A Study of Poly(hydroxyalkanoate)s by Quantitative ^{31}P NMR Spectroscopy: Molecular Weight and Chain Cleavage

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Recently, ^{31}P NMR has been introduced as a powerful tool for the elucidation of the different types of phenolic, alcoholic, and carboxylic structures that are present in lignins.[1,2] It was shown that the reaction of 1,3,2-dioxaphospholanyl chloride[2,3] or 2-chloro-4,4,5,5-tetramethyldioxaphospholane[4,5] (**I**) with the above labile proton-containing moieties produces phosphinic esters whose ^{31}P NMR chemical shifts are very sensitive to the chemical structure around the ^{31}P nucleus. Comparison with model compounds[3] provided valuable information regarding the highly complex structure of various types of lignins,[1,2,5] and the factors affecting the ^{31}P chemical shifts were studied in detail.[2,6] Consequently, Chan *et al.* applied the above methodology and proposed a facile qualitative and quantitative route for the determination of phenolic functional groups in poly(phenylene oxide) (PPO) resins.[7]

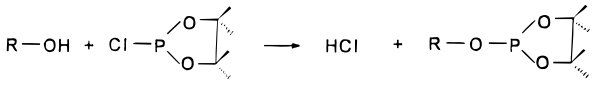
In this communication, we further explore the use of quantitative ^{31}P NMR for the accurate determination of number-average molecular weights (M_n) in poly(hydroxyalkanoate)s (PHAs). PHAs are a family of bacterially synthesized thermoplastic polyesters whose inherent biodegradability and biocompatibility have made them notorious.[8] The most recent advances are collected in a Supplement journal issue and edited by Page.[9] The chemical composition and M_n of PHAs can be controlled by varying the fermentation and isolation conditions,[10,11] leading to homologous polymers of varying physical properties. An absolute method for the facile and accurate determination of the molecular weight of polymers produced at various fermentation times and conditions would be advantageous with respect to presently used indirect methods, such as GPC or viscometry.

The proposed method involves the derivatization of the hydroxylic and carboxylic end groups of the PHA polymer chains with **I**, 2-chloro-4,4,5,5-tetramethyldioxaphospholane, according to Scheme 1. The reaction is simple and quantitative and can be readily performed in an NMR tube, thus facilitating the analysis of the derivatized material by ^{31}P spectroscopy.[1−4] The technique is applied to homopolymer, poly(*β*-hydroxybutyrate), and a random copolymer, poly(*β*-hydroxybutyrate-*co*-*β*-hydroxyvalerate), referred to as PHB and P(HB-*co*-% HV), respectively, where the % label is a "mol %".

Experimental Procedures. PHB samples BXG08 and acid-hydrolyzed ANNBXG08 samples were available from previous work.[12] A PHB homopolymer sample (BX IRD), a P(HB-*co*-6% HV) sample (BX P7/55), and a P(HB-*co*-20% HV) sample (BX P7/54) were provided by Marlborough Biopolymers, Billingham, UK. ALDR was a PHB homopolymer sample purchased from Aldrich Chemical Co.

Sample Preparation. A stock solution was prepared by dissolving approximately 400 mg of accurately weighed Bisphenol A (BPA) in pyridine, adding 5.5 mg of Cr(acac)$_3$, and subsequently bringing the total volume to 10 mL. The polymers were dissolved in $CDCl_3$, heating gently if necessary, with concentrations of 50−100 mg/mL. Care was taken to avoid making polymer solutions too viscous to be handled with the necessary accuracy. A 400 μL of the polymer solution and 40 μL of the stock solution were transferred into a 5 mm NMR tube. Subsequently, an excess of 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**I**) was introduced into the NMR tube (12−40 μL, depending on the sample), followed by shaking. The mixture was left to react overnight, and the ^{31}P spectroscopic analysis was carried out the following day.

Scheme 1



I

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were recorded on a Varian XL-300 instrument, operating at 121.4 MHz for the ^{31}P nucleus. The ^{31}P spin−lattice relaxation time (T_1) measurements of selected samples were performed by using the inversion−recovery pulse sequence. In a similar manner to previous accounts,[2] the presence of the relaxation agent Cr(acac)$_3$ was found to lower the ^{31}P T_1's to values in the range 5−7 s; thus the delay between acquisitions was set typically between 30 and 40 s. The parameters of a ^{31}P spectrum used for the molecular weight calculations (see Figure 1) were as follows: spectral width of 2.5 kHz, 90° pulse of 10.5 μs, broad-band proton decoupling, 3712 points zero-filled to 8K, line broadening of 1 Hz before Fourier transformation, 128 transients (∼1 h). The high molecular weight PHA samples required 1500−2000 acquisitions to obtain a reasonable *S*/*N* ratio. The ^{31}P peak of unreacted reagent **I**, reported[4] to appear at 176 ppm in $CDCl_3$, was used as a chemical shift reference. Molecular weights were calculated by integrating the ^{31}P signals resulting from phosphitylated BPA and the various types of labile proton moieties present in the polymers.

Results and Discussion. Model Compounds. The reaction of **I** with a series of model compounds was examined at first, in an attempt to establish a link between chemical structure and ^{31}P chemical shifts. These results, along with the ^{31}P chemical shifts of the phosphitylated products of the various polymers studied, are presented in Table 1. The model compounds were chosen for their similarity with the repeat unit structure of PHB, or other structures possibly present on the hydrolyzed PHB polymers. This approach is necessary, because of the well-known dependence of ^{31}P chemical shifts on the solvent system.[6] Although a considerable amount of work has already been done using model compounds for lignins,[3,4] that work was done using high concentrations of pyridine in the solvent system. In the present study, only the necessary stoichiometric amount of pyridine was introduced into the solvent system for solubility reasons. As expected, this had a strong effect on the ^{31}P chemical shifts of the phosphitylated compounds.[6] For example, the two phosphitylated carboxylic groups of (+)-3-methyladipic acid appear at 135.69 and 135.64 ppm (Table 1). This is about 1 ppm downfield from the values reported by Jiang et al.[4] when an excess of pyridine is present.

Examination of the data of Table 1 shows that the variation of the ^{31}P chemical shift with structure is more

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Table 1. ^{31}P Chemical Shifts[a] for Model Compounds and PHA Polymers[b]

sample	OH	COOH
3- hydroxybutyric acid	146.16	135.65
methyl 3-hydroxybutyrate	146.25	
(+)-3-methyladipic acid		135.69
		135.64
crotonic acid		135.61
poly(ϵ-caprolactone)	147.83	135.60
poly(β-propiolactone)	147.84	135.64
BXG08	146.42	135.61
ANNBXG08	146.42	135.57
BX IRD	146.41	135.60
ALDR	146.41	135.60
BX P7/55	146.41	135.60
BX P7/54	146.42	135.60

[a] Referenced to BPA peak at 138.57 ppm. [b] BXG08, ANNBXG08, BX IRD, and ALDR are homopolymer PHB samples; BX P7/55 and BX P7/54 are P(HB-*co*-HV) samples. See the experimental section for details.

pronounced for hydroxyl than for carboxyl groups. In addition, the downfield shift observed for the phosphitylated product of the hydroxyl group when going from 3-hydroxybutyric acid to its methyl ester (146.16 → 146.25 ppm) is consistent with the ^{31}P chemical shift of the phosphitylated OH end group of the polyester (146.41−146.42 ppm). The linear backbone structure of poly(ϵ-caprolactone) and poly(β-propiolactone) shifts the position of the ^{31}P peaks of their OH end group ~1.4 ppm downfield from PHB, at 147.83 ppm. This is in accord with the downfield shift observed for secondary alcohols with respect to primary alcohols by Archipov et al.[3]

In the case of the carboxyl end groups, the range covered by the respective phosphitylated products is extremely small. All the model compounds and polymer samples of Table 1 gave ^{31}P peaks at 135.5−135.7 ppm, regardless of the local chemical structure in the vicinity of the carboxylic group. The small chemical shift range for the carboxyl compared to hydroxyl groups in effect restricts the use of the former for extracting information about the local chemical structure in PHA polymers.

Hydrolyzed PHB Samples. As previously reported,[12] an annealed bacterial PHB sample (ANNBXG08) was hydrolyzed with 3.0 N HCl at 104.5 °C as a function of time, producing PHB polyesters with decreasing number-average molecular weights. These polyesters were subjected to phosphitylation with **I**, and the resulting products were analyzed by quantitative ^{31}P NMR according to the experimental protocol described previously. Some representative ^{31}P NMR spectra of hydrolyzed PHB are shown in Figure 1. Care was taken to ensure that the spectra were quantitative, by properly adjusting the delay between acquisitions to be at least $5T_{1max}$, and by switching off the decoupler during this long delay to avoid complications due to possible differences in the nuclear Overhauser effect (NOE).

The calculation of number-average molecular weights from spectra similar to Figure 1 is straightforward,[2] since the amount of the phosphitylated product of BPA with **I**, producing a peak at 138.57 ppm in Figure 1, is exactly known. The results of the M_n calculations are presented in Figure 2. Error bars were calculated by repeating the experiment three times, each time using fresh polymer samples. The ^{31}P spectra of the hydrolyzed PHB samples indicate that the number of OH end groups present in each sample is consistently smaller than COOH, regardless of the hydrolysis time. In addition, the number-average molecular weights obtained from the COOH integrals agree with those obtained by GPC. However, M_n's obtained from OH integrals are consistently higher than those measured by GPC.

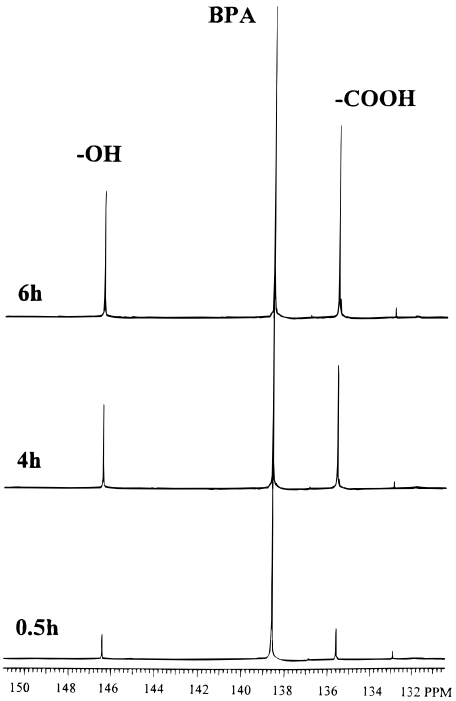

Figure 1. ^{31}P NMR spectra of HCl-hydrolyzed bacterial PHB, as a function of hydrolysis time, after derivatization with **I** to afford the respective phosphite esters. The peak at 132.9 ppm is due to the reaction of **I** with traces of water.

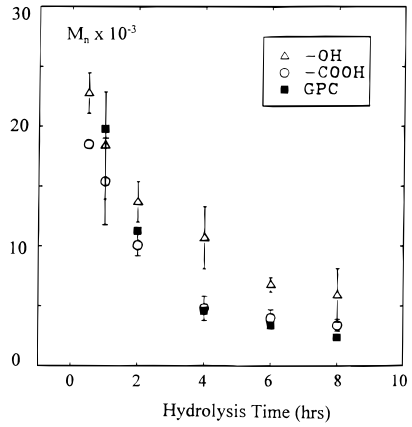

Figure 2. Number-average molecular weights, M_n, of hydrolyzed PHB samples as determined by quantitative ^{31}P NMR spectroscopy. Open symbols refer to M_n's obtained by OH and COOH end groups; solid symbols represent M_n's obtained by GPC from ref 12).

These observations suggest that the hydrolysis mechanism leads to a lower concentration of OH groups than COOH. Random chain scission is widely accepted as the dominant mechanism of cleavage occurring in the case of PHB.[13,14] It has been shown to operate during the pyrolysis of bacterial PHB and P(HB-*co*-HV),[13] the thermal[15] and hydrolytic[16] degradation of PHB and P(HB-*co*-HV), and the methanolysis of PHB.[17] Studies of the pyrolysis and thermal degradation of PHB revealed that random chain scission involves a β-CH hydrogen transfer through the formation of a six-membered-ring indermediate.[14] Such a reaction will induce the formation of a crotonate instead of a hydroxyl

Table 2. Number Average (M_n) and Viscosity-Average (M_v) Molecular Weights of Various PHA Polymers As Measured by Quantitative ^{31}P NMR Spectroscopy, GPC, and Viscometry

		M_n		
sample	composition	^{31}P NMR	GPC	M_v viscometry[b]
BX G08	PHB	137 000	174 000	
BX IRD	PHB	181 000	161 000	293 000
ALDR	PHB	510 000	670 000	
BX P7/55	P(HB-*co*-6% HV)	142 000		164 000
BX P7/54	P(HB-*co*-20% HV)	180 000	51 700	197 000

[a] GPC and viscometry data taken from ref 18, except BXG08 (ref 12) and ALDR (provided by Aldrich Chemical Co.).

end group. To account for our quantitative ^{31}P NMR data, one needs to assume that the β-CH hydrogen transfer mechanism is partly operative during the 3.0 N HCl acid hydrolysis of PHB at 104.5 °C. Since these conditions are not as severe as in the case of pyrolysis or thermal degradation studies, this mechanism contributes to, but does not dominate, hydrolytic degradation (in that case, no hydroxyl group peak would be observed). For the experimental conditions employed, this analytical technique showed that the contribution of the mechanism involving a β-CH hydrogen transfer, leading to a crotonate end group, was approximately 30%.

High Molecular Weight PHA's. To study the limits of the applicability of the technique, a number of high molecular weight samples of PHB and P(HB-*co*-HV) were derivatized with **I**. The results of the quantitative ^{31}P analysis and the molecular weights calculated are listed in Table 2. In addition, the molecular weights of the samples as measured by GPC and/or viscometry are also shown.[18] The results obtained using quantitative ^{31}P NMR are within experimental error of those measured by other techniques. At this point, however, it is not easy to determine which technique provides more reliable number-average molecular weights. GPC suffers inherently from the necessity of using polystyrene standards, and viscometry results are known to be imprecise when the molecular weight distribution is uncontrolled. Since the ^{31}P method presented herewith is an absolute value, independent of the distribution of molecular weights in the polymer, it is a significant characterization number when mechanistic interpretations are needed.

The molecular weights M_n of the polymers presented in Table 2 were calculated from the COOH derivative integrals of the ^{31}P NMR spectra. This was done because, surprisingly, the high molecular weight polymers also exhibited a smaller than expected amount of OH compared to COOH end groups. We believe this difference is real, and not an artifact of the technique. Differences in the respective T_1 relaxation times have to be excluded as a possible cause of this discrepancy, because the derivatized OH and COOH peaks exhibited similar T_1 values, and in all cases the delay between acquisitions was long enough to ensure that quantitative ^{31}P NMR spectra were acquired. Preferential phosphitylation of the COOH groups also has to be excluded, since the ^{31}P NMR spectrum of the derivatized products of 3-hydroxybutyric acid contained equivalent COOH and OH derivative peaks. One possible cause for the reduced amounts of OH moieties in the high molecular weight PHAs could arise as a result of the purification procedure.[19] During one or more stages of this procedure, the polymers are subjected to rather intense heating,[20] sometimes higher than 150 °C.[19] These conditions, although not strong enough to trigger extensive degradation, might lead to reactions that partially eliminate some of the OH end groups. Similarly, spray-drying of the final product could have effects equivalent to pyrolysis. It is stressed though that these assumptions should be further investigated through the analysis of appropriate PHA samples before any definite conclusions can be made. Seebach *et al.*[21] have made a serious study of the mechanism of this reaction for PHB and have compared molecular weight (MALDI MS and GPC) for the PHB with concentration of crotonate end groups.

Conclusions. Quantitative ^{31}P NMR spectroscopy has been found to be a valuable tool for the determination of number-average molecular weight of poly(hydroxyalkanoate) homo- and copolymers. Due to the 100% natural abundance of ^{31}P, the technique is highly sensitive and can be used for high molecular weight PHAs with satisfactory results. Furthermore, it was demonstrated that quantitative ^{31}P NMR can contribute to the understanding of the mechanism of acid hydrolysis of PHAs and the microstructure of the hydrolysis products. This is the first reported use of this technique for the PHA species and we believe it has the potential to become a useful tool in the study of PHA chemistry. More work in this direction is underway in this laboratory.

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