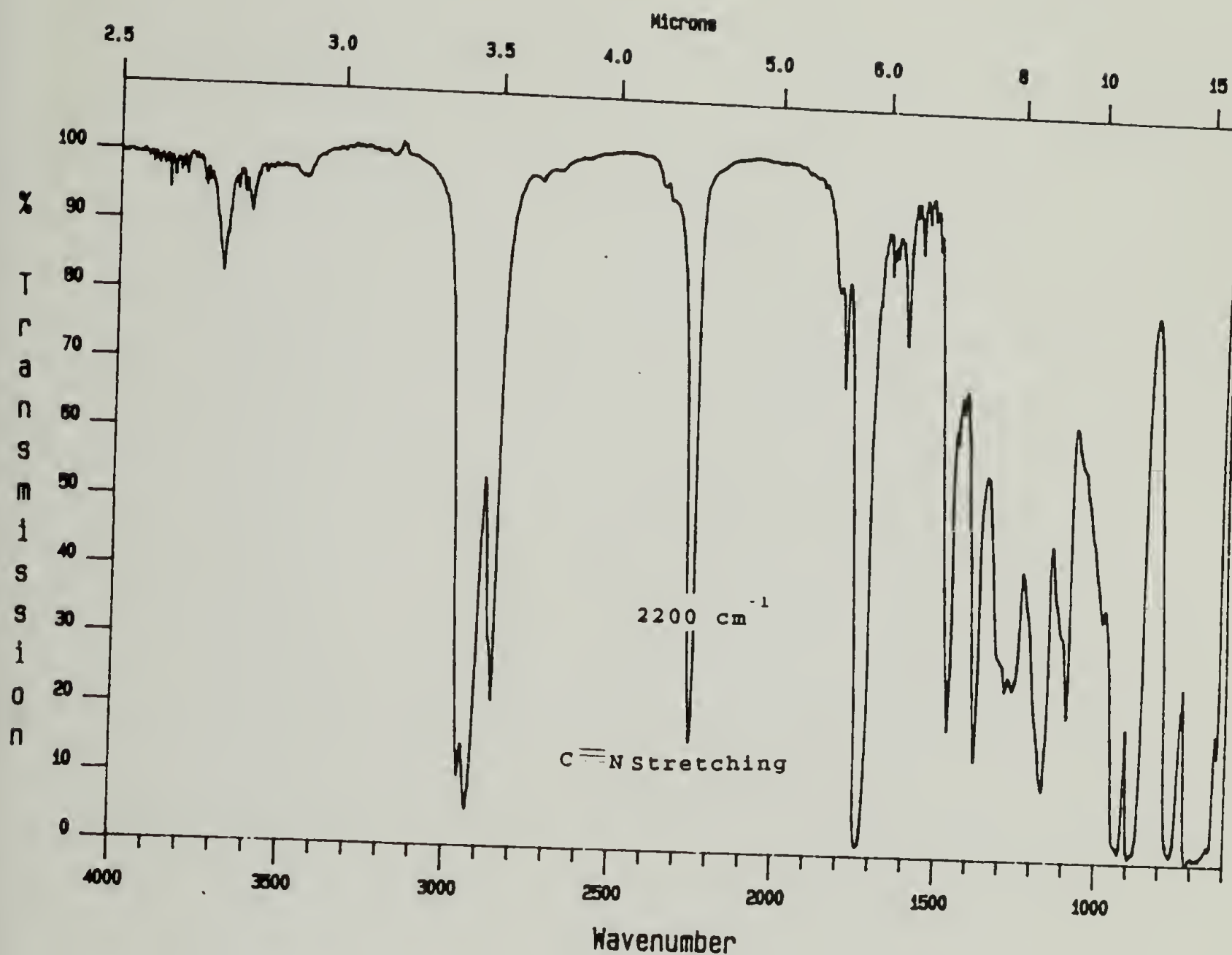


Figure 97. IR spectrum of the PHA produced on an equimolar mixture of nonanoic acid and 11-cyanoundecanoic acid.

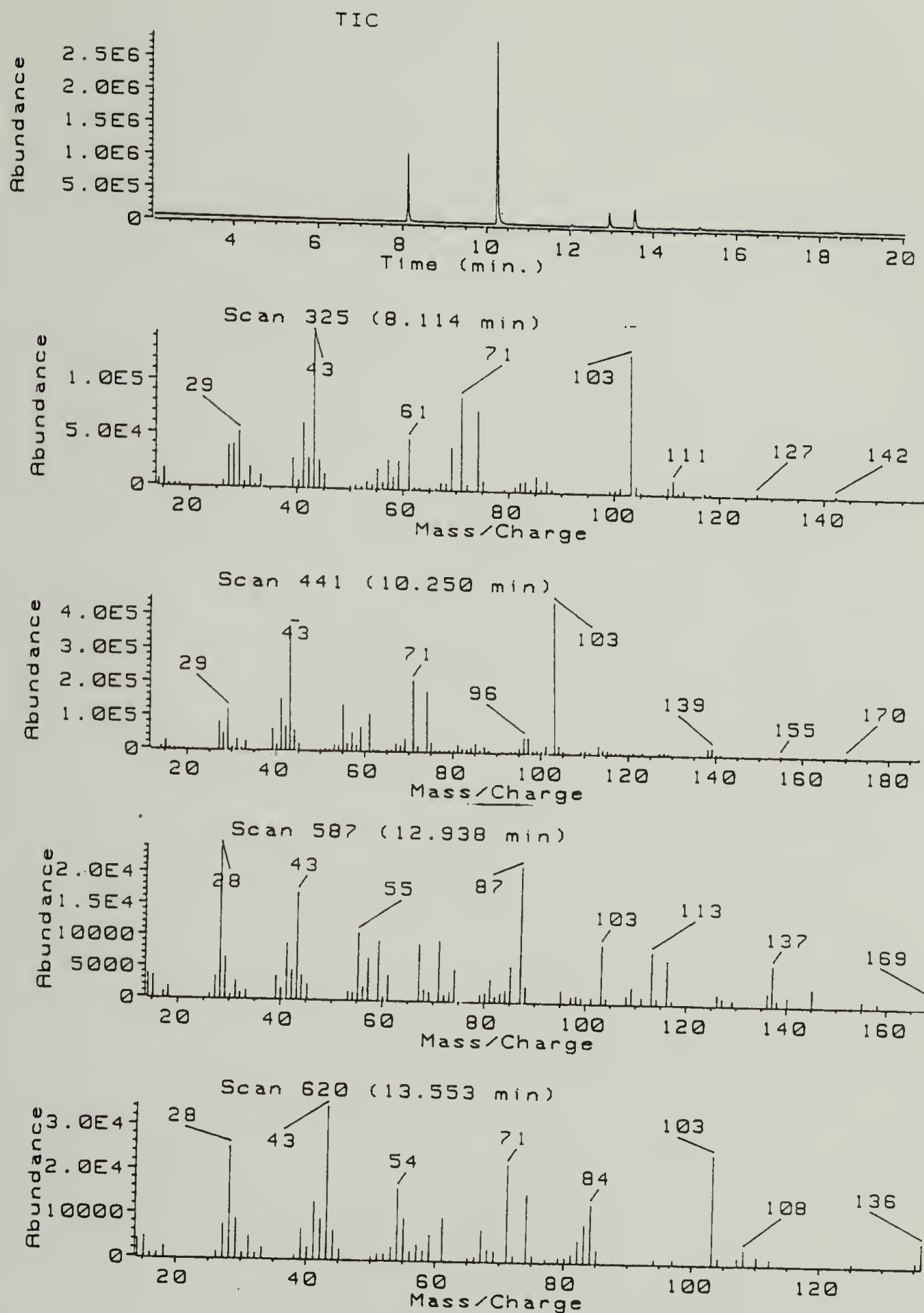


Figure 98. TIC of the methanolized sample of the PHA produced on an equimolar mixture of nonanoic acid and 11-cyanoundecanoic acid.



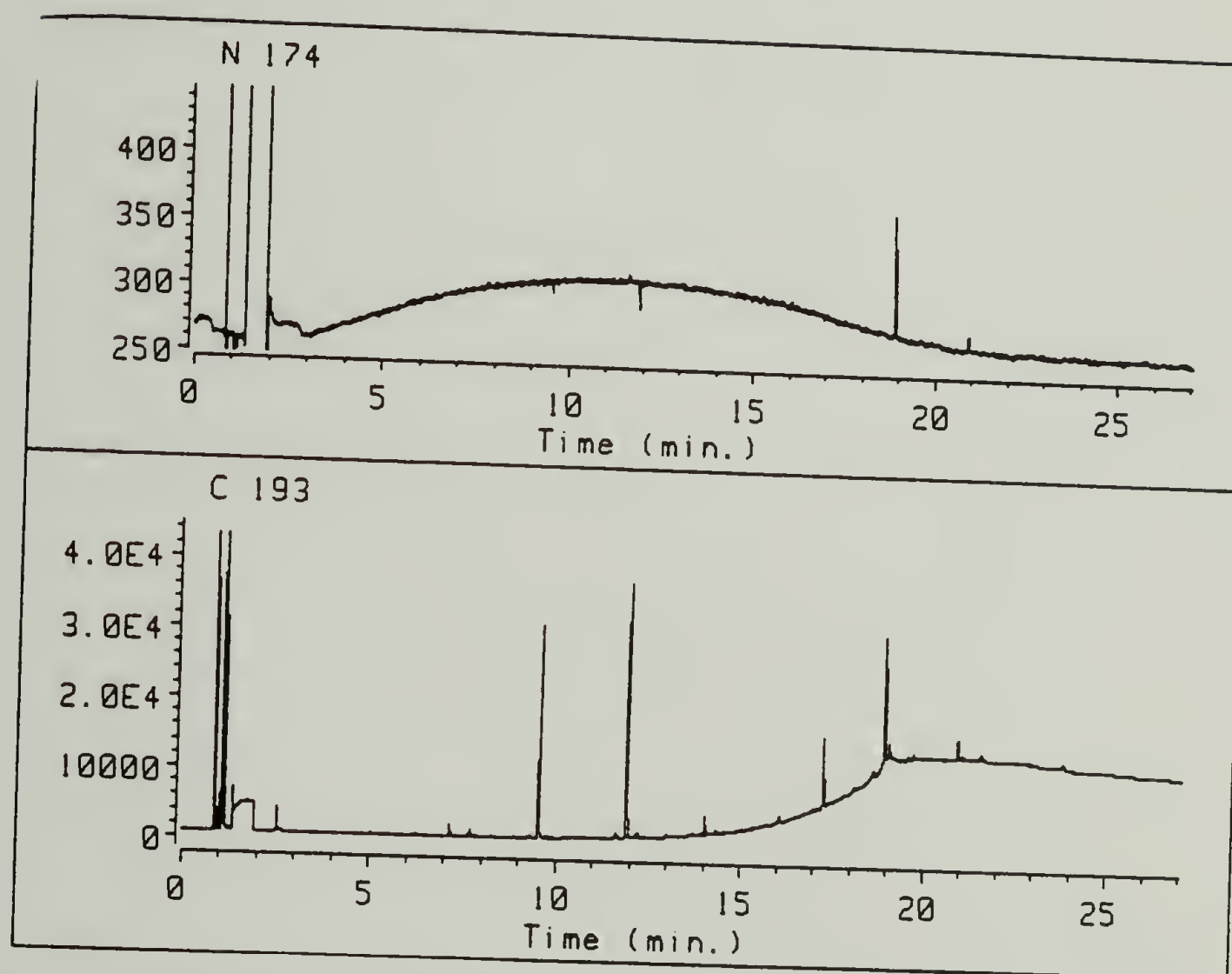
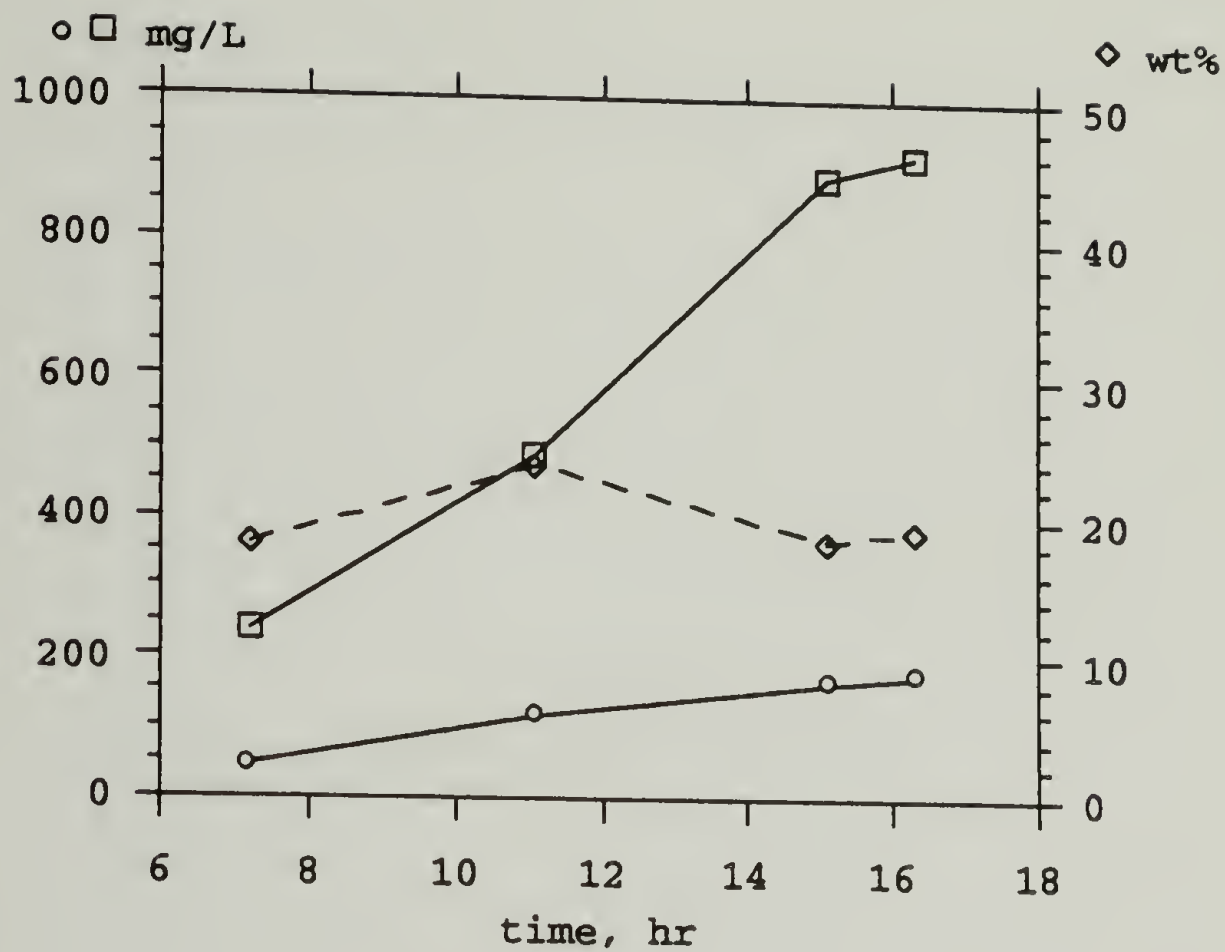


Figure 99. GC/AED chromatogram of the methanolized sample of the PHA produced from an equimolar mixture of nonanoic acid and 11-cyanoundecanoic acid.



○ PHA (mg/L)  
 □ Biomass yield (mg/L)  
 ◇ PHA Content (Wt%)

Figure 100. Biomass yield, PHA yield, and PHA content vs. growth time, growth with an equimolar mixture of nonanoic acid and 11-cyanoundecanoic acid.

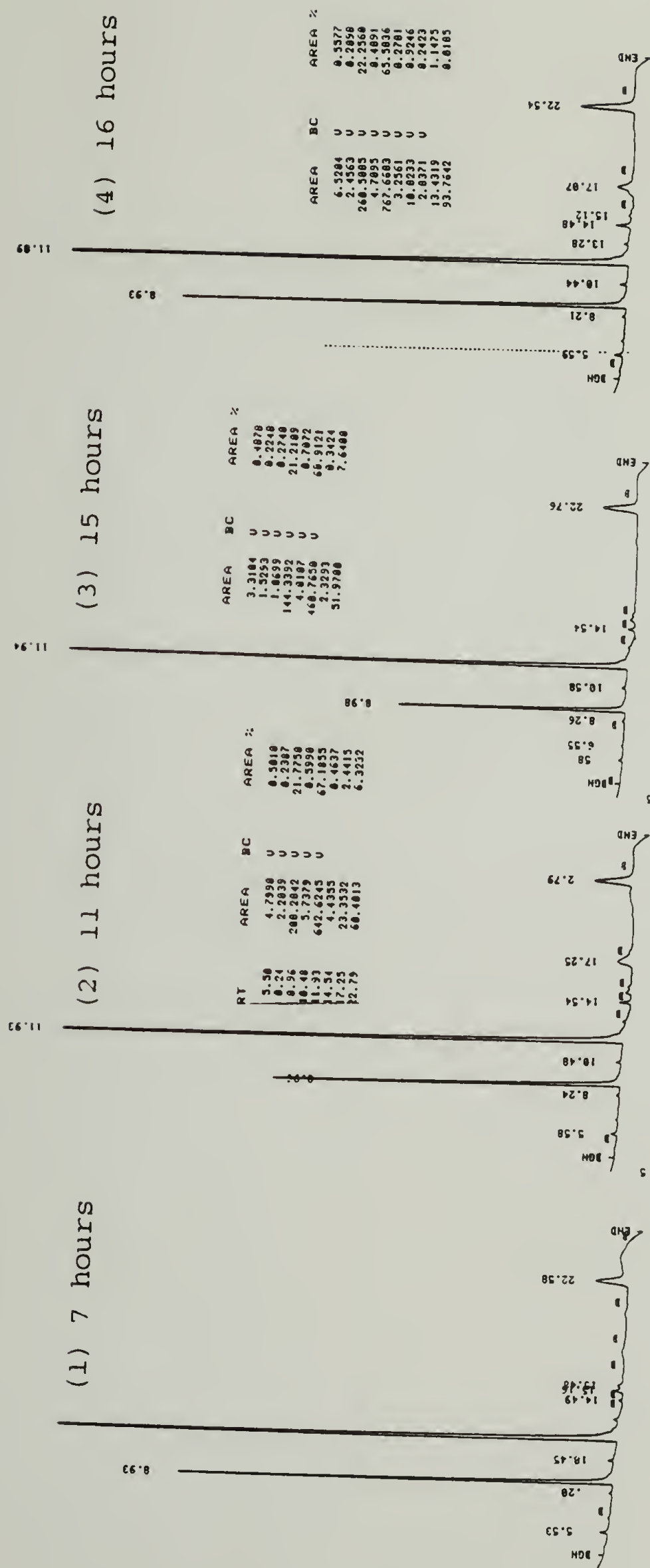
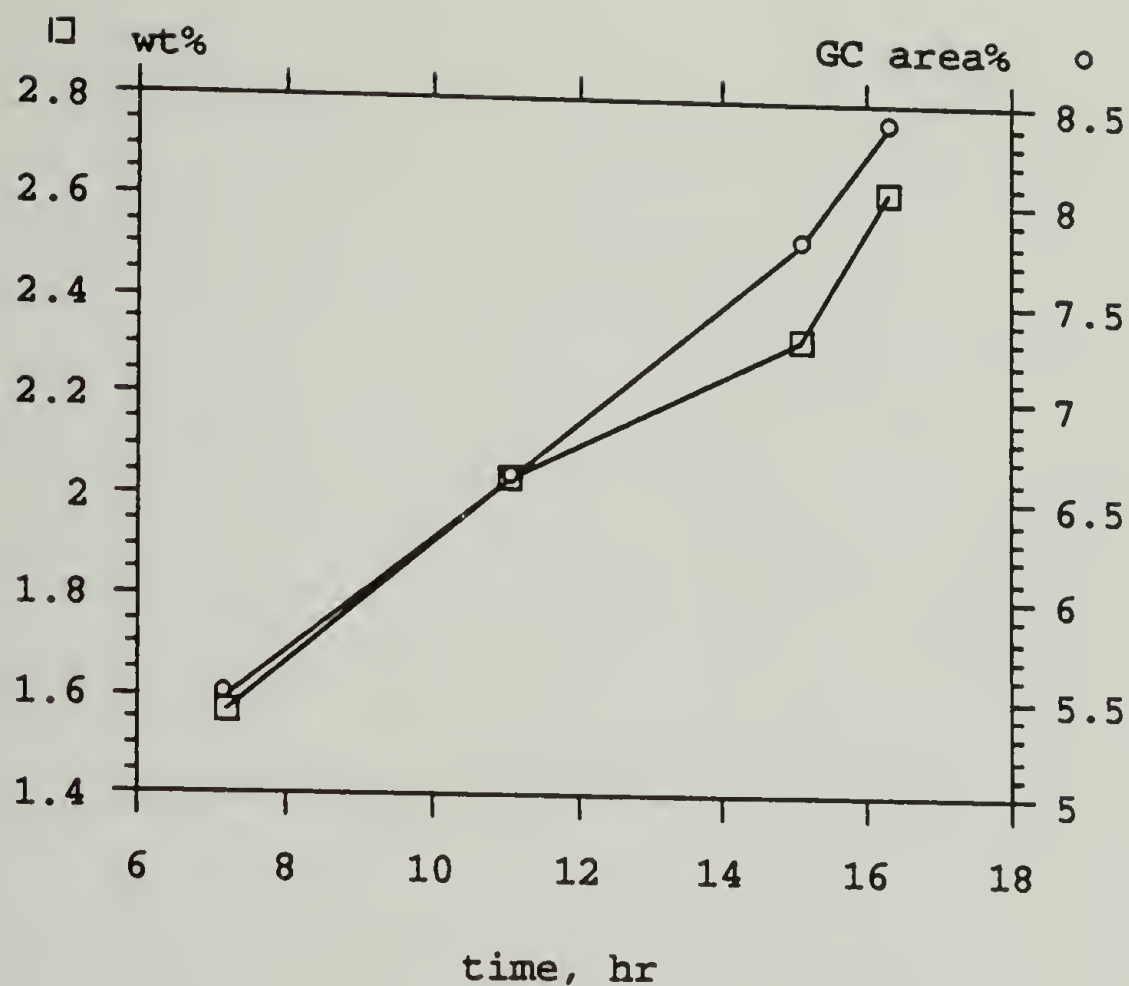


Figure 101. The gas chromatograms of methanolized samples of PHAs isolated from cells grown with an equimolar mixture of nonanoic acid and 11-cyanoundecanoic acid at different times.





□ Wt% of nitrogen

○ GC area % of cyanounit

Figure 102. Weight percent of nitrogen and GC area percent of the repeating units containing cyano group vs. growth time (growth with an equimolar mixture of nonanoic acid and 11-cyanoundecanoic acid).

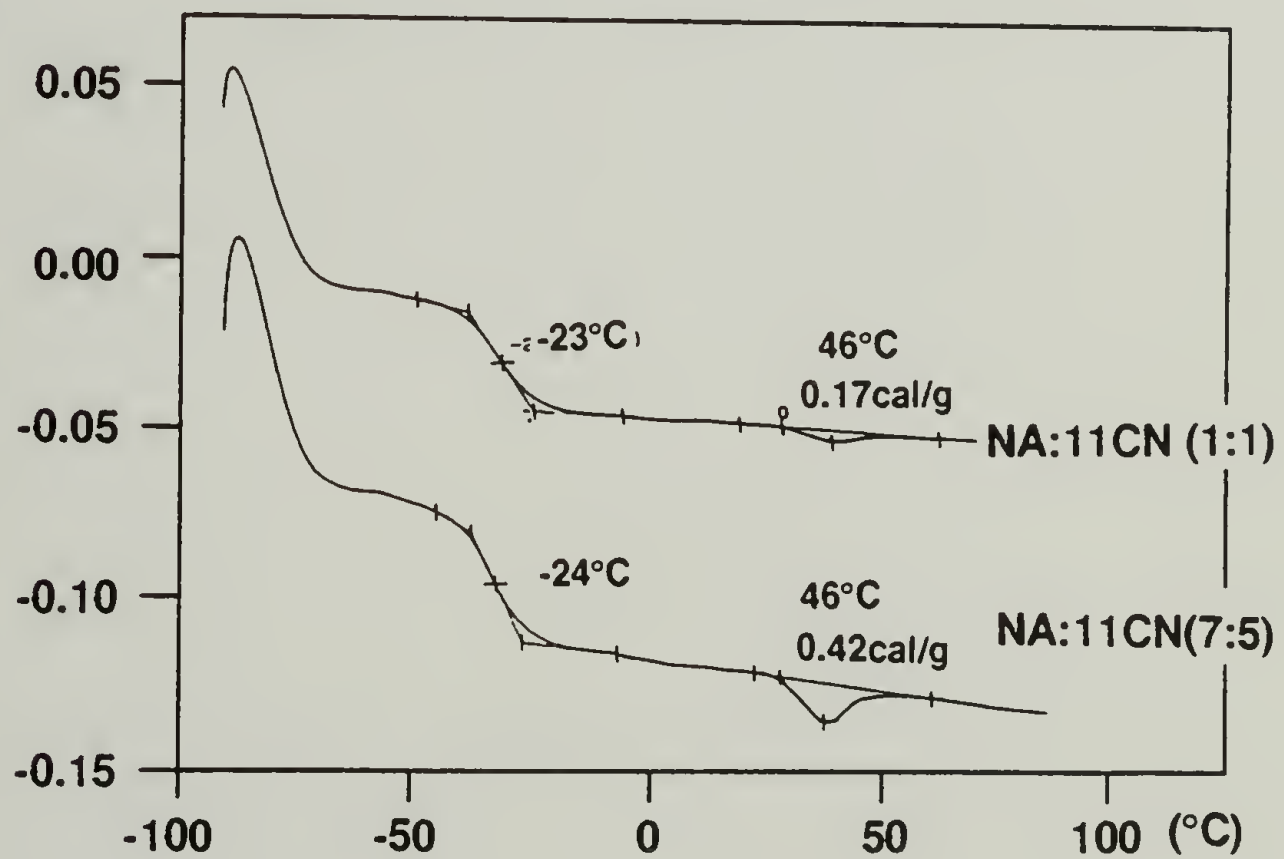
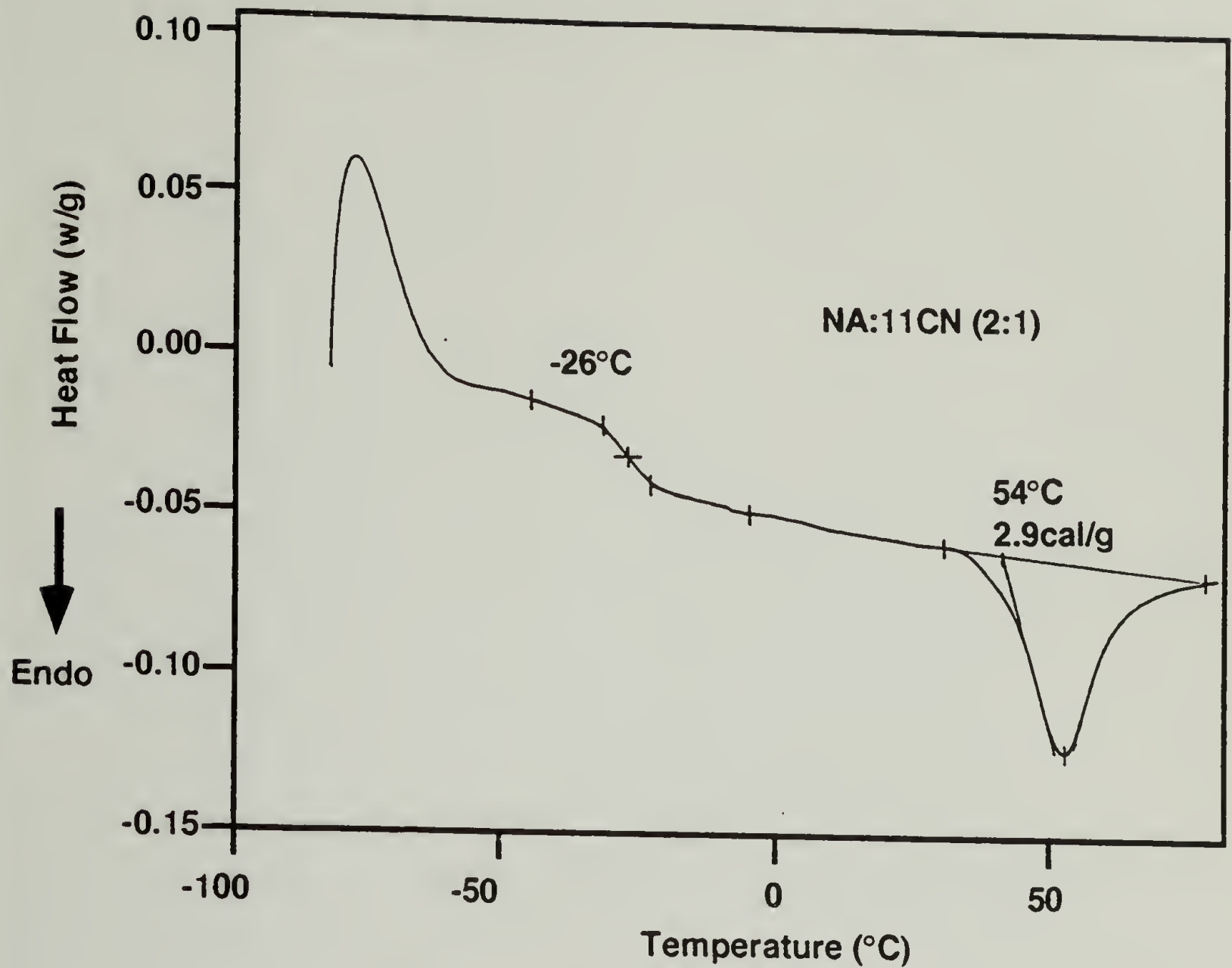


Figure 103. DSC thermogram of the PHA produced on mixtures of nonanoic acid, NA, and 11-cyanoundecanoic acid.

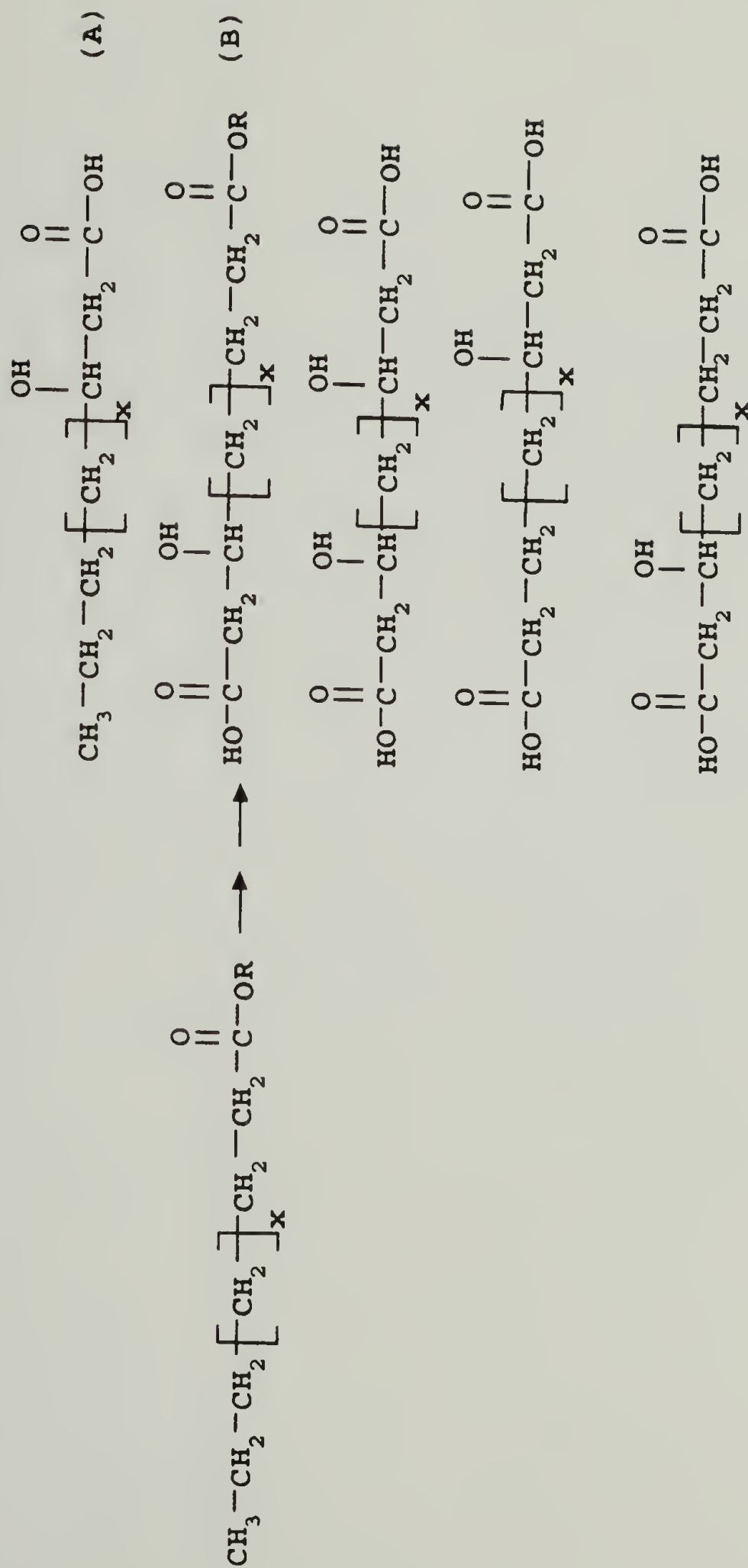


Figure 104. Possible products from esters of alkanolic acid when utilized by *P. oleovorans*.

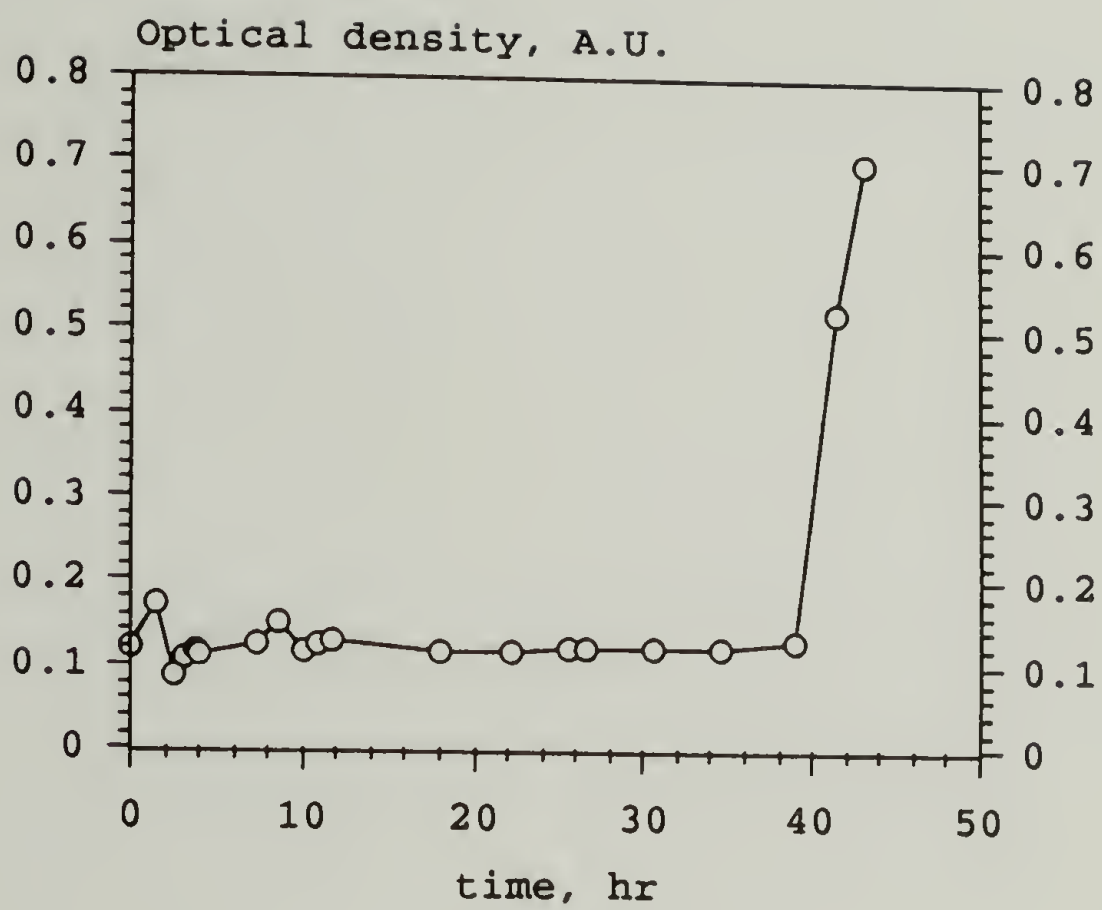


Figure 105. Growth plot of *P. oleovorans* grown with methyl caprylate.



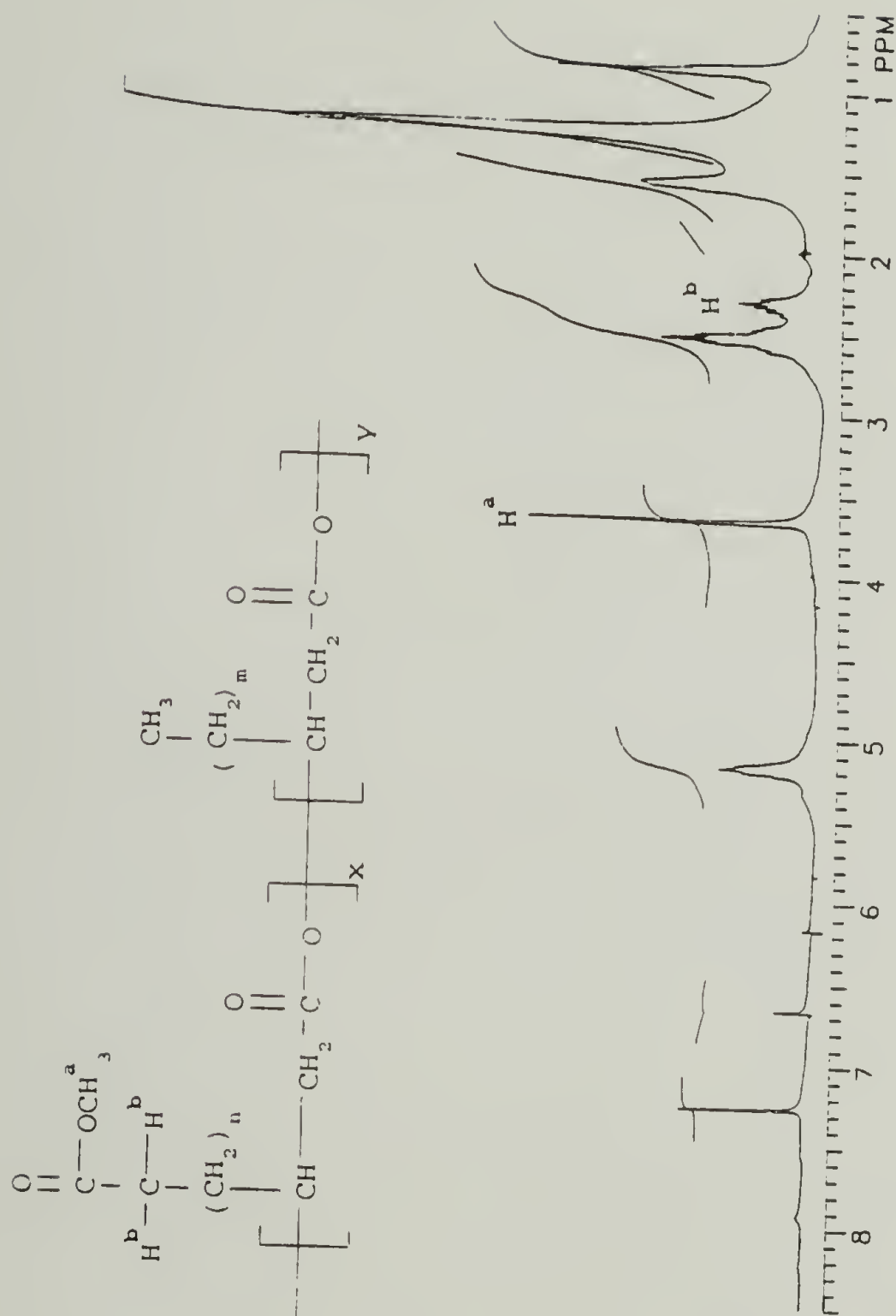


Figure 106.  $^1\text{H}$  NMR spectrum of the PHA produced with methyl caprylate.

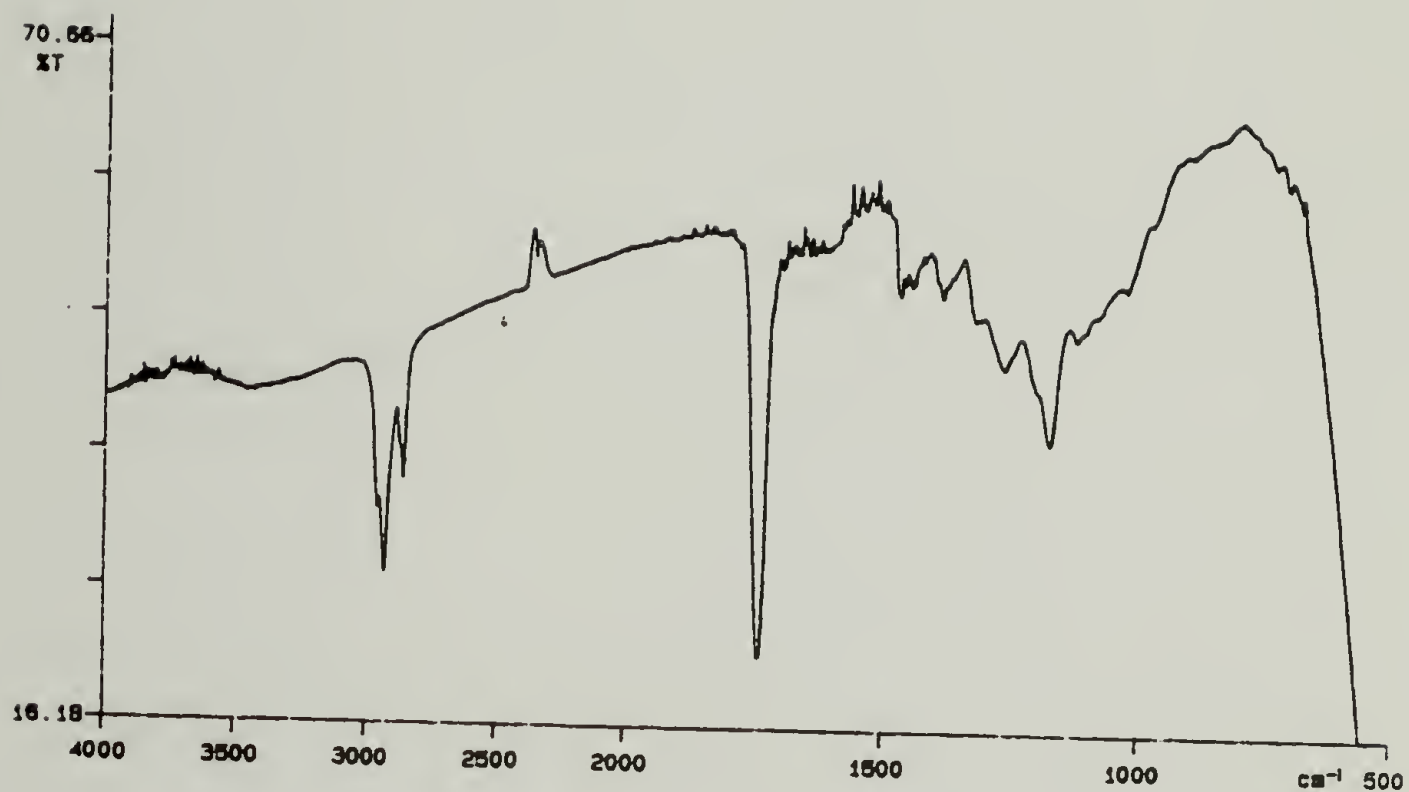


Figure 107. IR spectrum of the PHA produced with methyl caprylate.

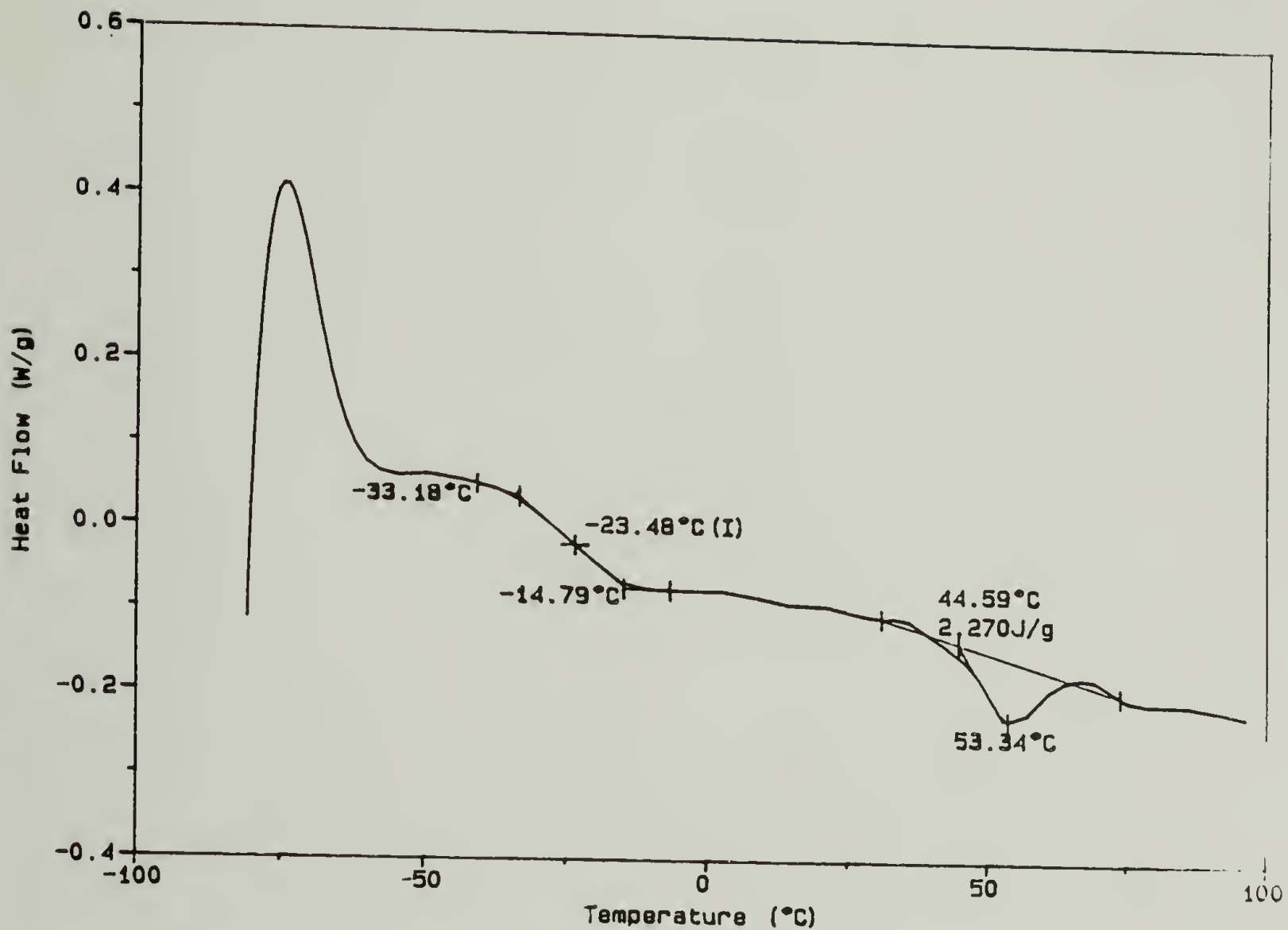


Figure 108. DSC thermogram of the PHA produced with methyl caprylate.

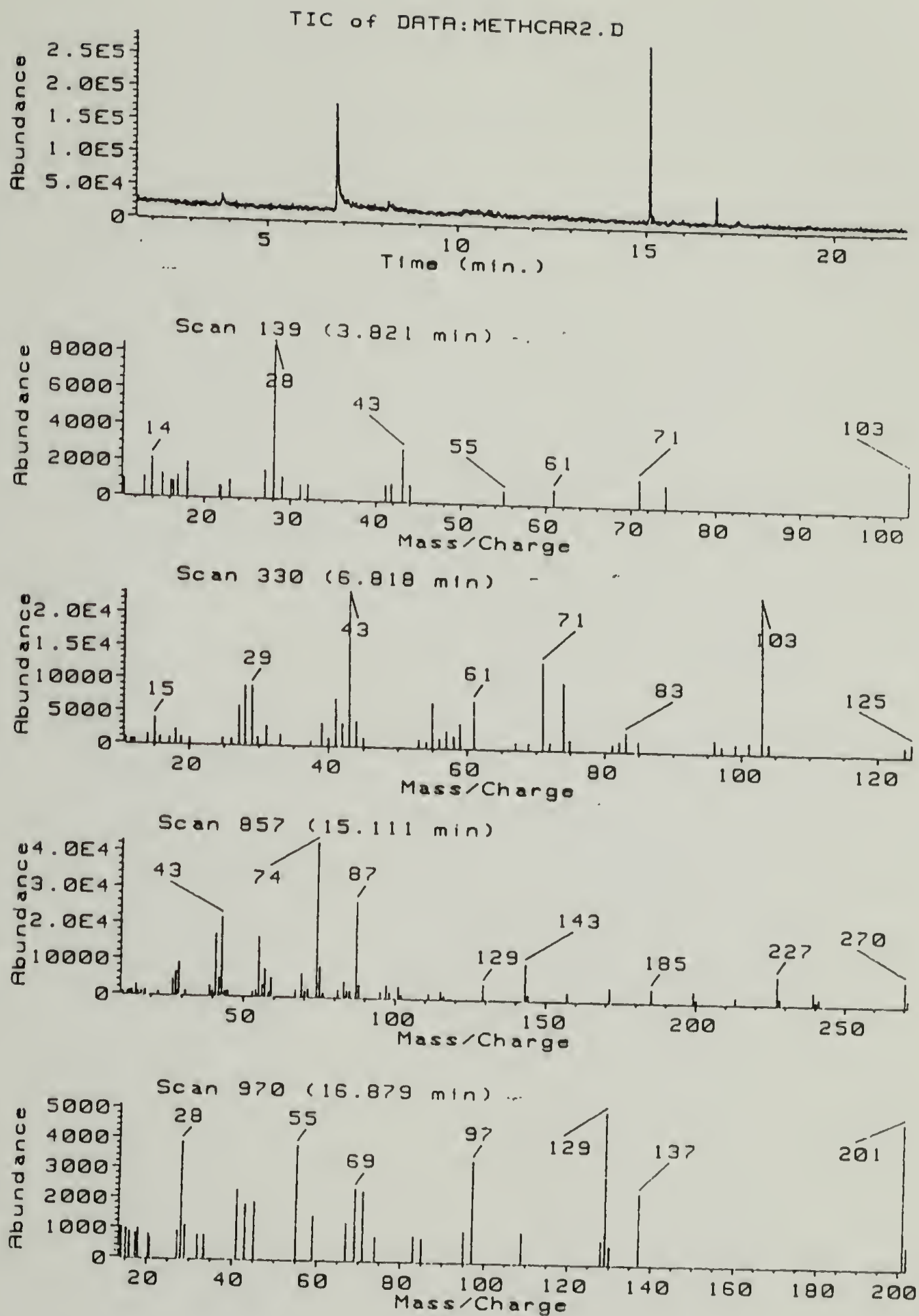


Figure 109. TIC and mass spectra of the methanolized sample of the PHA produced on methyl caprylate.



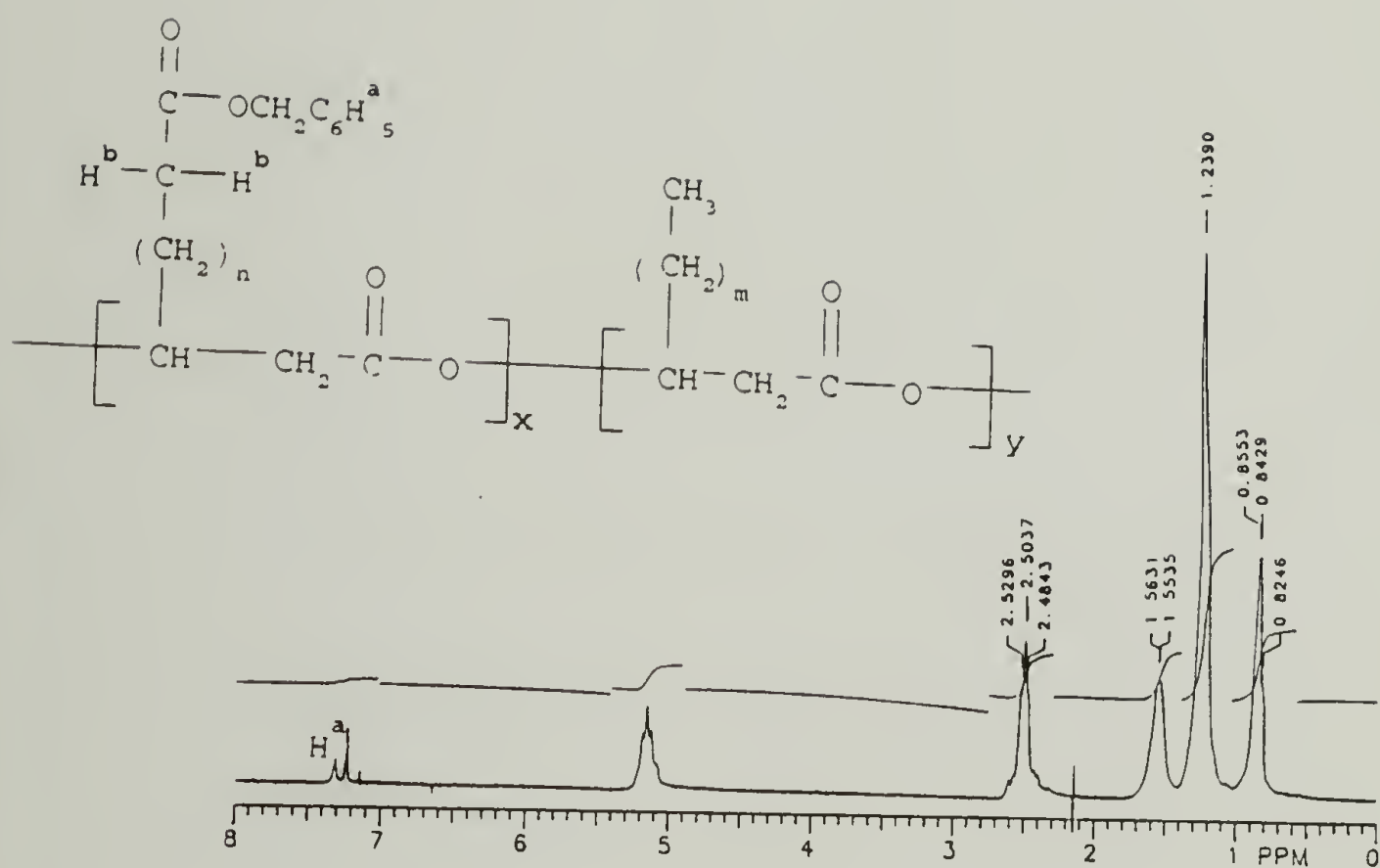


Figure 110.  $^1\text{H}$  NMR spectrum of the PHA produced on a mixture of nonanoic acid and the monobenzyl ester of sebacic acid.

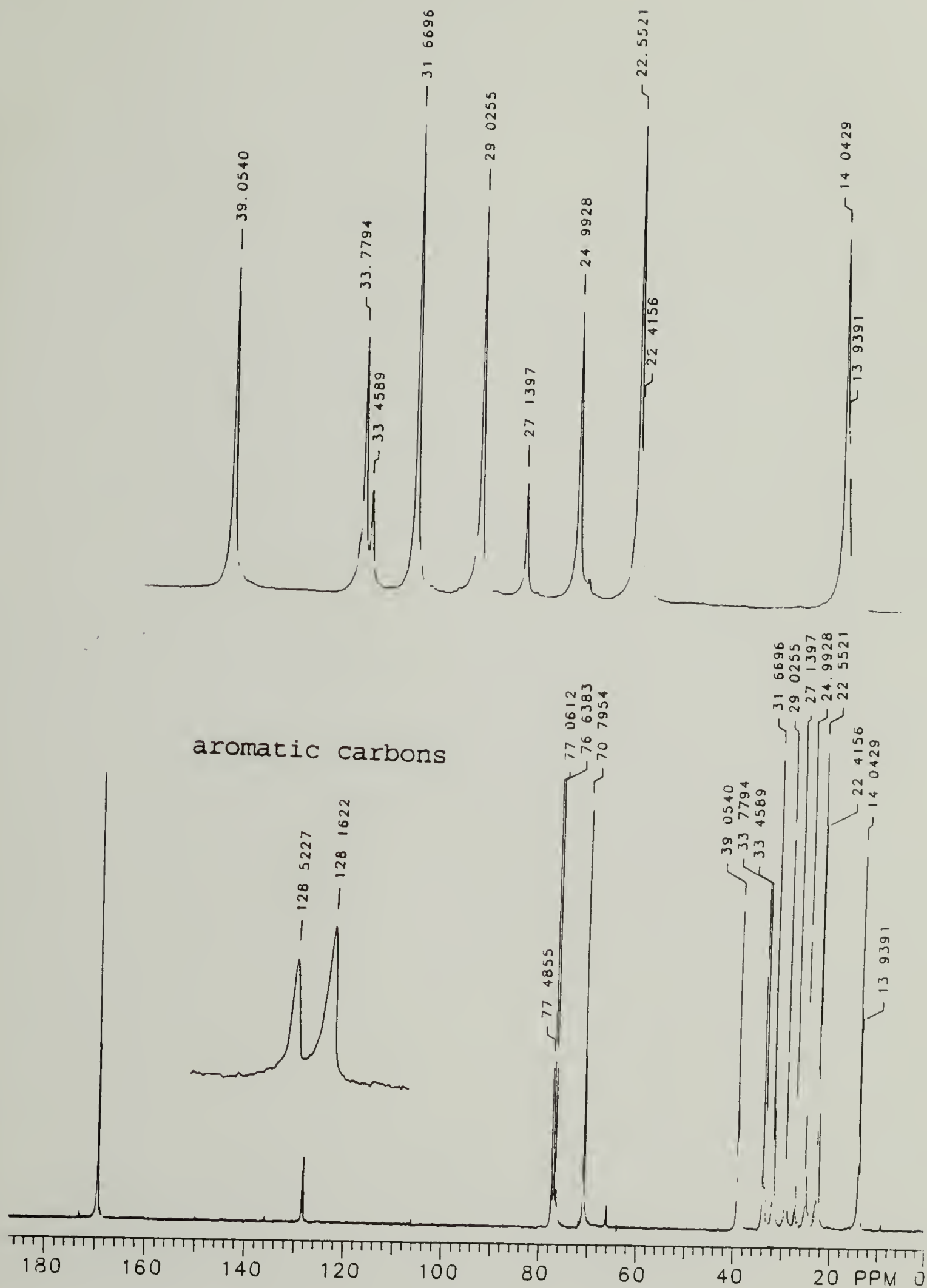


Figure 111.  $^{13}\text{C}$  NMR spectrum of the PHA produced on a mixture of nonanoic acid and the monobenzyl ester of sebacic acid.

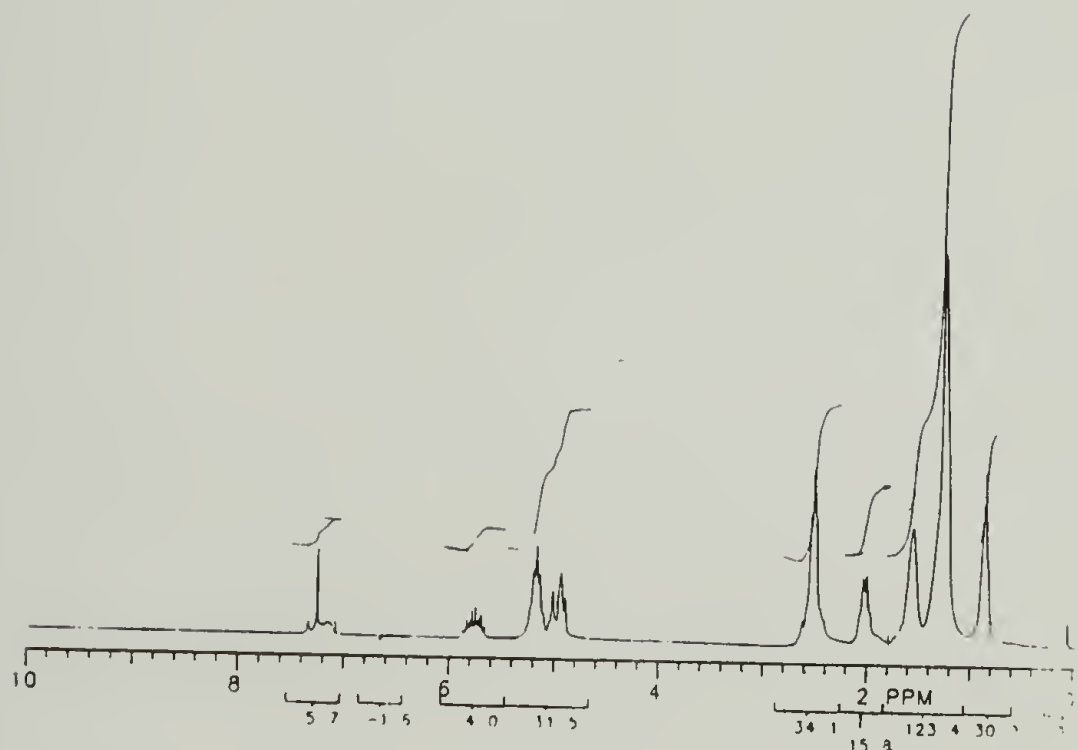


Figure 112.  $^1\text{H}$  NMR spectrum of the PHA produced on a mixture of nonane and benzyl-10-undecenoate.

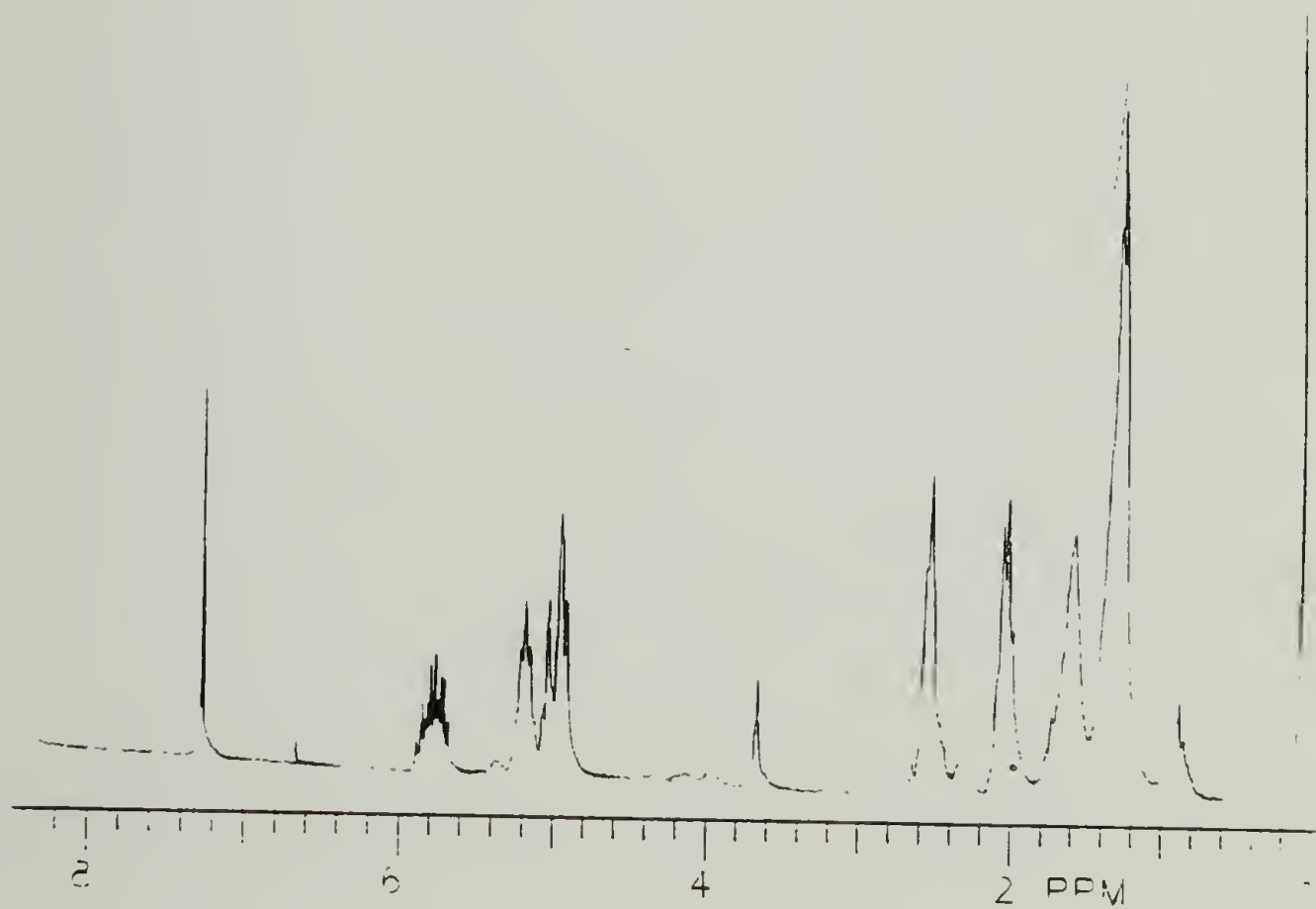


Figure 113.  $^1\text{H}$  NMR spectrum of the PHA produced on a mixture of nonane and methyl-10-undecenoate.



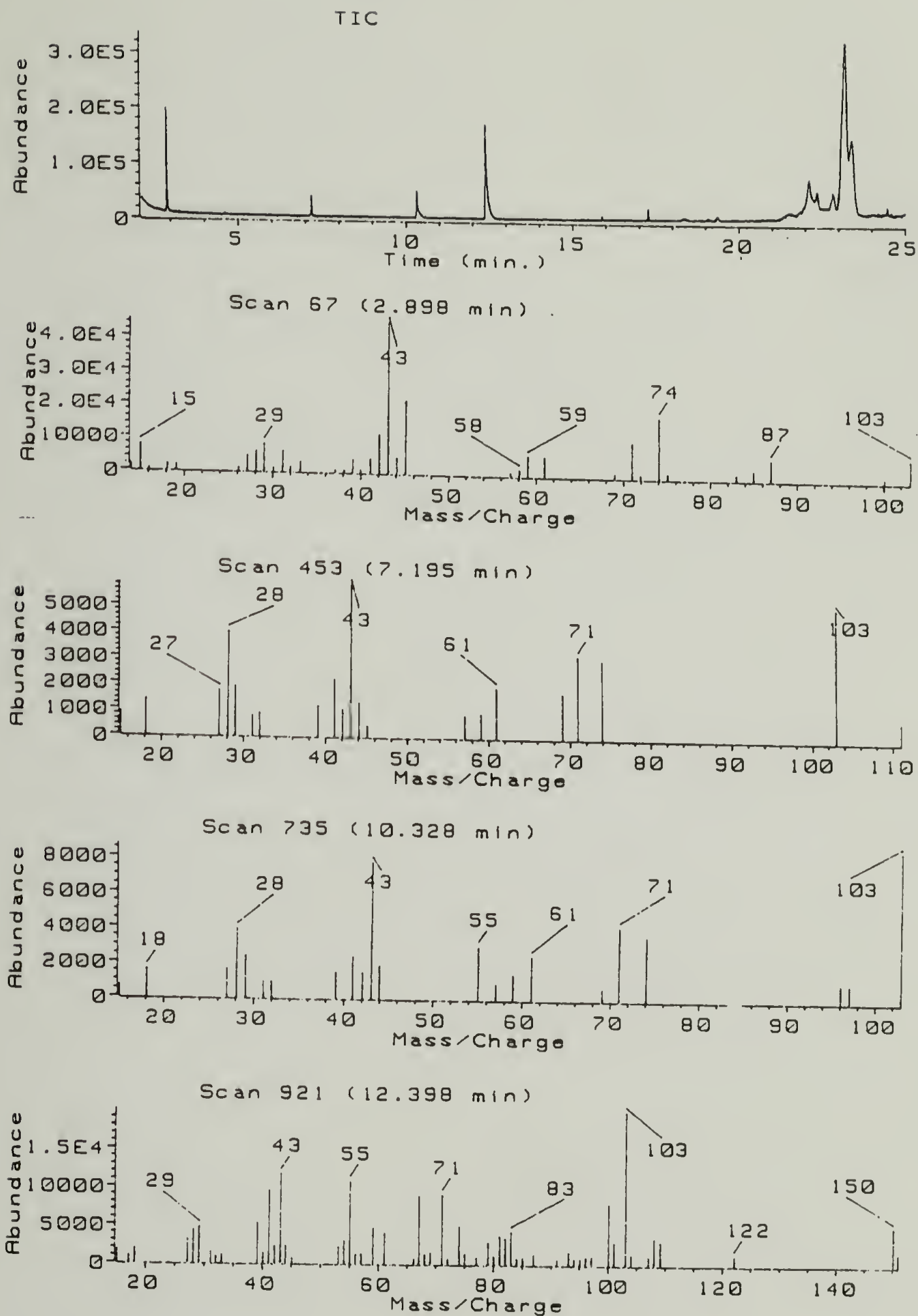


Figure 114. TIC and mass spectra of the methanolized sample of the PHA produced from 4-cyclohexylbutyric acid.

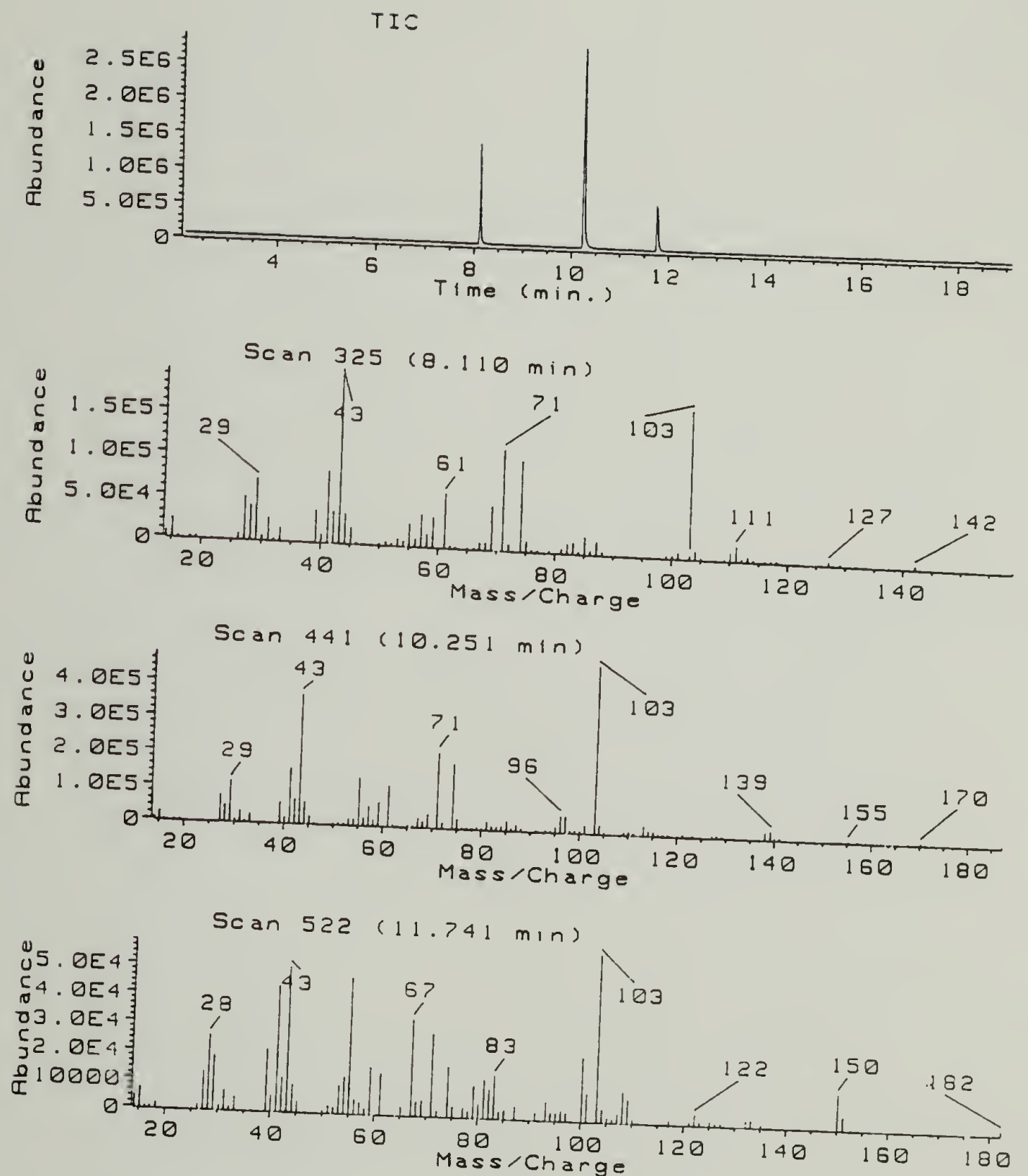


Figure 115. TIC and mass spectra of the methanolized sample of the PHA produced from an equimolar mixture of nonanoic acid and 4-cyclohexylbutyric acid.

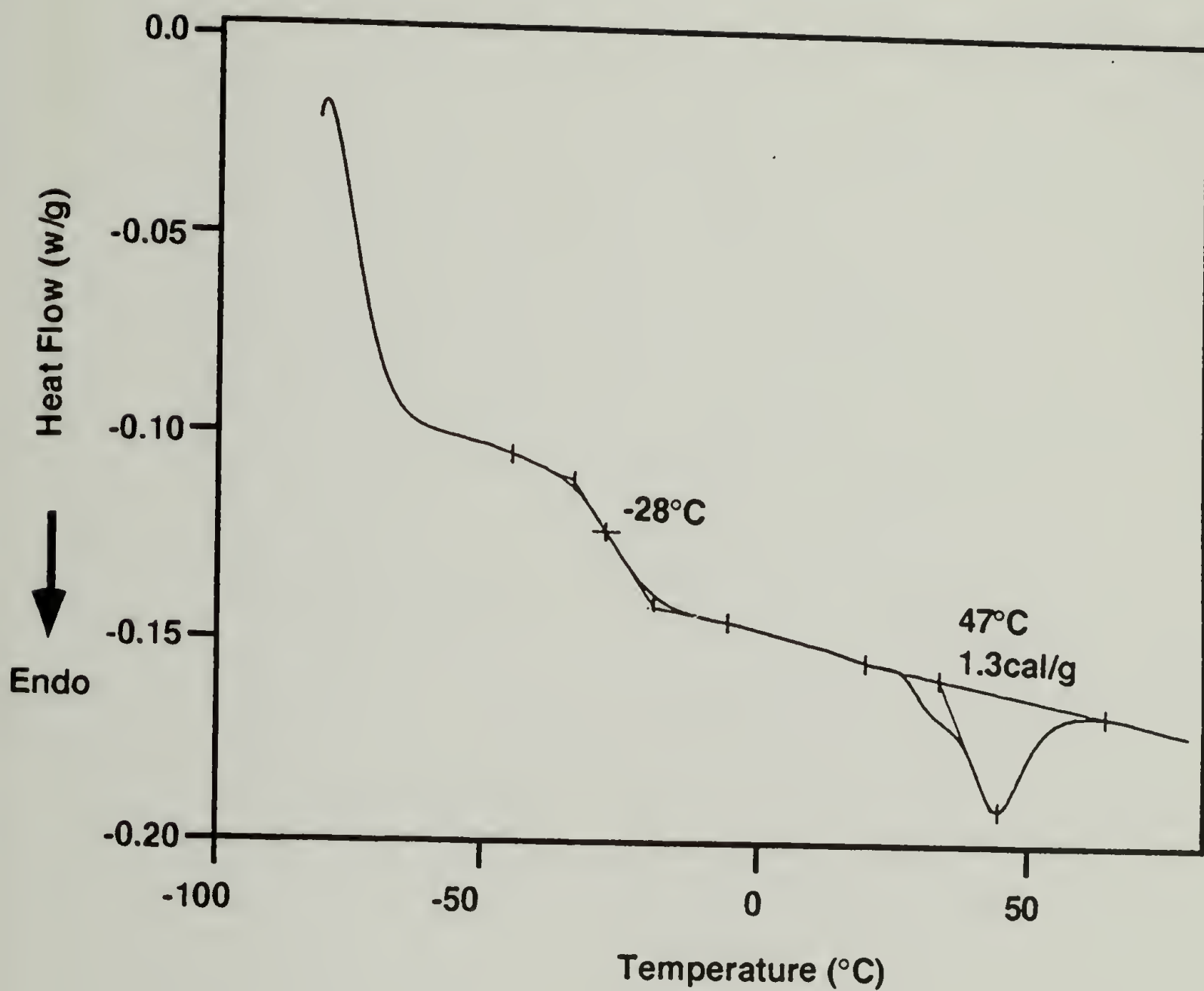


Figure 116. DSC thermogram of the PHA produced on an equimolar mixture of nonanoic acid and 4-cyclohexylbutyric acid.

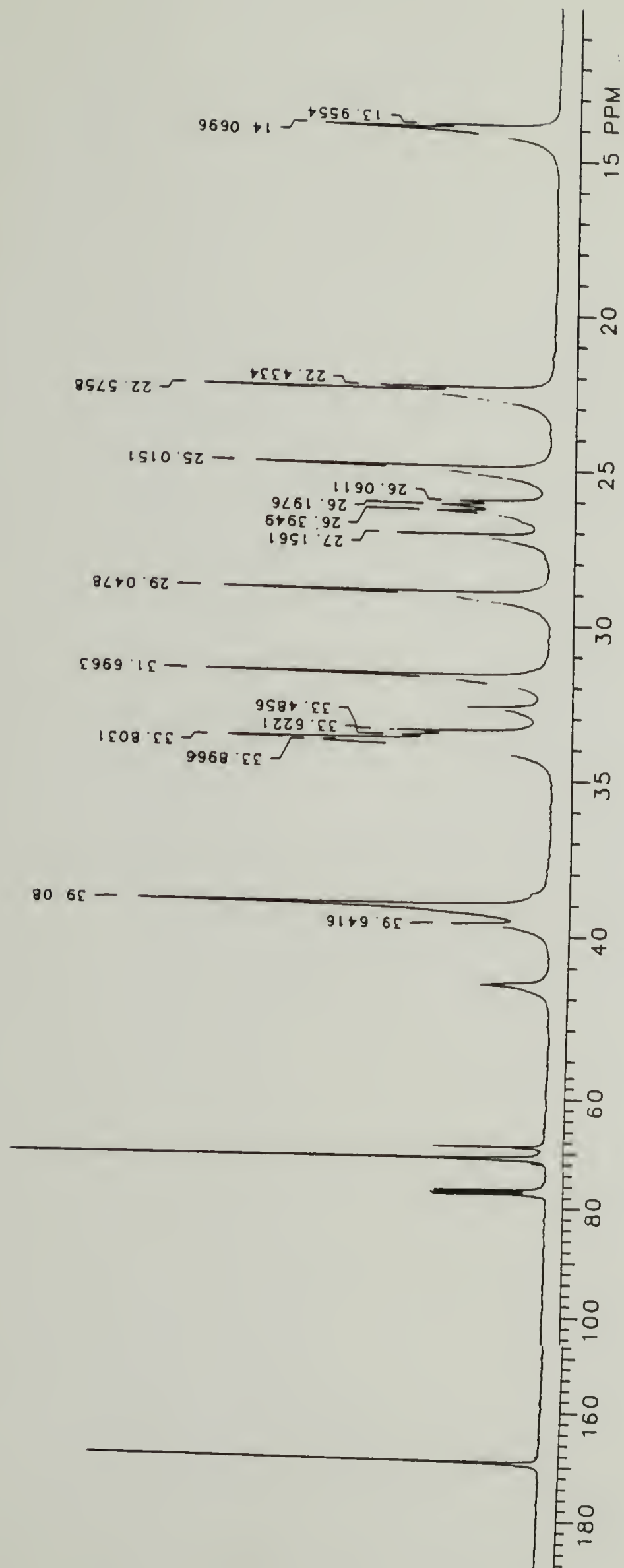


Figure 117.  $^{13}\text{C}$  NMR spectrum of the PHA produced on an equimolar mixture of nonanoic acid and 4-cyclohexylbutyric acid.



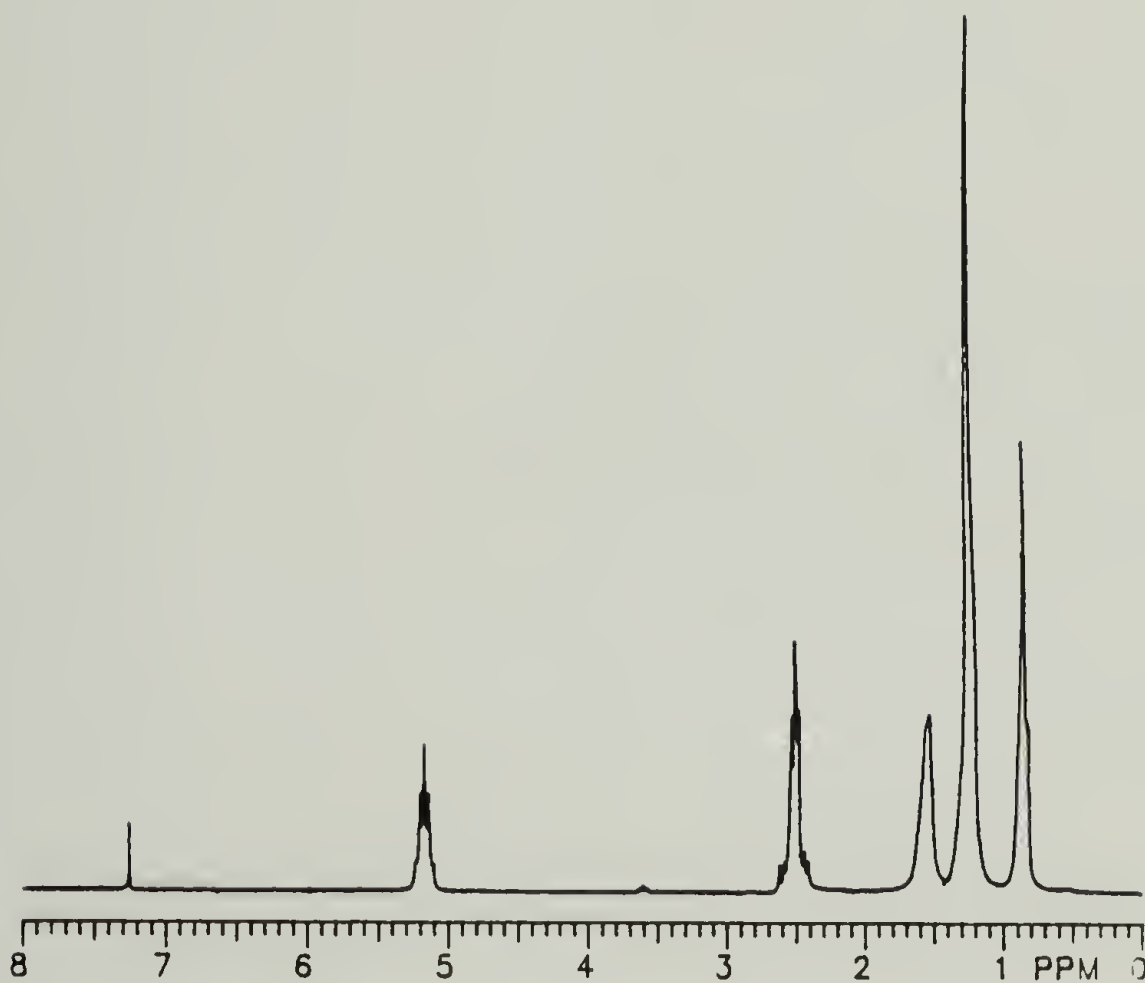


Figure 118.  $^1\text{H}$  NMR spectrum of the PHA produced from a 2.4:1 mixture of nonanoic acid and 12-hydroxydodecanoic acid.

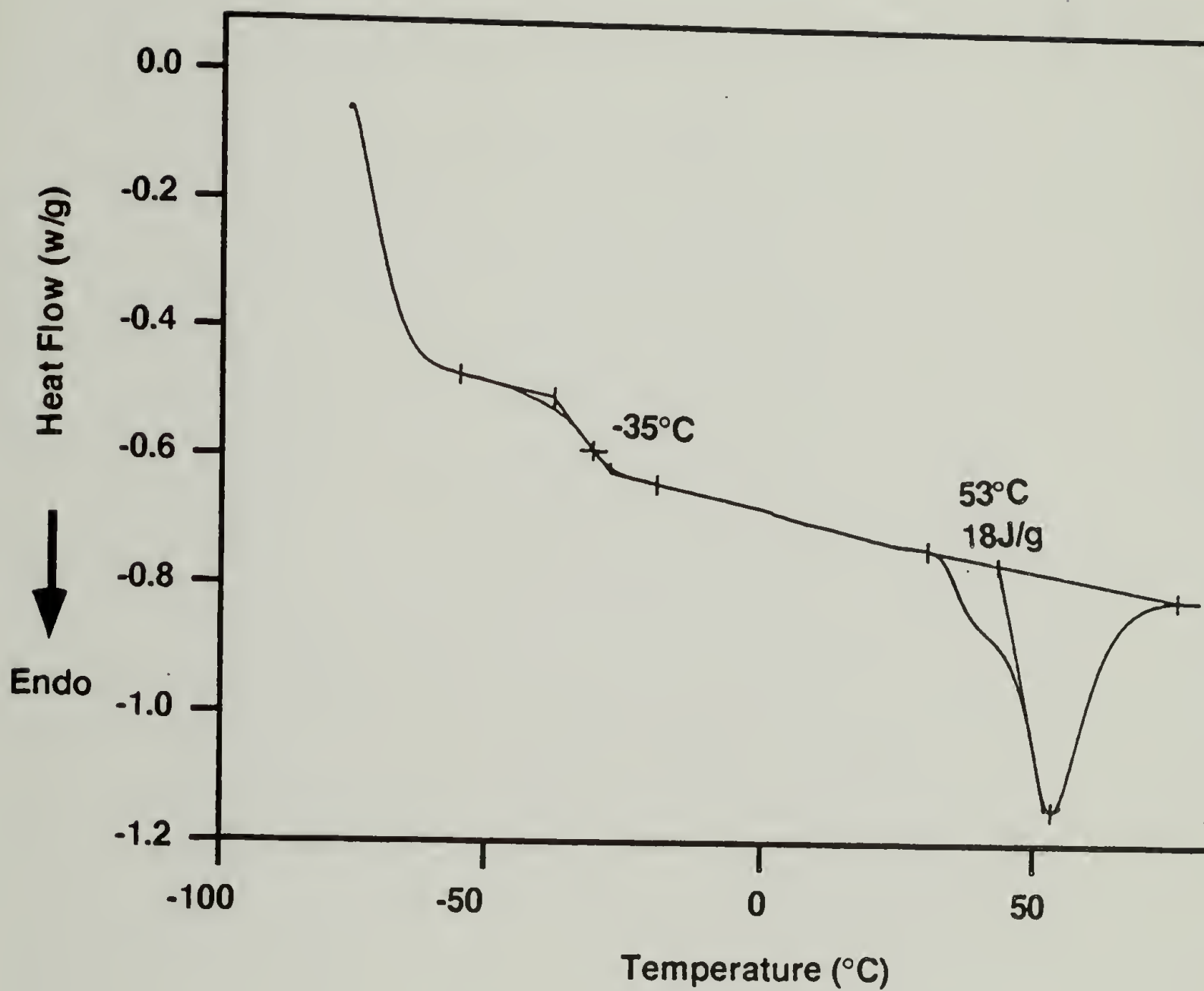


Figure 119. DSC thermogram of the PHA produced on a 2.4:1 mixture of nonanoic acid and 12-hydroxydodecanoic acid.

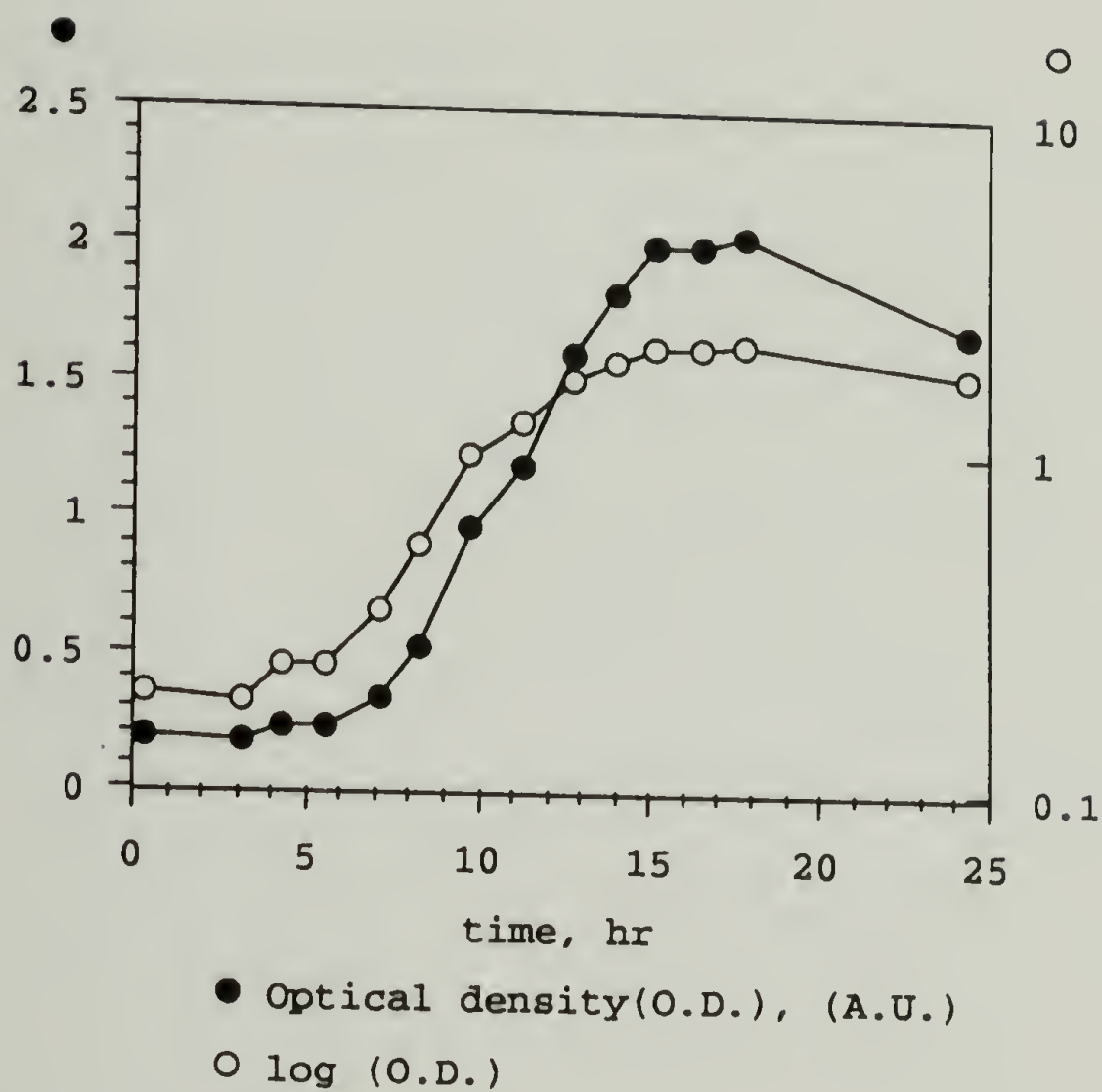


Figure 120. Growth curves of *P. oleovorans* grown with an equimolar mixture of nonanoic acid and 1,12-dodecanediol.

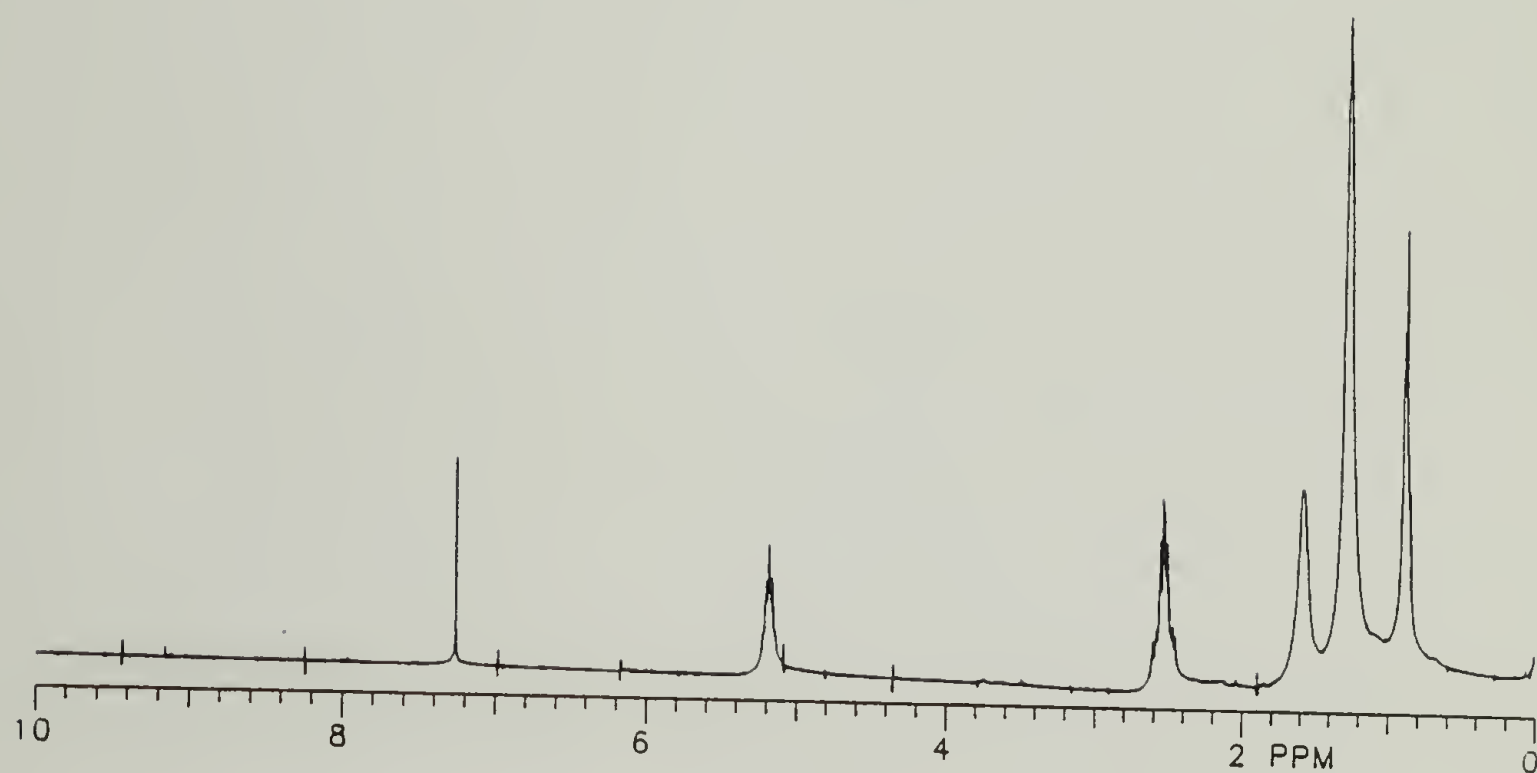


Figure 121.  $^1\text{H}$  NMR spectrum of the PHA produced on an equimolar mixture of nonanoic acid and 1,12-dodecanediol.



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