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^{13}C n.m.r. spectroscopy in living cells of *Pseudomonas oleovorans*

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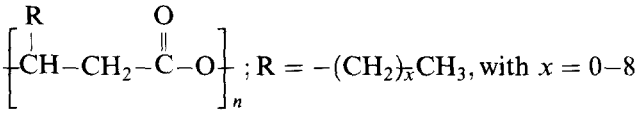
Natural abundance solution ^{13}C nuclear magnetic resonance (n.m.r.) spectroscopy was used to monitor the intracellular production and degradation *in vivo* of two poly(3-hydroxyalkanoates) (PHAs), produced by *Pseudomonas oleovorans*, poly(3-hydroxynonanoate), (PHN), poly(3-hydroxyphenylvalerate) (PHPV) and a blend of both PHN and PHPV. PHN was produced and degraded faster than PHPV when it was either the sole intracellular polyester or a component of a blend with PHPV. However, the rate of PHPV degradation was increased dramatically when PHN was present. Comparison of the n.m.r. method for quantitative monitoring of the PHAs with that of extracting and weighing the polymers from freeze-dried cells, showed that the ^{13}C n.m.r. method was faster, easier, non-destructive (to the cells) and more accurate, while giving additional valuable information on the mobility and the composition of the PHAs present. This method was also particularly useful for determining optimal harvest times because PHPV formation continued long after the maximum optical density was reached. © 1997 Elsevier Science Ltd.

(Keywords: bacterial polyesters; intracellular PHA production and degradation; ^{13}C n.m.r. spectroscopy *in vivo*)

INTRODUCTION

Poly(3-hydroxyalkanoates) (PHAs) are accumulated by many types of bacteria as an intracellular energy source and carbon storage material when the cells experience metabolic stress, such as a limitation of nitrogen, oxygen or other essential nutrients, in the presence of excess of carbon[1–3]. PHAs are an ideal storage material because they are in a highly reduced form and are water insoluble so no osmotic pressure effects are induced inside the cell. For practical applications, poly(3-hydroxybutyrate) has received the most interest to date largely because it is completely biodegradable and biocompatible, but a copolymer containing both 3-hydroxybutyrate and 3-hydroxyvalerate units has also been produced on a large scale for use as a biodegradable thermoplastic. Naturally occurring PHAs have the following general structure depending on the growth substrate used and the bacteria involved:



; R = $-(CH_2)_xCH_3$, with $x = 0-8$

The PHAs are produced and stored as granules or inclusion bodies in the bacterial cells. In the case of *Pseudomonas oleovorans* the granules contain two polymerases, a depolymerase and a structural protein on the surface of the granule[4,5]. Each repeating unit of a PHA has a chiral centre with an [R] configuration at the 3-position. This stereochemistry is essential to the inherent degradability of these biopolymers. Chemically synthesized PHAs which have similar structures, but have the opposite chirality, are much less biodegradable than the natural PHAs[6]. Recent work in our laboratories has focused on the bacterial production of functionalized PHAs containing alkene[6,7], bromine[8], phenyl[9,10], cyano[3] and ester[11,12] terminal groups on the R substituent.

When *P. oleovorans* is fed nonanoic acid (NA) it grows well and produces a high yield of copolyester[10], which contains mostly 3-hydroxynonanoate units, and for this reason it is referred to as poly(3-hydroxynonanoate). When *P. oleovorans* is fed 5-phenylvaleric acid (PVA) it grows very poorly and after a lag time of several days produces a homopolymer containing only 3-hydroxy-5-phenylvalerate units in low yield. Generally, when *P. oleovorans* is grown on a mixture of substrates, a random copolymer is produced which contains repeating units from both substrates by co-metabolism[1,13]. Co-metabolism (co-oxidation) was first described by Leadbetter and Foster[14] over 30 years ago as a metabolic process utilized by certain bacteria. In the present study, however, *P. oleovorans* grown on an equimolar mixture of NA and PVA, produced two different PHAs, including the normal random copolymer, poly(3-hydroxynonanoate), (PHN) and the normal homopolymer, poly(3-hydroxyphenylvalerate) (PHPV). When both NA and PVA are fed to *P. oleovorans*

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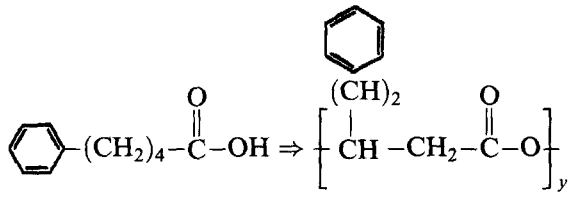
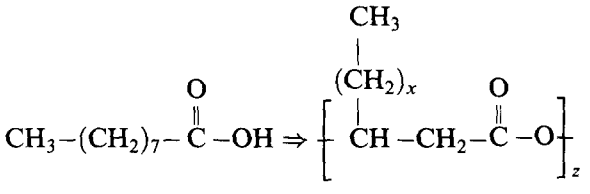
simultaneously, both PHPV and PHN were produced in good yields and these two PHAs were produced in the same cells and granules[10,15]. The structures of these substrates and the corresponding PHAs are shown below:



PVA PHPV



NA PHN, with $x = 3, 5$ and 7

One objective of the present study was to determine whether the cells could degrade synthetic functionalized polymers such as PHPV, and for this purpose a quantitative technique using natural abundance ^{13}C nuclear magnetic resonance (n.m.r.) spectroscopy was developed to monitor the concentration *in vivo* of PHAs produced by *P. oleovorans*. This method was used to monitor both the rates of production and degradation of PHPV as well as of PHN when these PHAs were produced either as the sole polymers or jointly in the cells. The accuracy of the n.m.r. method was compared to the usual method for determining polymer yields by extraction and weighing.

EXPERIMENTAL

Polymer production

Stock cultures of *P. oleovorans* (ATC20347) were prepared using conditions developed in our laboratories[2]. The culture medium was E* medium which consisted of: 1.1 g² L $(NH_4)_2HPO_4$, 5.8 g^{-1} L K_2HPO_4, 3.7 g^{-1} L K_2HPO_4 10 mls^{-1} L of a 100 mM solution $MgSO_4$ (in distilled water), 1.0 mls^{-1} L of a microelement solution. The microelement solution consisted of the following in 1 M HCL: 2.78 g^{-1} L $FeSO_4.7H_2O$, 1.67 g^{-1} L $CaCl_2.2H_2O$, 0.17 g^{-1} L $CuCl_2.2H_2O$, 0.29 g^{-1} L $ZnSO_4.7H_2O$, 1.98 g^{-1} L $MnCl_2.4H_2O$, 2.81 g^{-1} L $CoSO_4.7H_2O$. The cells were grown in E* medium on the following carbon substrates: (1) NA (10 mM), (2) PVA (10 mM), and (3) an equimolar mixture of NA/PVA (10 mM/10 mM) in a 121 New Brunswick Microfermentor with an air flow of 5 l min^{-1}, at 30°C and a stirring speed of 400 rev min^{-1}. The optical density (o.d.) of the bacterial cultures was monitored at 660 nm with a Spectronic Bausch and Lomb Spectrometer, and the cells were harvested when the o.d. was at a maximum. For the degradation studies, the culture was centrifuged for 20 min at 4000 rev min^{-1} and the pellet of cells was resuspended in E* medium containing no carbon source. One litre aliquots were taken at various time intervals and divided into two equal portions for quantitative analysis either by n.m.r. spectroscopy or extraction. The cells in these aliquots were centrifuged at 4000 rev min^{-1} for 20 min and frozen until used.

Because of the low cell and polymer yields obtained when the bacteria were grown on PVA as the sole carbon source, the procedure was modified. After resuspension of the cells in E* medium containing no carbon source, the culture was harvested periodically over 100 h, centrifuged, used for the n.m.r. spectroscopic method and later freeze-dried for the extraction method.

N.m.r. analysis

The frozen cells from 500 ml of growth medium were resuspended as a slurry in deuterium oxide (D_2O) with a total volume of 6 ml and transferred to an 10 mm n.m.r. tube equipped with a coaxial insert containing DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate [$(CH_3)_2SiCH_2CH_2CH_2SO_3$ Na], the n.m.r. spectroscopy standard. In the case of PHPV, the n.m.r. spectra were also obtained by placing the slurry in a cylindrical insert in a n.m.r. tube containing DSS. The coaxial insert and cylindrical insert were necessary to prevent the bacterial cells from metabolizing the DSS. The ^{13}C n.m.r. spectra were all obtained at 67.925 MHz with a Bruker IBM n.m.r. 270 MHz spectrometer. Spectra were obtained at 318 K for PHN and the PHN/PHPV blend, but spectra of cells with PHPV as the sole intracellular polymer were obtained at 345 K. This temperature of 345 K was necessary because of PHPV's decreased degree of mobility in bacterial cells, and the higher temperature gave a much better signal-to-noise ratio and sharper peaks. Spectra of PHN, PHPV and PHN/PHPV samples were obtained using an excess of scans (25 000 scans) taken over a 24 h period. PHN spectra could be obtained with 2000 scans, and PHPV with 8000 scans. Parameters used for n.m.r. spectra acquisition were the following: line broadening of 10 Hz, pulse width of 6 μs and delay between pulses of 2 s and 32 k data block, using a spectral width of 20 kHz. The ^{13}C 90° pulse was 9.5 μs in all experiments.

Extraction methods

The cells were freeze-dried and the polymer was extracted by refluxing in boiling chloroform (10 × cell dry weight) for 8 h. The biomass was removed by filtration and the volume of chloroform was reduced by rotary evaporation to approximately 5 ml. The polymer in chloroform was slowly added to rapidly stirring methanol, and the resulting precipitate was filtered, dried and weighed. In the case of the PHN/PHPV blend, the PHN-to-PHPV ratio was determined by integration of the ^1H n.m.r. spectrum. This spectrum was obtained in $DCCl_3$ with a Bruker 200 MHz ^1H n.m.r. spectrometer at 17°C and 7.45 MHz, (acetone-d_6, tetramethylsilane) at a polymer concentration of 10 mg ml^{-1}.

RESULTS AND DISCUSSION

Production and degradation studies of the intracellular bacterial polyesters, PHN and PHPV, were conducted using natural abundance ^{13}C n.m.r. spectroscopy. The ^{13}C n.m.r. spectra of these two polymers, when present as the sole intracellular PHAs, and the spectrum of the PHN/PHPV blend can be seen in *Figure 1*: (a) PHPV homopolymer, (b) PHN heteropolymer, and (c) PHN/PHPV blend. Peaks due to the DSS standard are

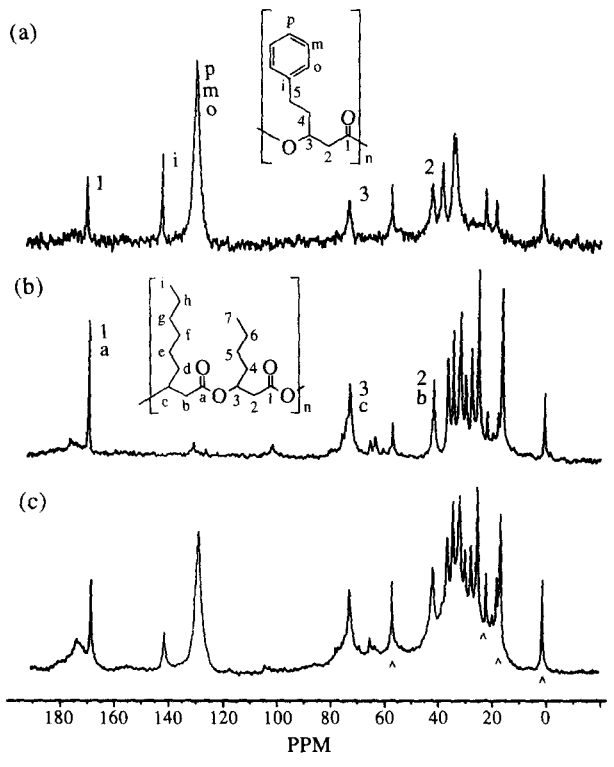


Figure 1 ^{13}C n.m.r. spectra of *P. oleovorans* cells which contained: (a) PHPV as a sole intracellular polymer; (b) PHN as the sole intracellular polymer; (c) PHN and PHPV as intracellular polymers (peaks due to the DSS standard are indicated with the symbol ^)

indicated with the symbol ∧ in *Figure 1c*. When PHN is the sole intracellular polymer, the carbonyl peak at 170 ppm was relatively sharp, presumably due to less dipolar and scalar broadening from the hydrogens. The peaks due to the backbone carbons, the methyne (–CH–) peaks at 74 ppm and the methyene ($-CH_2-$) peak at 47 ppm, as well as the peaks due to the alkyl carbons in the side chain (R), which occur between 15 and 40 ppm, are much broader than the carbonyl peak. The sharpness of the carbonyl peak may be due to less dipolar and scalar broadening from the protons. In all of the ^{13}C n.m.r. spectra of the cells containing PHN or PHPV, only peaks due to these PHAs were observed. This result is similar to the results observed by Amor *et al.*[16] for the ^{13}C spectra of PHB *in vivo*.

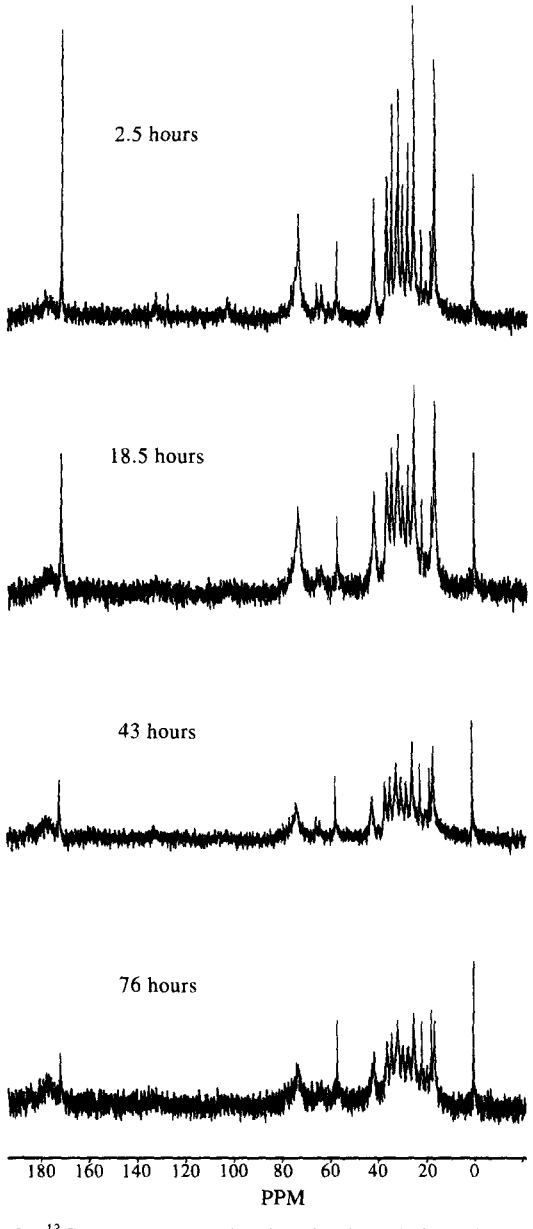


Figure 2 ^{13}C n.m.r. spectra showing the degradation of PHN in *P. oleovorans* at increasing times over a 76 h period

Table 1 PHN contents of cells at different times during the degradation cycle when PHN was the sole storage polymer present as determined by both the extraction and the ^{13}C n.m.r. spectroscopic method

	Determined by polymer extraction				Determined by ^{13}C n.m.r. spectroscopy	
Cell harvest time (h)	Cell yield ($g l^{-1}$)	PHN yield ($g l^{-1}$)	Polymer yield (% dry wt)[a]	% PHN remaining	C=O/DSS[b]	% PHN remaining
0	2.54	0.61	24.0	100	1.80	100
0	2.11	0.38	18.3	100	1.27	100
0	2.35	0.53	22.7	100	1.42	100
3	2.24	0.53	23.7	104.5	1.90	90.6
19	2.19	0.41	18.5	67.2	1.02	48.6
23	2.03	0.32	16.0	52.6	0.97	46.3
43	2.03	0.17	8.5	27.8	0.56	26.7
50	1.81	0.12	6.6	19.7	0.44	19.7
74	2.07	0.13	6.4	21.3	0.38	21.3
90	2.10	0.10	4.7	16.4	0.44	16.4

[a] PHN yield (g) as % of the cell dry weight
[b] The ratio of the carbonyl peak at 170 ppm to that of the DSS standard at 0 ppm

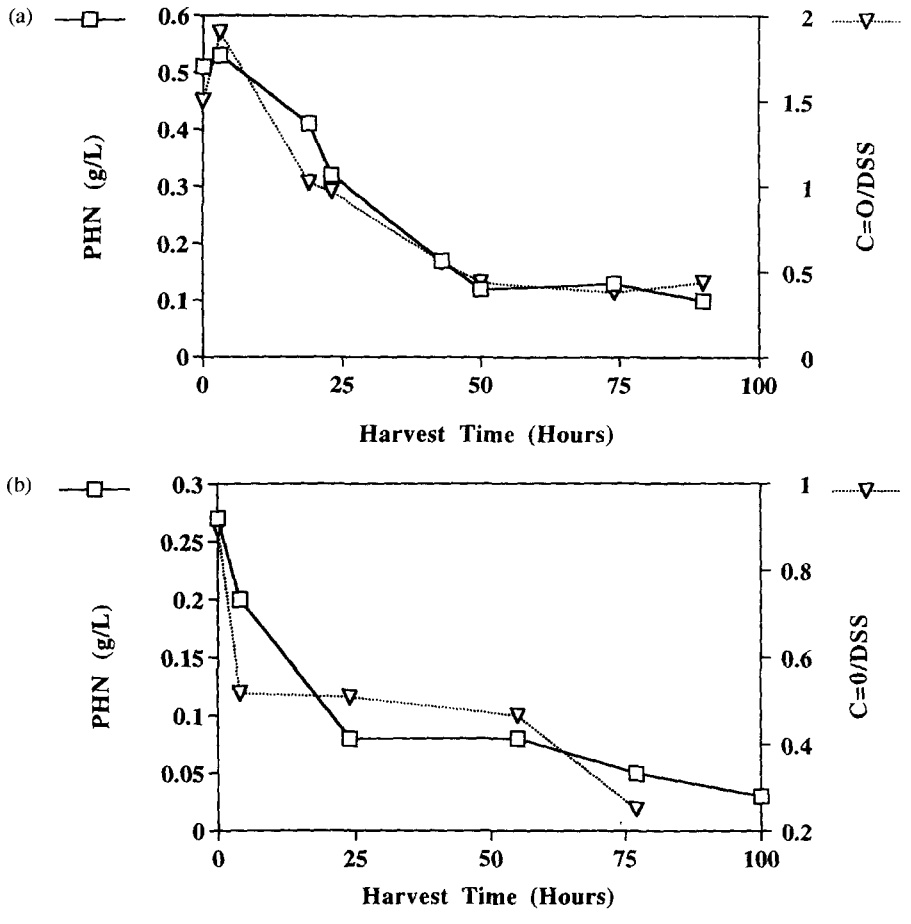


Figure 3 PHN remaining as determined by both the extraction method ($g l^{-1}$) (□) and the n.m.r. method (C=O/DSS) (▽) at increasing times when: (a) PHN was the sole intracellular polymer; (b) when both PHN and PHPV were present as intracellular polymers

The ratios of the integrated peak areas of the carbonyl-to-DSS peaks gave the same value as the peak height ratios, so the ratio of the peak heights (C=O/DSS) was used for comparison with the polymer yields. The ratio of the height of the peak at 170 ppm relative to that of the DSS standard at 0 ppm was used to evaluate the change in polymer concentration of PHN over a 74 h period during the degradation cycle after the cells were resuspended in a medium containing no carbon substrate (*Figure 2*). The yields were obtained by using the extraction method on samples taken at the same time intervals as those obtained for the n.m.r. measurements (*Table 1* and *Figure 3*). The peaks due to DSS and to the polymer have very different relaxation times as expected. The spectrum of the small DSS molecule had very sharp peaks, which were insensitive to temperature changes. The ratio of alkyl peak height to DSS peak height and carbonyl peak height to DSS peak height varied very little with changes in pulse width from 2 to 9 μs.

The phenyl peaks in PHPV were very sensitive to temperature. The signal-to-noise ratio for the phenyl group increased from 30 and levelled off at 60 with changes in pulse width from 4 to 8 μs. For this reason it was important to keep both the temperature and the pulse width constant during all the measurements. The different effects of line broadening (LB) on alkyl, aryl and DSS peaks were also evaluated. The effect of LB changes on the ratio of the peak heights of alkyl/DSS, aryl/DSS or C=O/DSS was quite linear and consistent

Table 2 PHPV contents of cells at different times during the degradation cycle when PHPV was the sole storage polymer present as determined by both the extraction and ^{13}C n.m.r. spectroscopy method

	Determined by polymer extraction				Determined by ^{13}C n.m.r. spectroscopy
Cell harvest time (h)	Cell yield ($g l^{-1}$)	PHPV yield ($g l^{-1}$)	Polymer yield (% dry wt)[a]	Ar/DSS[b]	% PHPV remaining
0	0.068	0.018	26.0	2.41	41.7
20	0.081	0.037	45.9	–	–
46	0.271	0.091	33.5	4.01	69.4
67	0.276	0.096	34.6	5.78	100
91	0.294	0.102	34.6	2.91	50.3
115	0.077	0.026	33.9	1.70	29.4

[a] PHPV yield (g)/cell yield (g)
[b] Ratio of the aromatic peak at 128 ppm to that of the DSS standard at 0 ppm

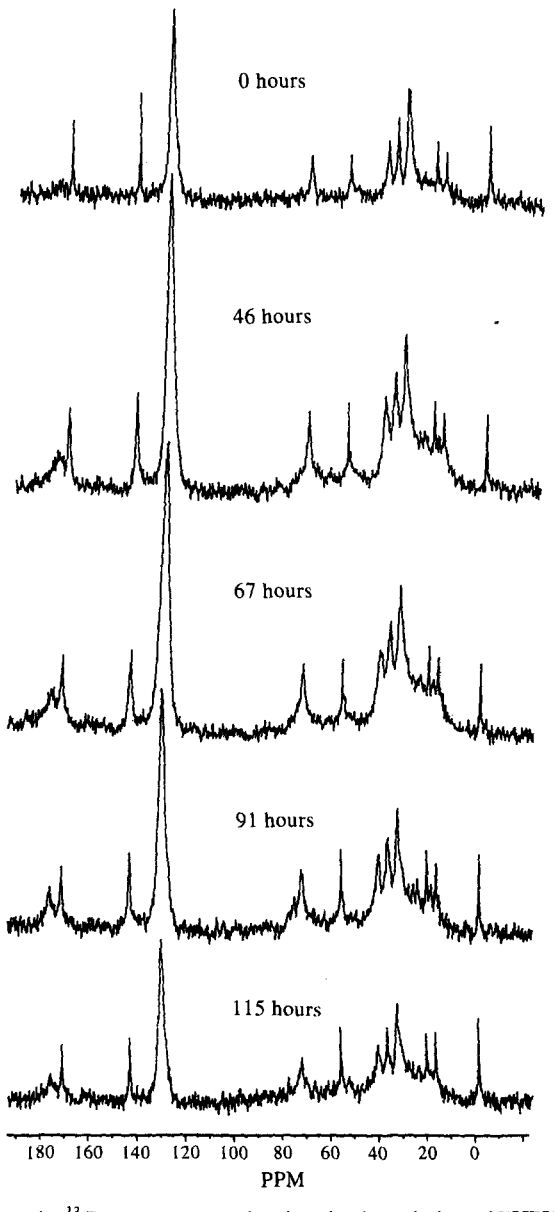


Figure 4 ^{13}C n.m.r. spectra showing the degradation of PHPV in *P. oleovorans* at increasing times over a 115 h period

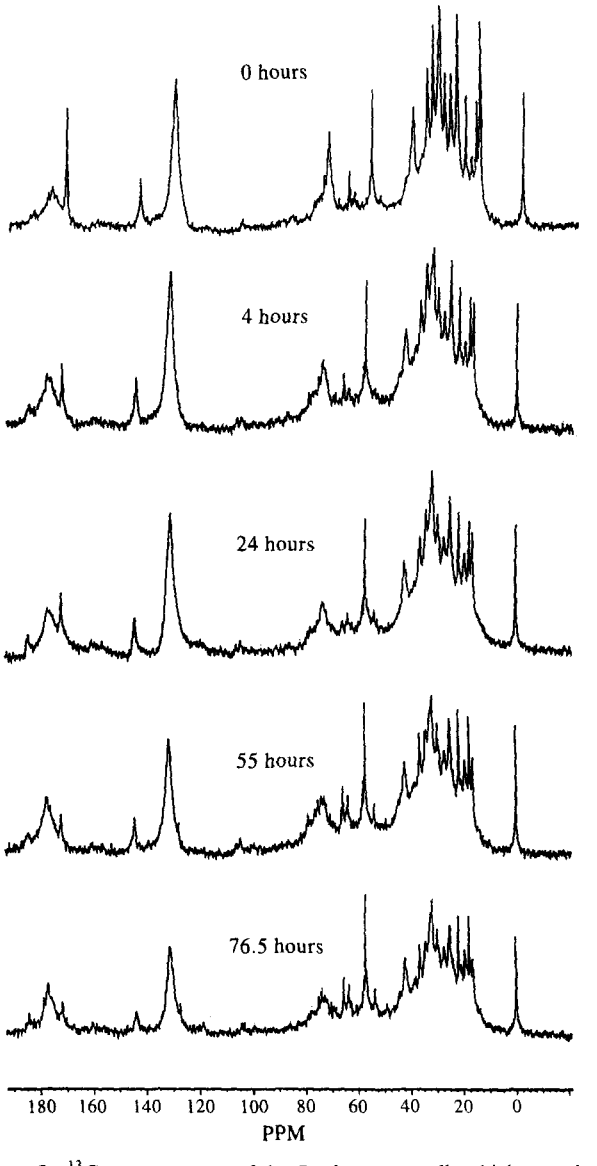


Figure 5 ^{13}C n.m.r. spectra of the *P. oleovorans* cells which contained both PHN and PHPV at increasing times over a 76.5 h period

above LB = 10 Hz, so this LB value was used in all n.m.r. measurements. To obtain quantitative values for polymer concentrations *in vivo*, a standard curve must be prepared from extractions, but relative concentrations of polymer production during fermentations can be obtained from ^{13}C n.m.r. measurements alone.

The ratio of the height of the phenyl peak of PHPV at 128 ppm to that of DSS at 0 ppm (Ar/DSS) was used to obtain relative changes in PHPV content over a 115 h

Table 3 PHN/PHPV contents of cells at different times during the degradation cycle when both PHN and PHPV were present as determined by the extraction and the ^{13}C n.m.r. spectroscopic methods

Cell harvest time (h)	Cell yield (gl^{-1})	Total polymer yield (gl^{-1})	% PHPV[a]	PHPV yield (gl^{-1})[b]	AR/DSS[c]	% PHN[a]	PHN yield[d] (gl^{-1})	C=O/DSS[e]
0	1.480	0.42	40	0.17	1.05	60	0.26	1.07
0	1.640	0.44	40	0.18	1.12	60	0.26	0.90
0	1.470	0.44	35	0.12	1.09	65	0.22	0.88
4	0.186	0.34	39	0.13	1.23	61	0.20	0.52
24	0.271	0.18	58	0.11	1.11	52	0.08	0.51
55	0.276	0.20	62	0.12	0.89	48	0.08	0.47
77	0.294	0.13	65	0.09	0.70	35	0.05	0.25
100	0.600	0.10	65	0.07	ND	35	0.03	ND

[a] Determined by ^{1}H n.m.r.
[b] Total polymer yield × % PHPV
[c] Ratios of the aromatic peak at 128 ppm to that of the DSS standard at 0 ppm in the ^{13}C n.m.r. spectra
[d] Total polymer yield × % PHN
[e] Ratios of the carbonyl peak at 170 ppm to that of the DSS standard at 0 ppm in the ^{13}C n.m.r. spectra

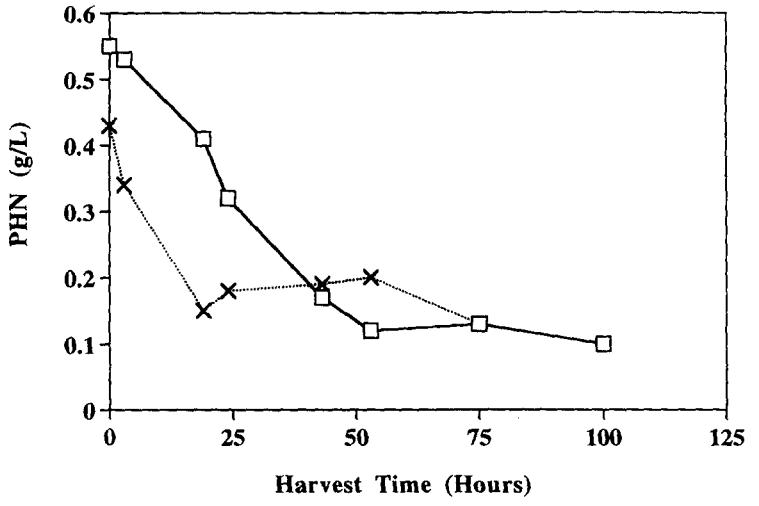


Figure 6 Comparison of the PHN content in *P. oleovorans* over a 100 h period when it was the sole intracellular polymer (□) and when it was present with PHPV (×) as determined by polymer extraction

period (*Figure 4*). The aromatic-to-DSS peak height ratios (Ar/DSS) were compared to PHPV yields obtained from extraction procedures (*Table 2*). In the case in which both PHN and PHPV were produced, the n.m.r. spectra were obtained at 318 K (*Figure 5*). At this temperature the carbonyl peak of PHPV disappeared due to peak broadening (PHPV is much less mobile than PHN), and the 170 ppm peak was used to monitor the PHN present with no interference from the PHPV carbonyl peak. The (C=O/DSS) and the (Ar/DSS) peak height ratios were compared to polymer yields obtained from extraction procedures (*Table 3*).

In the PHPV degradation studies, it is clear that even at 0 h, when the cells were resuspended in a carbon-free medium, polymer formation had not reached a maximum, even though the o.d. of the bacterial culture had reached a maximum (*Figure 4* and *Table 2*). Therefore, these n.m.r. results confirm those of Fritzsche *et al.*[9] that o.d. measurements can be inaccurate as an indication of the optimal time to harvest a PHA. This possibility is an important consideration for fermentations using *P. oleovorans* because this bacterium can begin to rapidly degrade its intracellular PHA as soon as polymer production is completed[17].

Figure 6 compares the rate of degradation of PHN when it was present as the sole intracellular polymer and when it was present as a component of the blend. For the former, PHN degradation began 19 h after the cells were resuspended in a carbon-free medium, and 80% of the polymer was degraded after 90 h. The rate of degradation of PHN when it was present as a component of a blend was relatively unchanged compared to the degradation rate when it was the sole intracellular storage polymer. That is, 89% of the stored polymer was degraded compared to 80% of the stored polymer when PHN was the sole polymer present during a 90 h period.

Figure 7 compares the rate of degradation of PHPV when it was present as the sole intracellular polymer and when it was present as the component of a blend. In the case where PHPV was present as the sole intracellular storage polymer, degradation was not observed until 91 h after the bacteria was resuspended in a carbon-free medium. Degradation was still in progress 115 h after the resuspension of cells in the carbon-free medium. At that point the experiment was terminated, but only 26% of the intracellular stored PHPV was degraded. In the case where both PHN and PHPV were present the rate of PHPV degradation was more than twice that observed when the PHPV was the sole intracellular storage polymer. This change in the rate of degradation of PHPV in the presence of PHN is one of the most interesting aspects of this study because only one

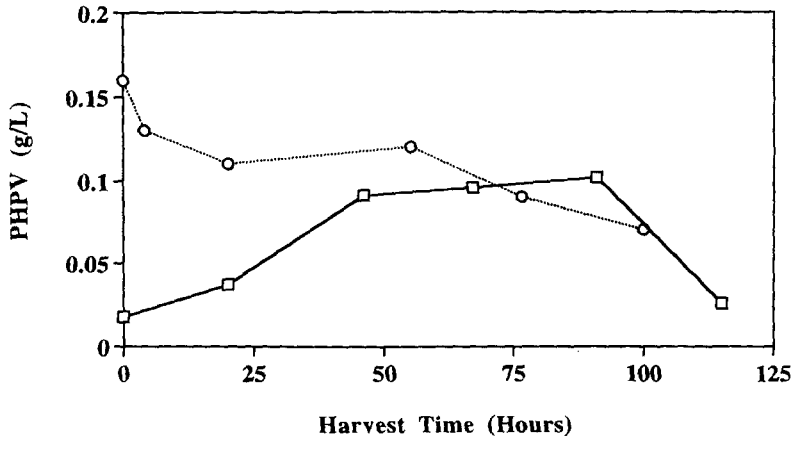


Figure 7 Comparison of the PHPV content in *P. oleovorans* over a 115 h period when it was the sole intracellular polymer (□) and when it was present with PHN (○) as determined by polymer extraction

depolymerase has been observed to be present in cells of *P. oleovorans* when grown either on NA, PVA or a mixture of NA and PVA[4,5]. This result suggests that the same intracellular depolymerase was responsible for the degradation of PHN and PHPV, but the reason the degradation of PHPV was faster in the presence of PHN is unknown.

The results obtained from the analysis of the ^{13}C n.m.r. spectra correlated closely with the results from the extraction studies in all cases (e.g. see *Figure 3*). Therefore, the ^{13}C n.m.r. spectroscopic method is clearly an accurate and very sensitive technique for monitoring the intracellular formation and degradation of bacterial polyesters. Indeed, PHA production can be detected and identified *in vivo* using ^{13}C n.m.r. spectroscopy before optical microscopy can detect granule formation and before Nile Blue fluorescence shows that any polymer is present. The signal-to-noise ratio obtained for PHPV in a cylindrical insert (holding 0.5 ml of the slurry of bacterial cells) was 34 for a concentration of 2 mg of polymer in 1 l of cells (*Figure 2*). The presence of PHPV *in vivo* could be detected at concentrations at least 100-fold lower than this concentration. The sample preparation involved in the n.m.r. method was minimal, and the technique was non-destructive to the cells even at a temperature of 345 K. After extended periods of time at 318 K, *P. oleovorans* from the slurry used in the n.m.r. experiments grew on agar plates.

CONCLUSIONS

Natural abundance ^{13}C n.m.r. spectroscopy was used to monitor the *in vivo* degradation of two PHAs, PHN and PHPV, produced by *P. oleovorans*. ^{13}C n.m.r. spectroscopy is a valuable non-destructive method for monitoring polymer formation and degradation *in vivo*. It has the advantages of accuracy, speed, sensitivity and, in addition, can be used to identify the polymers present as well as their mobility *in vivo*.

In this study, it was shown that the synthetic PHA, PHPV, was degraded by *P. oleovorans* in a similar manner to that of the natural substrate, PHN. In the case in which the cells contained both PHN and PHPV, both underwent intracellular degradation. The rate of degradation of PHPV was slower than that of PHN. The rate of degradation of PHPV was much greater in the presence of PHN than when PHPV was the sole intracellular polymer, but the rate of degradation of PHN was not significantly affected by the presence of the PHPV. It is very likely therefore, that PHN and PHPV are degraded by the same intracellular depolymerase, and that the enzyme operates more efficiently in the presence of PHN.

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