Thermal Degradation of Microbial Poly(4-hydroxybutyrate)

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ABSTRACT: We obtained by microbial synthesis an almost pure poly(4-hydroxybutyrate) (P(4HB)) sample (i.e., a P(3HB-*co*-4HB) copolymer containing only 3% of 3HB units). The thermal degradation of this material was investigated using thermogravimetry, direct pyrolysis mass spectrometry and also by preparative pyrolysis and subsequent NMR analysis of the pyrolyzate. Previous studies[7] on P(3HB-*co*-4HB) copolyesters have reported that the thermal degradation process occurs by a β-CH hydrogen transfer, similar to the well-known case of P(3HB). However, the latter work did not distinguish between the thermal degradation fate of 3HB and 4HB units. Our findings indicate that the thermal decomposition of P(4HB) yields a series of cyclic oligomers (i.e., γ-butyrolactone and its higher homologs) by an intramolecular exchange mechanism. Detailed mass spectrometric and ^{1}H-NMR evidence is presented to prove this point.

Introduction

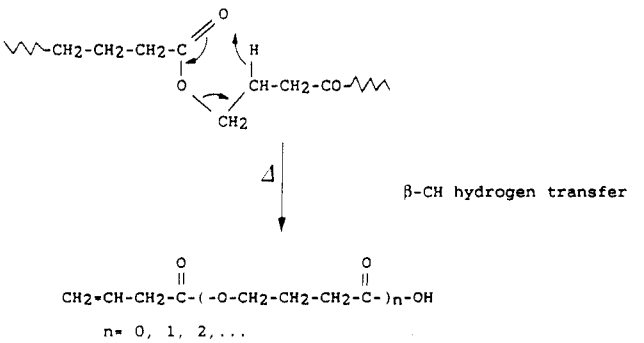
Poly(3-hydroxyalkanoate)s (PHAs) are a novel class of polyesters that can be produced by fermentation of a large number of bacterial species. PHAs bear many attractive properties: they are thermoplastic materials that are obtained from non-oil-based sources; being produced biosynthetically, they possess optical purity and are biodegradable and biocompatible; by careful choice of bacterial species and strains, growth conditions, and carbon sources feed, a large variety of comonomers can be introduced in the polyester main chain.[1-6] This last aspect has been used by the British firm ICI to circumvent the problem associated with the industrial processing of poly-3-hydroxybutyrate P(3HB), the longest term known and most studied among the PHAs. In fact, P(3HB) is thermally unstable at temperatures near its melting point (180 °C). By copolymerizing 3HB with 3-hydroxyvalerate (3HV), a new polyester P(3HB-*co*-3HV) is obtained with a lower degree of crystallinity and melting point. P(3HB-*co*-3HV) is not thermally degraded at the processing temperature.[3]

Recently, Doi et al.[6-8] developed a fermentation process to produce a new copolyester of 3HB and 4-hydroxybutyrate (4HB) from *Alcaligenes eutrophus*. The latter copolymer (a linear polyester) shows a rate of biodegradation higher than that of P(3HB) and P(3HB-*co*-3HV).[6]

The same authors[7] studied the thermal degradation of microbial P(3HB-*co*-4HB) in the temperature range 100–200 °C by monitoring the time dependent change in molecular weight of melt samples and determined the activation energy and the rate constant of the copolymer chain thermal cleavage. No appreciable weight loss was observed in the copolyesters for 20 min up to a temperature of 200 °C.

They concluded that the thermal decomposition occurs in P(3HB-*co*-4HB) by a random chain scission process and proposed a mechanism of degradation involving a β-CH hydrogen transfer process, similar to the widely accepted mechanism of cleavage occurring[9-11] in the case of P(3HB). Furthermore, the β-CH hydrogen transfer process was assumed to occur also in P(3HB-*co*-4HB) copolymers containing a very high amount (82%) of 4HB units. This result was quite unexpected for us, since we have studied the thermal decomposition mechanisms of several classes of polyesters and have established that (with exception of poly(β-lactones)) the pyrolysis of linear aliphatic polyesters produces cyclic oligomers.[12-15] There is no literature on the thermal degradation of P(4HB), neither on the pyrolysis products originating from poly(γ-lactones), and since the previous authors[7] did not distinguish between the thermal degradation fate of 3HB and 4HB units in the copolymer and did not identify the products originated in the pyrolysis of their P(3HB-*co*-4HB) copolymers, we decided to reinvestigate the matter before drawing any conclusion.

Scheme 1



We obtained by microbial synthesis an almost pure P(4HB) sample (i.e., a P(3HB-*co*-4HB) copolymer containing only 3% of 3HB units, as detected by NMR). The synthesis was performed by using *A. eutrophus* as described by the previous authors.[8] In this report we study the thermal degradation mechanism of our P(4HB) sample, comparing it with that of P(3HB) by using the direct pyrolysis mass spectrometry technique[12] (DPMS) and ^{1}H-NMR.

Our evidence shows that 4HB units do not decompose like 3HB units by a β-CH hydrogen transfer reaction (Scheme 1) and indicates instead an intramolecular ester exchange process (Scheme 2), leading to the formation of cyclic oligomers (macrocyclic lactones).

Our work suggests that P(3HB-*co*-4HB) polymers containing significant amounts of 3HB will undergo chain

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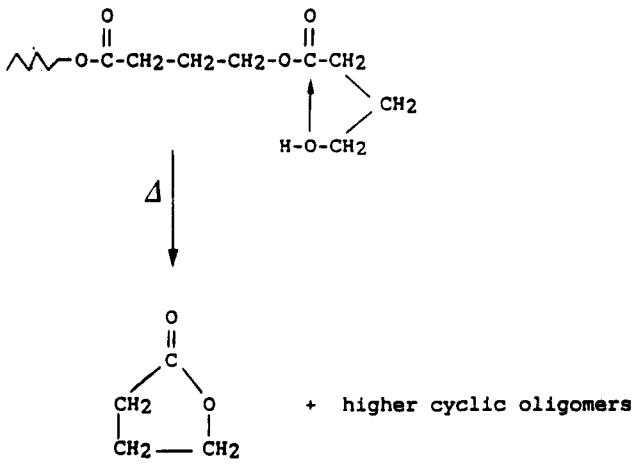
Scheme 2



scission via β-CH transfer process in competition with the formation of cyclic oligomers, and this will form the subject of further investigations.

Direct Pyrolysis Mass Spectrometry

Since the mechanism of thermal cleavage of a polymer chain can be often inferred from the structure of the primary pyrolysis products formed, thermal degradation studies ought to focus on techniques that allow detection of the latter. Under this aspect, the characterization of polymers by DPMS yields unique information.[12-14] A specific advantage of this technique is that polymer pyrolysis is accomplished on-line under high vacuum at the microgram level. Therefore, the pyrolysis products formed are quickly volatilized and the low probability of molecular collision reduces the occurrence of secondary reactions, and thus primary products can be detected. The pyrolysis is achieved adjacent to the electrons emitter, so that the pyrolysis products, once formed, are immediately ionized, thus preventing any further thermal rearrangement. The flight time of the ions from the ion source to the detector is on the order of microseconds. Therefore ions of high mass, which are often essential for understanding the thermal degradation mechanism of the polymer, can be detected on-line, whereas they are often lost using other techniques. Many studies, however, have been based on techniques which only allow the off-line analysis of the pyrolysis products,[13] and therefore thermally labile pyrolysis products, that may have very short lifetimes at high temperatures, may escape detection. A relatively long residence time of the pyrolysis products in the hot zone may cause a further degradation of the compounds primarily formed, and thermal degradation processes may also occur during the transport time (the time elapsing from product formation to detection). In DPMS residence and transport times are on the order of milliseconds for probe pyrolysis (electron impact (EI), chemical ionization (CI)) and even less for filament pyrolysis (desorption chemical ionization (DCI)). Sample size is also relevant, ranging from milligram amounts used in off-line techniques, to micrograms used in DPMS. Therefore, it happens that different techniques may see different products, and workers should consider that the lifetimes of pyrolysis products at the temperatures used in their experiments can be very short and that labile and higher molecular weight compounds may remain undetected.

Ionization methods that minimize the fragmentation processes of the molecular ions such as CI and DCI (where the pyrolysis is achieved inside the ion source) are very valuable in DPMS, since the resulting mass spectra contain intense molecular ions and therefore the identification of pyrolysis compounds is easier. It has been observed[13] that EI-MS studies of the thermal decomposition of aliphatic polymers are hampered by the lability of aliphatic molecules toward EI (i.e., the aliphatic compounds generated in the pyrolysis of these polymers do not exhibit significant molecular ions). On the contrary, the CI and DCI mass spectra show intense molecular ions corresponding to the compounds originating from aliphatic polymers. A comparative EI/CI/DCI study is often appropriate in order to detect the primary pyrolysis products originating from the latter polymers.[17]

The main problem connected with the DPMS technique is the identification of the products in the spectrum of the multicomponent mixture produced by thermal degradation. In fact, in the mass spectrum of a polymer, the molecular ions of the thermal products will appear mixed with the fragment ions formed in the ionizing step.

Several methods are available today in the standard mass spectrometric practice in order to identify compounds present in mixtures: (a) comparison with the mass spectra of authentic compounds; (b) tandem mass spectrometry to compare daughter and parent ion spectra; (c) soft ionization techniques, such as CI, DCI, or fast atom bombardment (FAB) to simplify complex ion fragmentations present in EI mass spectra.

We have used these techniques in order to investigate the thermal decomposition of P(4HB).

Experimental Section

Biopolymer Synthesis. P(3HB) and P(4HB) were obtained by fermentation of *A. eutrophus* H16 (ATCC 17699), according to Doi procedures.[7,16] Polyesters were extracted from lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by precipitation in hexane. The procedure used to obtain P(4HB) yielded a mixture of polyesters whose total composition was estimated by ^{1}H-NMR to be 61% 3HB and 39% 4HB. This mixture was extracted with hot acetone, and the soluble part was found to consist of a polyester containing 97% 4HB and 3% 3HB on a molar basis. The preparation of P(3HB) yielded instead a pure homopolymer.

Molecular Weight Measurement. A Waters 6000 A solvent delivery system equipped with a series of four Ultrastyragel columns (in the order 1000-, 500-, 10 000-, and 100-Å pore size) was used for gel permeation chromatography (GPC) analyses. A Model R 401 differential refractometer from Waters was used as the detector. Analyses were performed at 25 °C using chloroform as eluent at a flow rate of 1 mL/min. The columns were calibrated with polystyrene molecular weight standards. The GPC molecular weight was 710 000 for P(4HB) and 800 000 for P(3HB).

NMR Spectroscopy. The 200-MHz ^{1}H-NMR spectra were recorded at 25 °C in $CDCl_3$ (20 mg/mL) on a Bruker AC 200 spectrometer with a 4-s pulse repetition, a 2000-Hz spectral width, 16K data points, and a 256 scan accumulation. The ^{1}H noise decoupled 50-MHz ^{13}C NMR spectra were recorded on the same samples with a 1.6-s pulse repetition, a 10 000-Hz spectral width, 32K data points, and a 30 000 scan accumulation.

Thermal Analysis. Thermal gravimetry was performed with a Perkin-Elmer TGS/2 apparatus in a nitrogen atmosphere (60 mL/min) at a heating rate of 10 °C/min. The melting points of the polyester samples were measured by differential scanning calorimetry with a Mettler TA 3000 apparatus at a heating rate of 10 °C/min. These measurements referred to first heatings and gave 178 °C for P(3HB) and 52 °C for P(4HB).

Preparative Pyrolysis. P(4HB) (20 mg) was placed in a sublimation apparatus under a rotative pump vacuum. Pyrolysis was carried out from 250 to 320 °C in about 2 h. Pyrolysis products condensed at 0 °C were analyzed by NMR and FAB MS.

Mass Spectrometry. A double focusing Kratos MS 50 mass spectrometer equipped with the standard electron impact (EI), desorption chemical ionization (DCI), or fast atom bombardment (FAB) ion sources and with an Eclipse (Data General) data system running with DS90 acquisition software was used to obtain mass spectra. When operating with the EI ion source, the instrument was scanned from m/z 1400 to 20 at a scan rate of 10 s/decade.

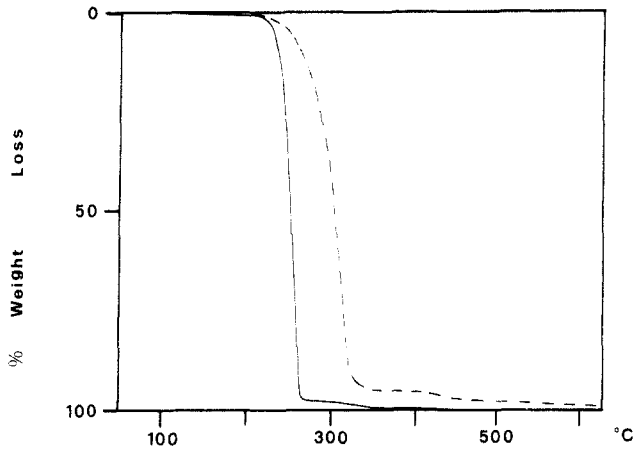


Figure 1. Weight loss curves for P(3HB) (—) and for P(4HB) (- - -) under a nitrogen atmosphere (heating rate 10 °C/min).

Polymers were pyrolyzed directly in the ion source using the standard Kratos probe heated from 30 to 400 °C at a heating rate of 10 °C/min. When operating with the DCI ion source, the instrument was scanned from m/z 1400 to 20 at a scan rate of 1 s/decade. Ammonia (99.998%, Matheson, Union Carbide, Oevel, Belgium) was used as the reagent gas. Samples, dissolved in chloroform, were deposited on the standard DCI tip and heated from 50 to 600 °C at a heating rate of 500 °C/min. Both in EI and in DCI mode the ion source was maintained at 150 °C and PFK or Fomblin was used for mass calibration. When operation was in the FAB mode, the cesium ion gun was operated at 20 kV. The instrument was scanned from m/z 2200 to 60 at a scan rate of 10 s/decade at an accelerating potential of 8 kV. Cesium and rubidium iodides (50:50 by weight) were used for computer calibration. The resolution was approximately 3000. B/E linked scans (daughter ion spectra) were performed in the EI mode using a linked scan unit at a scan rate of 20 s/decade and recorded on an UV oscillograph.

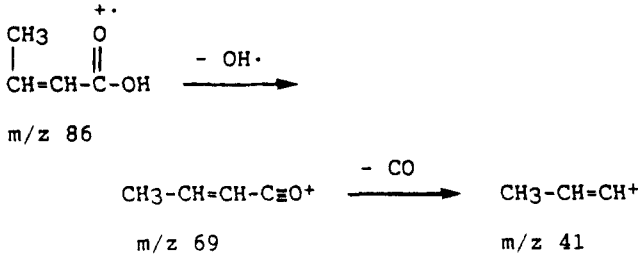
Results and Discussion

The integral thermogravimetric curves relative to the two isomeric polyesters P(3HB) and P(4HB) are shown in Figure 1. They are quite different, and the maximum volatilization rate determined on the differential thermogravimetric curves (DTG) is observed at about 270 °C for P(3HB) and 320 °C for P(4HB).

In order to compare the structure of the products generated in the pyrolysis of the two polymers, the ammonia DCI positive mass spectra of P(4HB) and P(3HB) were recorded. In Figure 2a is shown the DCI mass spectrum of P(4HB). The peaks at m/z 104, 190, 276, and 362 correspond to pseudomolecular ions $M_nNH_4^+$ (n = 1–4), thus confirming that P(4HB) thermally degrades giving a series of oligomers. In Figure 2b is reported the DCI mass spectrum of P(3HB), which shows essentially a similar peak pattern. In fact, the spectra show two intense molecular ion series at masses M_n (or M_n + H) and M_n + NH_4, corresponding to the isobar oligomers generated in the pyrolytic process. Although the mass spectrum of P(3HB) (Figure 2b) allows one to discern oligomers up to the octamer, whereas the spectrum of P(4HB) (Figure 2a) shows only peaks up to the tetramer, and the two spectra differ in several other details, the overall pattern of the two DCI spectra in Figure 2 does not allow a structural differentiation of the oligomers produced in the pyrolysis of P(4HB) and P(3HB).

In order to achieve the structural differentiation wanted, we decided to investigate in detail the ion fragmentation processes occurring in the two series of isomeric molecular ions produced in the pyrolysis of the two microbial polyesters. Figure 3a shows the EI (18-eV) mass spectrum, taken at a probe temperature of 270 °C, of the pyrolysis products evolved from P(3HB).

The products originate from a β-CH hydrogen transfer process[9-11] and are oligomers bearing carboxyl and olefin end groups. In fact, we observe the molecular ion peak corresponding to the monomer (crotonic acid, m/z 86) and its EI fragments:



Higher oligomers up to the tetramer (peaks at m/z 172, 258, and 344, respectively) are detectable in the spectrum in Figure 2a, but they are accompanied by much more intense peaks at m/z 87, 173, 259, and 345 (M + 1) that could be formed in an ion-molecule reaction common to this class of compounds. Also these products show fragmentation patterns similar to the monomer.

Figure 3b shows the EI (18 eV) mass spectrum at a probe temperature of 320 °C of the pyrolysis products evolved

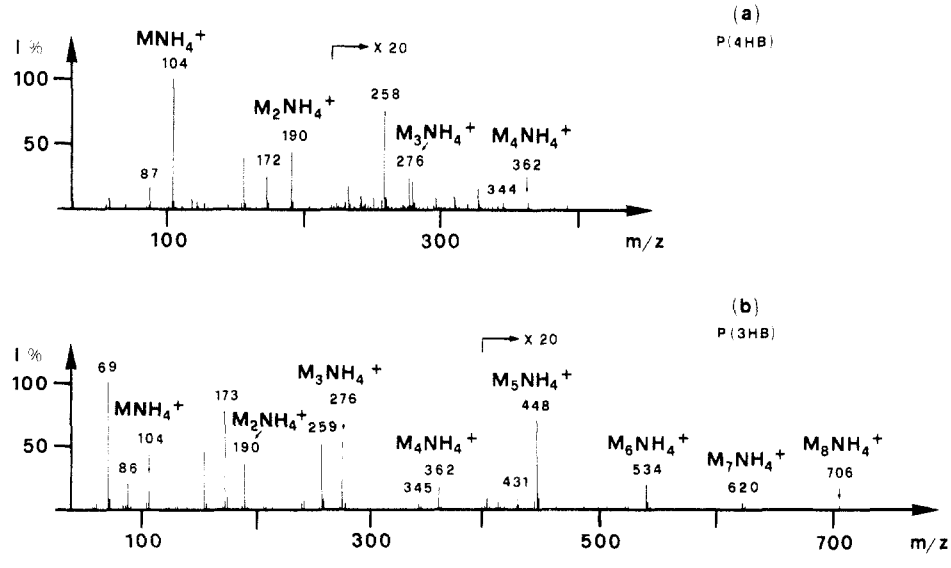


Figure 2. Positive ion pyrolysis DCI (ionizing gas NH_3) mass spectra of (a) P(4HB) and (b) P(3HB).

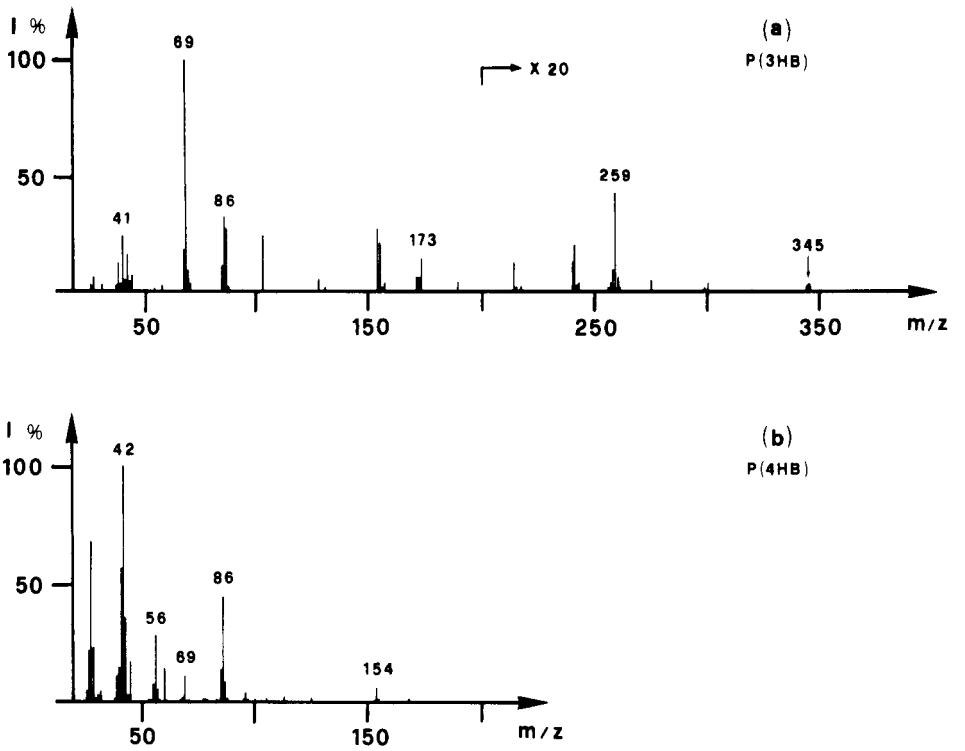
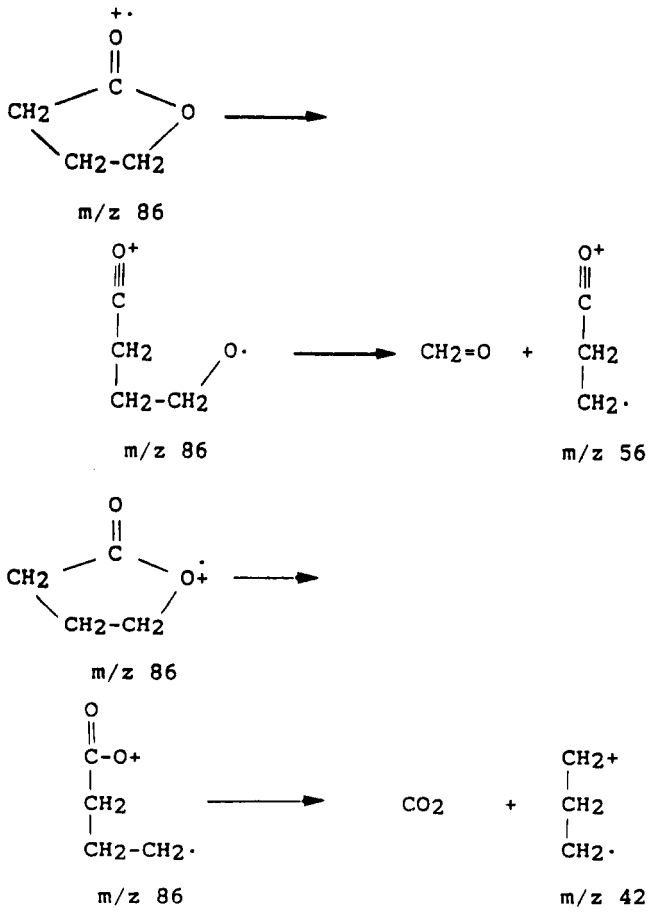


Figure 3. Direct pyrolysis EI mass spectra (18 eV) of (a) P(3HB) and (b) P(4HB) at 270 and 320 °C, respectively.

from P(4HB). Although the two polymers are isomeric, the spectrum in Figure 2b is considerably different from that of P(3HB), and the formation of γ-butyrolactone can be hypothesized. In fact, if we observe (Figure 3b) the peak at *m/z* 86 and its EI fragmentation peaks, the overall pattern can be interpreted as supporting the assignment of the molecular ion at *m/z* 86 to the γ-butyrolactone structure.



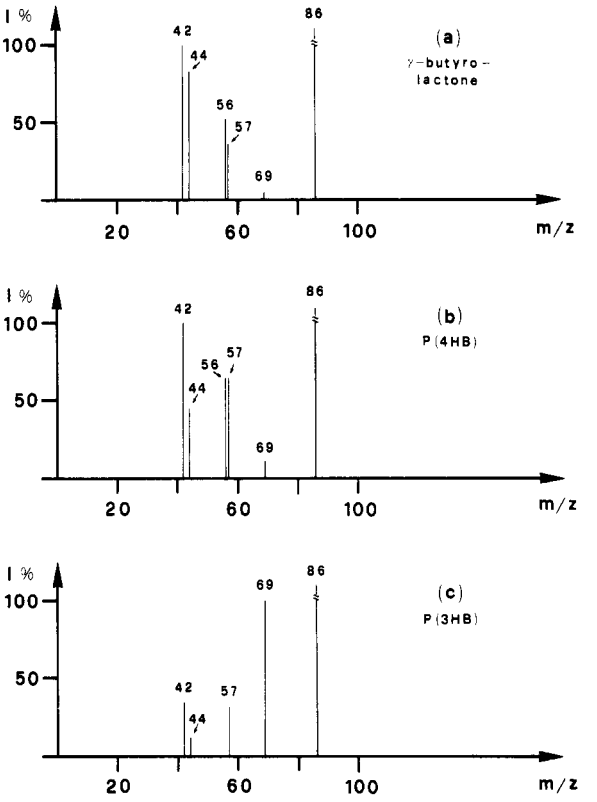


Figure 4. EI (70-eV) daughter ion spectra of (a) γ-butyrolactone, (b) the ion at *m/z* 86 from direct pyrolysis MS of P(4HB), and (c) the ion at *m/z* 86 from direct pyrolysis MS of P(3HB).

In order to verify that the peak at *m/z* 86 belongs to the molecular ion of γ-butyrolactone and not to 3-butenoic acid, coming from a β-CH hydrogen transfer process as in the case of P(3HB), we performed some daughter ion (B/E linked scans) measurements.

The EI daughter ion spectrum of an authentic sample of γ-butyrolactone in Figure 4a has been compared with the daughter ion spectra of the ions at *m/z* 86 obtained in the pyrolysis of P(4HB) (Figure 4b) and P(3HB) (Figure 4c), respectively.

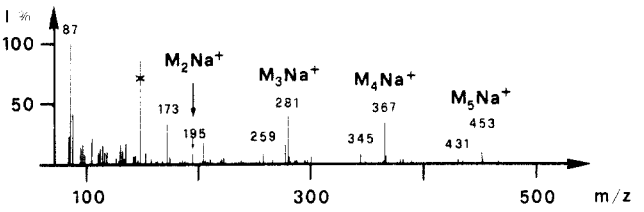


Figure 5. Positive ion FAB mass spectrum (3-nitrobenzyl alcohol matrix doped with NaCl) of the pyrolysis products obtained by thermal degradation of P(4HB) in a sublimation apparatus. The peak crossed with an "x" is due to residual matrix after computer background subtraction.

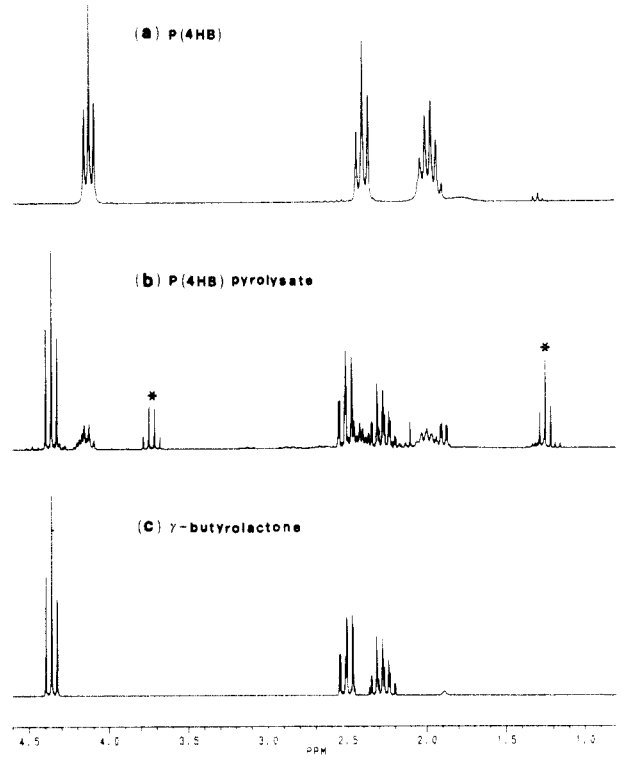


Figure 6. 200-MHz ^{1}H-NMR spectra in $CDCl_3$ of (a) P(4HB), (b) pyrolysis products obtained by thermal degradation of P(4HB) in a sublimation apparatus, and (c) γ-butyrolactone. Starred signals in trace b are due to ethyl alcohol present as a stabilizer in the chloroform used to collect the pyrolyzate. Chemical shifts are in ppm from internal tetramethylsilane.

The first two spectra are identical. On the contrary, in the spectrum in Figure 4c we observe a very intense peak at m/z 69 and the absence of the peak at m/z 56, thus confirming that the pyrolysis product at m/z 86 from P(4HB) is γ-butyrolactone.

In order to doublecheck the results obtained by the DPMS investigation, we then performed a preparative pyrolysis of P(4HB), as described in the Experimental Section.

The FAB mass spectrum of this pyrolyzate is shown in Figure 5. Protonated molecular ions corresponding to m/z values of 87, 173, 259, 345, and 431 and sodiated adducts at m/z values of 195, 281, 367, and 453, clearly show the formation of oligomers up to the pentamer. However, as in the DCI experiments, also in this case we cannot discern between cyclic or open chain products.

Therefore we recorded the 200-MHz ^{1}H-NMR spectrum of the pyrolysis products of P(4HB) and compared it with those of an authentic sample of γ-butyrolactone and with a sample of the pure polymer.

The spectra are shown in Figure 6a–c, and we can observe that in the spectrum of the pyrolyzate (Figure 6b), besides the three multiplets centered at 4.36, 2.50, and 2.27 ppm corresponding to the methylene protons of γ-butyrolactone (Figure 6c), are present also three other multiplets of lower intensity centered at about the same ppm as those present in P(4HB) (Figure 6a) (4.12, 2.39, 1.97 ppm).

This result indicates that the P(4HB) pyrolyzate consists of a mixture of γ-butyrolactone and of its higher (cyclic) homologs. The ring strain causes a downfield shift of the proton signals in γ-butyrolactone, which levels gradually off in the higher lactones to reach about the same chemical shift values observed in the polymer. This strain effect has been already observed to occur in other macrocyclic esters.[16]

Furthermore, it is to be noted that a β-CH hydrogen transfer process is ruled out by the absence in the spectrum of the P(4HB) pyrolyzate of the characteristic NMR signals of 3-butenoic acid (multiplets at 3.1, 5.1, and 5.8 ppm) and of its higher homologs.

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