MICROBIOLOGICAL REVIEWS, Dec. 1990, p. 450–472
0146-0749/90/040450-23$02.00/0

Vol. 54, No. 4

Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates

ALISTAIR J. ANDERSON AND EDWIN A. DAWES*

Department of Applied Biology, University of Hull, Hull, HU6 7RX, United Kingdom

INTRODUCTION

Seventeen years have elapsed since we last reviewed extensively the topic of the microbial reserve polyester poly(3-hydroxybutyrate) (PHB) (35). During this period the subject has developed tremendously in several areas. The first has been the recognition that PHB is but one example, albeit the most abundant, of a general class of optically active microbial polyesters which contain hydroxyacyl monomer units other than 3-hydroxybutyrate (3HB), and are termed polyhydroxyalkanoates (PHA). These compounds embrace copolymers which possess properties differing from PHB in ways that make them especially attractive for

* Corresponding author.

commercial exploitation, which is the second major area of research expansion. Two recently published (but not recently written) substantial reviews, of bacterial growth and survival (34) and of intracellular reserves (132), have considered PHB, although the latter article does not include any mention of PHAs. The review of Holmes (80) considers in detail the physical properties of PHB and copolymers, their commercial significance, and their applications.

Although the properties of PHB as a biodegradable thermoplastic material have captured attention for more than 20 years, and patents were originally filed in the United States by J. N. Baptist in 1962, the first industrial production of PHB and PHA did not occur until 1982, when ICI plc marketed them under the trade name Biopol. Marchessault et al. (97) have also drawn attention to the potential role of PHAs as so-called biomass transducers, i.e., their use in the microbial transformation of carbohydrate feedstock via PHA into chiral depolymerization products (152, 153) or small-molecule organic chemicals by pyrolysis. Underlying these developments has been the need to study the structure of PHB and of PHA copolymers, their physical state, the metabolic pathways involved, and the regulation of their synthesis in the microbial cell, endeavors which represent a third major area of activity.

The current sharp focus of attention on the environmental pollution caused by discarded petrochemical plastics has given added impetus to studies of the biodegradation of PHAs in the natural environment. However, against these important applied aspects there has also been an increasing appreciation of the role of such polymers in the life of microorganisms in their natural habitats.

The present review aims to survey some of the important developments that have occurred since 1973.

OCCURRENCE OF PHAs

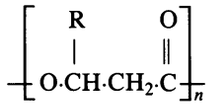
Discovery of PHAs

The first indication that the polymer discovered by Lemoigne (93) may contain proportions of 3-hydroxyacids other than 3HB was given by Wallen and Rohwedder (174), who, in 1974, reported heteropolymers in chloroform extracts of activated sewage sludge. They noted the presence of 3HB and 3-hydroxyvalerate (3HV; 3-hydroxypentanoate) as major constituents with C_6 and possibly C_7 3-hydroxyacids as minor components. This heteropolymer had a lower melting point than PHB and, unlike the homopolymer, was soluble in hot ethanol.

Findlay and White (55), using capillary gas-chromatographic (GC) analysis, detected at least 11 short-chain 3-hydroxyacids, the principal ones being 3HB and 3HV, in polymer extracted from marine sediments. Purified polymer extracted from monocultures of *Bacillus megaterium* contained approximately 95% 3HB, 3% 3-hydroxyheptanoate, 2% 3-hydroxyoctanoate, and trace amounts of three other 3-hydroxyacids. Subsequently, Odham et al. (117) found PHAs containing C_4, C_6, and C_8 components in sewage sludge.

A significant development was the report in 1983 by De Smet et al. (36) that *Pseudomonas oleovorans*, when grown on 50% (vol/vol) *n*-octane, accumulated granules that resembled PHB inclusions by freeze-fracture electron microscopy but corresponded to an empirical formula of $C_8H_{14}O_2$ on analysis and appeared to consist principally of a polyester of 3-hydroxyoctanoic acid. It was suggested that a minor, unidentified fatty acid present in hydrolysates was a degradation product of 3-hydroxyoctanoate. Subsequently a more detailed investigation (R. G. Lageveen, Ph.D. thesis, University of Groningen, Groningen, The Netherlands, 1986) and Lageveen et al. (91) disclosed that growth on *n*-octane yielded polymer containing (*R*)-3-hydroxyoctanoate and (*R*)-3-hydroxyhexanoate as major and minor components, respectively. *P. oleovorans* did not synthesize PHB from either *n*-alkanes or glucose.

Most of the known polyhydroxyalkanoates are polymers of 3-hydroxyacids possessing the general formula



The majority of the published research on PHAs other than PHB has concentrated on two bacteria: *Alcaligenes eutrophus* and *P. oleovorans*.

PHAs Produced by *A. eutrophus*

A glucose-utilizing mutant of *A. eutrophus* accumulates up to 80% (wt/wt) PHB with glucose as the carbon source (79). When propionic acid is included in the medium, a random copolymer containing both 3HB and 3HV monomer units is produced (80; P. A. Holmes, L. F. Wright, and S. H. Collins, European patents 0 069 497, January 1983, and 0 052 459, December 1985). The 3HV content of the PHA is dependent on the ratio of propionic acid to glucose in the medium during the polymer accumulation stage, and these authors have described conditions under which the bacterium accumulates 70% (wt/wt) copolymer containing 33 mol% 3HV monomers. With propionic acid as the sole carbon source, a 3HV content of 43 mol% has been achieved (41) but the total polymer content of the bacteria was relatively low (35%, wt/wt). As a cosubstrate with glucose, butyric acid produces only PHB (Holmes et al., patent, 1985). A copolymer containing 90 mol% HV units can be produced by *A. eutrophus* with valeric (pentanoic) acid as the sole carbon source (48), and as a cosubstrate with glucose, this compound yields PHA containing a higher proportion of 3HV units than obtained under comparable conditions with glucose plus propionic acid (74).

In addition to the well-studied accumulation of polyesters of C_4 and C_5 3-hydroxyacids, *A. eutrophus* is capable of producing PHAs containing 4-hydroxybutyrate and 5-hydroxyvalerate monomers; Doi and colleagues have demonstrated that these monomers are incorporated into copolymers and terpolymers when *A. eutrophus* is supplied with various single or mixed carbon sources (Table 1). The composition of such polyesters is determined by the relative concentrations of the carbon sources available during polymer accumulation, and these monomer units are, in each case, derived from a substrate of the same carbon chain length.

PHAs Produced by *Pseudomonas* Species

P. oleovorans. De Smet et al. (36) observed the presence of intracellular granules consisting of poly(3-hydroxyoctanoate) in *P. oleovorans* ATCC 29347 grown in two-phase medium containing 50% (vol/vol) octane. Subsequently, Lageveen et al. (91) extended this study and demonstrated the accumulation of polymers containing two or more 3-hydroxyacids from single *n*-alkanes (C_8 to C_{12}) as the sole carbon source (Table 2), the greatest yields of polymer being obtained from C_8 to C_{10} alkanes. When the organism was supplied with 1-octene, 3-hydroxy-7-octenoate was incorpo-

TABLE 1. Composition of PHAs accumulated by *A. eutrophus* ATCC 17699 from various carbon sources

Carbon source	Concn (g/liter)	PHA (%, wt/wt)	Monomer composition (mol%)				Reference[a]
			3HB	4HB	3HV	5HV	
$CH_3(CH_2)_3COOH$	20	36	10	–[b]	90	–	48 (90)
$Cl(CH_2)_3COOH$	18	27	89	11	–	–	44
$HO(CH_2)_3COOH$	16.5	30	67	33	–	–	44 (89, 90)
$HO(CH_2)_3COOH$ $CH_3(CH_2)_2COOH$	9.6 8	43	82	18	–	–	90
$HO(CH_2)_4OH$	20	8	75	25	–	–	89 (44)
$Cl(CH_2)_4COOH$ $CH_3(CH_2)_2COOH$	15 5	29	63	37	–	–	89
$O(CH_2)_3CO$	20	21	83	17	–	–	46 (47, 89)
$O(CH_2)_3CO$ $CH_3(CH_2)_2COOH$	10 10	65	76	24	–	–	47
$Cl(CH_2)_4COOH$[c]	20	1	24	–	24	52	49
$Cl(CH_2)_4COOH$[c] $CH_3(CH_2)_3COOH$	5 15	19	26	–	65	9	49
$HO(CH_2)_3COOH$ $CH_3(CH_2)_3COOH$	17.5 2.5	18	32	45	23	–	90

[a] Other relevant references in parentheses.
[b] Monomer not reported.
[c] Data for *A. eutrophus* NCIB 11599.

rated as a major monomer unit, although a substantial amount of 3-hydroxyoctanoate was also present in the PHA. Polyesters containing a proportion of unsaturated monomer units were also produced with 1-nonene or 1-decene as the sole carbon source. This strain of *P. oleovorans* also produces substantial amounts of PHA (Table 2) from *n*-alkanoic acids (19, 68) and C_8 to C_{10} *n*-alcohols (74).

The carbon chain length of the substrate determines the range of monomer units incorporated into PHA, with the 3-hydroxyacid possessing the same carbon chain length as the substrate being at least a major component of the polymer in all cases. It is apparent from the incorporation of substantial proportions of other 3-hydroxyacids, differing in chain length from the carbon substrate by two carbon units, that the removal or addition of C_2 units is involved in the biosynthesis of PHA by *P. oleovorans*, and this is discussed below.

Fritzsche et al. (58) have shown that it is possible to incorporate a proportion of branched-chain 3-hydroxyacids into PHA produced by *P. oleovorans* when 5-, 6-, or 7-methyloctanoate is provided as a cosubstrate with octanoic acid. 7-Methyloctanoate sustained growth when provided as the sole carbon source and yielded PHA containing 86% methyl-3-hydroxyoctanoate and 10% methyl-3-hydroxyhexanoate monomers. These workers have also reported (57) the accumulation of unsaturated polyesters by *P. oleovorans* from 3-hydroxy-6-octenoic acid or 3-hydroxy-7-octenoic acid as the sole carbon source; as previously observed for PHA produced from 1-octene (91), the polymer contained a proportion of saturated monomers.

Other *Pseudomonas* Species. The accumulation of PHA from *n*-alkanoic acids is not restricted to *P. oleovorans*. Haywood et al. (74) examined various *Pseudomonas* species for growth and polyester accumulation with C_6 to C_{10} straight-chain alkanes, alcohols, and alkanoic acids as the sole carbon source. Polymer accumulation was demonstrated in *P. aeruginosa* (three strains), *P. putida*, *P. fluorescens*, and *P. testosteroni*; in each case the range of substrates that supported PHA synthesis was different and also differed from that in *P. oleovorans*. The pattern of PHA accumulation in these *Pseudomonas* species is clearly related to that in *P. oleovorans* since 3-hydroxyacids possessing the same carbon chain length as the substrate, or differing by multiples of two-carbon units, are the major

TABLE 2. Composition of PHAs accumulated by *P. oleovorans* from *n*-alkanes, *n*-alkanoic acids, and *n*-alcohols

Carbon source	PHA (%, wt/wt)	3-Hydroxyacid monomers in PHA (mol%)									Reference
		C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	
Alkane											
Hexane	2	–[a]	–	100	–	–	–	–	–	–	
Heptane	11.4	–	–	–	100	–	–	–	–	–	
Octane	25.3	–	–	11	–	89	–	–	–	–	
Nonane	24.3	–	–	–	37	–	63	–	–	–	91
Decane	21.9	–	–	10		66		24	–	–	
Undecane	14.3	–	–		23	–	63		14	–	
Dodecane	5.8	–	–	2	–	31	–	36	–	31	
Alkanoate											
Hexanoate	5	3	<1	72	–	22	–	3	–	–	
Heptanoate	22	–	7	<1	86	<1	7	–	–	–	
Octanoate	41	<1	1	6	–	75	–	17	–	–	68
Nonanoate	49	–	3	<1	20	5	72	–	–	–	
Decanoate	37	<1	1	7	–	44	–	47	–	<1	
Alcohol											
Octanol	15	–	–	6	–	91	–	3	–	–	
Nonanol	33	–	–	–	27	–	73	–	–	–	74
Decanol	6	–	–	–	–	63	–	37	–	–	

[a] Monomer not reported.

monomer units found in the polyesters and no 3HB monomer (characteristic of PHB) is present in the polymer.

The accumulation of PHAs containing medium-chain-length (C_6 to C_{12}) 3-hydroxyacids, but not 3HB, appears to be a characteristic of the fluorescent pseudomonads, and it has been suggested that the ability to accumulate these PHAs, which is not dependent on the presence of a plasmid, may be of taxonomic value (81).

A number of *Pseudomonas* species accumulate PHA containing 3-hydroxydecanoate as the principal monomer unit from gluconate as the sole carbon source (A. J. Anderson, E. A. Dawes, G. W. Haywood, and D. Byrom, European patent 90303119.3, March 1990). One such organism, *Pseudomonas* sp. strain NCIMB 40135, produces these polymers from various carbon sources, including glucose, fructose, acetate, glycerol, and lactate. The polymer produced from glucose (74a) contained 79, 20, and 1 mol% C_{10}, C_8, and C_6 3-hydroxyacids, respectively, and yielded similar ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra to those reported (68) for polymers produced by *P. oleovorans* from *n*-alkanoic acids. Timm and Steinbüchel (171a) have shown that many strains of *P. aeruginosa* and certain other *Pseudomonas* species are also capable of accumulating substantial amounts of PHAs containing 3-hydroxydecanoate with gluconate as the sole carbon source. Although the biosynthetic pathways that yield these PHAs from unrelated substrates remain to be elucidated, it is likely that the precursors for the polymer are derived from reactions involved in lipid biosynthesis.

PHAs Produced by Other Bacteria

Recently, Brandl et al. (20) have reported that the photosynthetic bacterium *Rhodospirillum rubrum* is capable of producing PHAs containing C_4 to C_6 3-hydroxyacids from a range (C_2 to C_{10}) of *n*-alkanoic acids. Terpolyesters containing these hydroxyacids were produced from C_6, C_7, and C_{10} substrates. The shorter-chain substrates yielded polyesters containing C_4 and/or C_5 3-hydroxyacids but less than 1% of the C_6 monomer. The accumulation of PHA by *R. rubrum* is evidently quite different from that seen in the *Pseudomonas* species.

PHB accumulation by carbohydrate-utilizing halophilic archaebacteria has been reported (54). Thus, *Halobacterium mediterranei* accumulated some 38% (wt/wt) PHB at 15% salts concentration; the PHB yield declined by half as the salts were increased to 30%.

Methanotrophs of both types I and II (which assimilate carbon via the ribulose monophosphate and the serine pathways, respectively) accumulate PHB. Asenjo and Suk (6) have studied aspects of the conversion of methane to PHB, including growth conditions and inoculum age, in three type II organisms. The overall equation for organisms using the serine cycle was stated (6) as follows:

$$8CH_4 + 12O_2 \rightarrow C_4H_6O_2 \text{ (PHB monomer)} + 4CO_2 + 12ATP + FPH_2$$

giving a theoretical yield of PHB of 67% (86 g/128 g of CH_4). *Methylocystis parvus* was their organism of choice for further work, and PHB contents of up to 68% (wt/wt) were obtained with older (70-h) inocula, although the growth rate was lower and it took longer for the culture to attain the stationary phase than with younger inocula. Nonetheless, this maximum was more than double the highest previously reported for methanotrophs, e.g., 20 to 25% PHB in *Methylosinus trichosporium* (150). Mathematical models were constructed for growth and PHB formation from methane under conditions which assumed that there was nitrogen limitation with an adequate supply of methane and oxygen, that polymer accumulation occurs under nongrowth conditions, and that the rate of accumulation is highest in the early stationary phase, declining as the PHB content approaches its maximum (5).

Copolymers of 3HB with up to 22 mol% 3HV have now been found in *Rhizobium meliloti*, which, according to conditions, accumulated as much as 58% (wt/wt) of the material (172).

Haywood et al. (74) screened a wide variety of bacteria to determine the composition of PHAs produced from various carbon sources. Some bacteria were, like *A. eutrophus*, capable of producing a copolymer containing C_4 and C_5 3-hydroxyacids when supplied with propionic or valeric acid as cosubstrate with glucose, whereas others accumulated copolymer with only valeric acid plus glucose.

Several bacteria, including *Rhodococcus*, *Corynebacterium*, and *Nocardia* strains, produce 3HB-co-3HV copolymers from glucose as the sole carbon source (A. J. Anderson, E. A. Dawes, G. W. Haywood, and D. Byrom, European patent 90304267.9, April 1990). Copolymers produced from carbohydrate substrates contain, typically, 80 mol% 3HV monomer (G. W. Haywood, A. J. Anderson, and E. A. Dawes, unpublished results). With valeric acid as the sole carbon source, *Rhodococcus* sp. strain NCIMB 40126 produces a substantial amount of 3HV homopolymer; this polyester cannot, at present, be produced by *A. eutrophus*.

Future Work

Undoubtedly, novel PHAs, produced in pure culture, will be discovered in the quest for biodegradable plastics with desirable physical properties. Although commercial production of PHAs from expensive substrates may prove untenable, or the maximum yield may be low, it is important that this fundamental research be pursued to advance our understanding of the metabolic capabilities of bacteria in relation to PHA synthesis. The application of recombinant DNA technology and acquisition of knowledge of the precise control mechanisms involved in polymer synthesis should lead to the production of novel polymers tailored for specific purposes.

DETECTION, ISOLATION, AND ANALYSIS OF PHB AND OTHER PHAs

Staining Reactions

Microbiologists have traditionally detected the presence of PHB granules in bacterial cells by staining with Sudan Black B (see, for example, reference 162). However, Ostle and Holt (121) have advocated the use of Nile Blue A, a water-soluble basic oxazine dye that has a greater affinity and higher specificity than Sudan Black for PHB, and that gives a bright orange fluorescence at a wavelength of 460 nm. Other inclusion bodies, such as glycogen and polyphosphate, do not stain with Nile Blue A, emphasizing its usefulness.

Isolation of Native Polymer Granules

Native PHB granules were first isolated from cell extracts of *B. megaterium* (106) and of *Azotobacter beijerinckii* (141) by repeated centrifugation of DNase-treated cell extracts

layered on glycerol, using swing-out rotors; the granules collect at the interface. Griebel et al. (66) used both differential and density gradient centrifugation with glycerol to purify *B. megaterium* granules and secured preparations which retained about 30% of their original PHB synthase activity.

Crude granules from *Zoogloea ramigera* could be collected from extracts of sonically disrupted cells by centrifuging at 10^5 *g* for 60 min (61). After suspension in 50 mM potassium phosphate (pH 7.0), they were further fractionated by sucrose density gradient centrifugation at $6.4 \times 10^4 \times g$. It was claimed that PHB synthase activity remained stable for at least 6 months when crude granules were stored at −20°C.

Nickerson (115) purified PHB granules from sporulated cultures of *Bacillus thuringiensis* by density gradient centrifugation in NaBr. Two bands, one with and one without associated protein, were seen, and it was suggested that the latter consisted of granules that had lost their bounding membrane during purification. The technique was capable of separating PHB granules from spores and the intracellular entomocidal protein crystals produced by *B. thuringiensis*.

Effect of PHB on Buoyant Density of Bacteria

The accumulation of PHB (from 0 to 1.699 pg $cell^{-1}$) by *A. eutrophus*, under nitrogen-limiting conditions with fructose as the carbon source was accompanied by increases in cell volume from 1.208 to 3.808 μm^3 and in buoyant density from 1.110 to 1.145 pg μm^{-3} (126). The cell volume was found to increase linearly with PHB content and was attributable principally to an increase in width and not in cell length. Buoyant densities were measured on Percoll gradients, and it was shown that sucrose gradients create artifacts in which PHB-containing cells display apparently lower densities than PHB-less mutants, an effect previously noted by Schlegel and Oeding (148) and probably due to the high osmolarity of sucrose gradients. Mas et al. have developed a general mathematical model for determining the effects of intracytoplasmic inclusions on the volume and density of microorganisms (100).

Flow Cytometry

Srienc et al. (164) have used flow cytometry to measure the distributions of single-cell light-scattering intensity, which increases as refractile PHB granules accumulate in *A. eutrophus*. This noninvasive technique is clearly useful, although the contributions to the observed scattering of changes in cell size as the polymer content increases remain uncertain.

Extraction of PHB and Other PHAs

PHB and other PHAs are readily extracted from microorganisms by chlorinated hydrocarbons. Refluxing with chloroform has been extensively used; the resulting solution is filtered to remove debris and concentrated, and the polymer is precipitated with methanol or ethanol, leaving low-molecular-weight lipids in solution. Longer-side-chain PHAs show a less restricted solubility than PHB and are, for example, soluble in acetone (19). However, the large-scale use of solvents commercially is not economic, and other strategies have been adopted (see below). Ethylene carbonate and propylene carbonate have been used by Lafferty and Heinzle (90a) to extract PHB from biomass. Scandola et al. (146) reported that 1 M HCl-chloroform extraction of *Rhizobium meliloti* yielded PHB of $M_w = 6 \times 10^4$ compared with 1.4×10^6 when acetone was used.

Analysis of PHB and Other PHAs

Methods are available for determination of the PHB or PHA contents of microorganisms, the composition of PHAs, and the distribution of the monomer units of which they are composed.

The formerly widely used spectrophotometric method of Law and Slepecky (92) for quantitative PHB analysis, depending upon conversion of the polymer to crotonic acid by heating with concentrated sulfuric acid, has been largely superseded by GC and high-pressure liquid chromatography, which are applicable to PHAs in general. Thus, Braunegg et al. (21) subjected cells to direct, mild acid or alkaline methanolysis, followed by GC of the 3HB methyl ester, in an accurate procedure that could be completed within 4 h, enabling concentrations as low as 10 μM to be assayed. Riis and Mai (140) have suggested that the method can be improved by the use of propanol and HCl, which causes less degradation, and Comeau et al. (27) have described a convenient GC method for PHA analysis of activated-sludge samples.

Findlay and White (55) quantitated chloroform-extracted PHAs from *B. megaterium* and from environmental samples by acid (HCl) ethanolysis and GC-mass spectrometric (MS) analysis of the resulting 3-hydroxyalkanoic acid ethyl esters; they were able to analyze routinely samples containing 100 ng of purified polymer. Karr et al. (84) have used ion exclusion (Aminex) high-pressure liquid chromatography for the rapid analysis of PHB in *Rhizobium japonicum* bacteroids following digestion with concentrated sulfuric acid. Because this last method did not detect repeating units of polymer longer than 3HB, Brandl et al. (20) determined the PHA content and composition by GC after methanolysis of lyophilized cells for 140 min at 100°C to yield the methyl esters of the constituent 3-hydroxyalkanoic acids.

Pyrolysis of PHAs under nitrogen at controlled temperatures between 250 and 600°C, followed by GC-MS of trimethylsilyl derivatives, was used by Morikawa and Marchessault (110), and oligomeric products were identified as "dehydrated" monomer, dimer, and trimer from both PHB and 3HB-co-3HV copolymers. Grassie et al. (63, 64) have also applied pyrolytic techniques, and a fast and direct method of analysis of PHAs by rapid pyrolysis-capillary GC has been developed by Helleur (76). Samples of PHA (50 to 100 μg) are pyrolyzed at 450°C for 10 s, and the volatile products are subjected to GC. PHB is converted to crotonic acid and isocrotonic acid, whereas 3HB-co-3HV copolymers are converted to crotonic acid plus *trans*-2-pentenoic acid, with a small amount of the *cis* isomer. Some dehydrated dimers and trimers, derived from the homopolymer and copolymers, respectively, are also formed. Comparison with results of NMR analysis showed a reasonable correlation between the two methods of analysis for the 3HV content of copolymers. The technique is applicable to lyophilized bacteria as well as to extracted PHAs.

Because pyrolysis, like NMR (see below), seems to furnish structural information only up to the trimer level, Ballistreri et al. (8) undertook a comparison of three different polymer degradation techniques, with PHB and 3HB-co-3HV copolymer, to discover whether higher oligomers can be detected to aid the determination of monomer sequences. Furthermore, there is the danger that rearrangement by

interchange may occur on heating these polymers, rendering interpretation of sequences uncertain. They reported that fast-atom-bombardment MS analysis of partial methanolysis products is preferable to the two methods involving pyrolysis that they investigated, namely, direct pyrolysis-MS and fast-atom-bombardment MS analysis of the partial pyrolysis products, when structural information is needed to determine the sequence distribution of monomer units. However, fast-atom-bombardment analysis of the partial pyrolysis products was valuable for securing detailed data on the selectivity of the thermal degradation processes occurring in these polymers.

The conformational structure of the PHB molecule in solution has been studied by viscometry, light scattering, optical rotatory dispersion, and ^{1}H NMR (2, 29, 41, 99), with the overall conclusion that the helical structure is retained in chloroform solution.

NMR techniques have been applied successfully to the investigation of PHAs. Analysis of ^{1}H NMR spectra permits the determination of polymer composition, and the distribution of monomer units can be deduced from the diad and triad sequences by ^{13}C NMR spectral analysis. Jacob et al. (83) showed that it was possible to use cross-polarization magic-angle spinning ^{13}C NMR spectra of lyophilized samples of *Pseudomonas* sp. strain LBr to monitor directly and nondestructively PHB accumulated and utilized by this organism. A similar solid-state technique was used by Doi et al. (42) for PHB isolated from *A. eutrophus*.

Doi et al. (40) analyzed the conformation of PHB in chloroform solution by 500-MHz ^{1}H NMR spectroscopy, and the sequence distribution of the monomeric units in 3HB-co-3HV copolymer from *A. eutrophus* was determined by analysis of 125-MHz ^{13}C NMR spectra; statistically random diad and triad sequences were observed (41). Bloembergen et al. (15) also used ^{1}H NMR and additionally reported that Fourier transform infrared spectroscopy is a sensitive method for determining the degree of crystallinity of such copolymers.

Molecular Weight Determination

Gel permeation chromatography in chloroform at 30°C with a set of five microstyragel columns was used by Barham et al. (10) to determine the viscosity-average molecular weight (M_v) of PHB from *A. eutrophus*. Polystyrene standards were used, and the results were corrected by using the Mark Houwink relationship, $[\eta] = KM^{\alpha}$, where $[\eta]$ is the intrinsic viscosity, M is the molecular weight, and K and α are constants for the particular solute-solvent-temperature combination. Suzuki et al. (168) carried out similar analyses to determine the molecular weight distribution of PHB from the methylotroph *Protomonas extorquens*, and Scandola et al. (146) used viscometry to compare the M_w of PHB samples extracted by different methods from *Rhizobium* spp.

Glass Transition and Melting Temperatures

PHAs are partially crystalline polymers, and the definition of their thermal and mechanical properties is normally expressed in terms of the glass-to-rubber transition temperature (T_g) of the amorphous phase and the melting temperature (T_m) of the crystalline phase. Differential scanning calorimetry is used for these determinations. PHB samples commonly exhibit a degree of crystallinity in the range of 60 to 80%, which decreases to 30 to 40% as the 3HV content of 3HB-co-3HV increases to 30 mol% (109). T_g for PHB has been recorded within the span −5 to 5°C (10) and 0 to 20°C (23, 146), decreasing slightly as the 3HV content of copolymer increases. Likewise, the T_m of PHB, measured by differential scanning calorimetry, decreases from about 174°C for the homopolymer to about 143°C for copolymer containing 30 mol% 3HV (109). Scandola et al. (145) found that 3HB-co-4HB copolymers are partially crystalline over the range of composition investigated (7 to 82 mol% 4HB) and noted that the T_g of PHB is decreased more markedly by copolymerization with 4HB than with 3HV.

PHYSICAL PROPERTIES OF PHAs

Marchessault and colleagues showed that PHB is a compact right-handed helix with a twofold screw axis and a fiber repeat of 0.596 nm (28, 119). It is optically active, with the chiral center of the monomer unit always in the *R* absolute configuration [D-(−) in the traditional nomenclature]. The similarity of the PHB structure to that of polypropylene, which also has a compact helical configuration and a melting point near 180°C, attracted the attention of ICI plc to the potential of PHB for fiber and plastics applications (154; Holmes et al., European patent, 1985), particularly in the biomedical field, where biocompatibility and biodegradability are important features. PHB and polypropylene display similar degrees of crystallinity and T_g, although their chemical properties are completely different, PHB possessing far inferior solvent resistance but better natural resistance to UV weathering. Physically, PHB is stiffer and more brittle than polypropylene (79).

However, when copolymer formation occurs with 3HB and 3HV monomer units, the properties of the material (Biopol; ICI) alter as a consequence of decreased crystallinity and T_m. This results, in mechanical terms, in a decrease in stiffness (Young's modulus) and an increase in toughness, producing more desirable properties for commercial application. Consequently, a range of properties is feasible from the hard and brittle homopolymer via a balance of stiffness and toughness to soft and tough for copolymers with a high incorporation of 3HV. Holmes (79) observed that Biopol is not a specialty engineering material for high-performance mechanical applications.

The crystalline chain conformation of poly(3-hydroxyvalerate) PHV is very similar to that of PHB (18, 176). It also is a 2_1 helix with a two-repeating-unit volume of 0.13 nm^3 and a fiber repeat of 0.556 nm compared with 0.11 nm^3 and 0.596 nm, respectively, for PHB. Copolymers of 3HB and 3HV are isodimorphic; they display a melting point minimum at a 3HV content of ca. 30 mol%, and for compositions on one side of this melting point minimum 3HV units crystallize in the PHB lattice while on the other 3HB units crystallize in the PHV lattice. It is isodimorphism which accounts for many of the interesting physical properties displayed by these materials (17, 18).

Bloembergen et al. (15) observed that the rate of crystallization decreased with increasing 3HV content; the rate could be significantly increased by the addition of nucleating agents, a procedure which simultaneously reduces brittleness and increases elongation at break. Barham (9) made an extensive study of the nucleation behavior of PHB and found that in the pure polymer nucleation is sporadic. Organ and Barham (120; S. J. Organ and P. J. Barham, Abstr. Biol. Eng. Polym. Conf., Cambridge, England, 1989) have investigated the crystalline morphology and nucleation of 3HB-co-3HV copolymers and blends by transmission electron microscopy after developing an etching technique by using

methylamine. They reported that the incorporation of 3HV units into PHB had a profound effect on crystallization behavior, influencing the nucleation rates, growth rates, degree of crystallinity, and subsequent aging effects which may result from particular crystallization conditions. The ability to control these factors, which influence the physical and mechanical properties of the material and hence its commercial applications, is obviously of paramount importance.

The topic of solid-state mechanical properties of PHAs lies outside the scope of this review, but it should be noted that Owen (122) examined the dynamic tensile modulus and measured X-ray scattering of 3HB-co-3HV copolymers and that Bauer and Owen (13) studied load elongation curves and thermomechanical softening of PHB and copolymers. The viscoelastic and thermal properties of PHB from *Rhizobium* spp. have been investigated by Scandola et al. (146), and Holmes (79) reported some of the earlier observations relating to mechanical properties.

Novel copolyesters of 3HB and 4HB, with a range of compositions varying from 0 to 49 mol% 4HB, have been obtained from *A. eutrophus* presented with 4-hydroxybutyric and 4-chlorobutyric acids (44) or with γ-butyrolactone (47, 89) as substrates. These copolymers were characterized and found to possess a random sequence distribution of monomer units. The X-ray crystallinity decreased with an increasing fraction of the 4HB units, e.g., from 55 to 14% as the 4HB content increased from 0 to 49 mol%, with a corresponding decrease in T_m from 180 to 150°C. Copolymers containing more than 40 mol% 4HB exhibited the mechanical properties of an elastic rubber (89). Comparative studies by these workers indicated that the biodegradability of 4HB-containing polyesters is greater than that of PHB or 3HB-co-3HV polymers.

The M_n (2.4×10^5 to 2.9×10^5) of the copolyesters derived from γ-butyrolactone and butyric acid at pH 7.5 was relatively independent of the proportions of the substrates in the feedstock but was influenced by the pH of the medium, e.g., increasing from 1.46×10^5 to 3.74×10^5 over the pH range 5.1 to 9.0. The polydispersities (M_w/M_n ratios) of these copolyesters were in the range of 1.8 to 2.7 (46).

The physical properties of PHAs containing long-chain 3-hydroxyacids, produced from *n*-alkanes or *n*-alkanoic acids by *P. oleovorans* (19, 91) and certain other pseudomonads (4, 74) differ markedly from 3HB-co-3HV in being elastomers. Nevertheless, these copolymers containing C_8 to C_{10} components crystallize as a 2_1 helix in an orthorhombic lattice with two molecules per unit cell and an average fiber repeat of 0.455 nm (cf. 0.596 nm for PHB). The hydrocarbon side chains form ordered sheets, and conformational analysis led Marchessault et al. (98) to propose two possible structures, namely herringbonelike or comblike, depending on the angle between the side chains and the axis of the PHA helix. A maximum crystallinity of about 25% was deduced from X-ray diffraction studies, i.e., less than half that of annealed 3HB-co-3HV samples.

NUMBER OF PHB GRANULES PER CELL AND THEIR NATIVE STRUCTURE

An intriguing question is the number of polymer granules in a bacterial cell and whether this is related to the level of polymer accumulation. Ballard et al. (7) determined the number and size of granules in nitrogen-limited *A. eutrophus* at intervals during PHB accumulation by means of freeze-fracture, transmission electron microscopy, and analysis of the resulting photographs by using a cylindrical cell model to interpret the results. In two different-scale experiments the average number of granules per cell remained constant at 12.7 ± 1.0 and 8.6 ± 0.6, respectively, and increased uniformly in average diameter from 0.24 to 0.50 μm to accommodate the PHB produced. It appears that the number of granules per cell is fixed at the earliest stages of polymer accumulation. These investigators discovered that deviations from the cylindrical cell model occurred at PHB cell fractions above 0.58, indicating that the cells became more spherical to accommodate additional polymer, coinciding with the time when the rate of PHB synthesis declined. Polymer production in *A. eutrophus* ceases when a PHB content of about 80% is attained, although PHB synthase activity remains high, suggesting that physical constraints operate and the cell is unable to accommodate more polymer within the fixed existing amount of cell wall material, despite the availability of substrate and active synthase (7).

Light-scattering measurements yield an average molecular weight for PHB granules of about 5×10^9 (52), and on the basis of the molecular weights recorded for extracted polymer (10^3 to more than 10^6 depending on the organism), each granule must contain a minimum of 1,000 polymer chains. Mas et al. (100) concluded from volume and density measurements that PHB granules from *A. eutrophus* contain some 40% water.

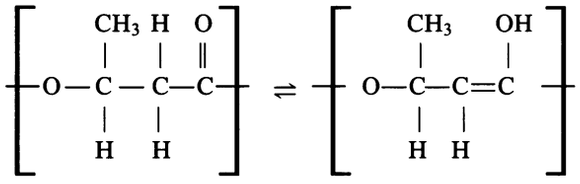
Granules are typically 0.2 to 0.5 μm in diameter and possess a membrane coat about 2 nm thick, composed of lipid and protein, representing some 0.5 and 2%, respectively, of the granule weight (95). The activities of the PHB synthase and depolymerase are associated with this membrane protein. Dunlop and Robards (51) used freeze-etching to study the ultrastructure of thin sections and isolated PHB granules from *Bacillus cereus*. No internal structures were seen within the granules, which consisted of an outer coat, a core that produced characteristic stretching patterns during freeze-fracture, and a bounding membrane.

A focus of interest is the structure of native PHB granules in vivo. From the earliest work with granules, it was recognized that unless mild isolation techniques were used, their properties were easily modified, e.g., loss of susceptibility to depolymerase action (95). It was also noted that the molecular weight of the polymer was affected by the extraction technique employed, neutral solvent extraction yielding higher values than alkaline hypochlorite or acid hydrolysis treatment.

The early X-ray diffraction studies of solid PHB made by Alper et al. (3) and Lundgren et al. (94) led to the conclusion that PHB granules in vivo are crystalline. Treatment of native granules with aqueous acetone to remove the membrane coat disclosed a fibrillar structure and long, parallel flexible fibers that coalesced to form ribbons which, in turn, yielded the characteristic lath-shaped crystals of PHB (52) previously reported by Alper et al. (3). These observations raised interesting problems concerning the synthesis and degradation of PHB. In the former process the synthase acquires a soluble substrate from the cytoplasm and deploys it to elongate a polymer chain within the highly hydrophobic crystalline milieu of the granule. Or is the nascent polymer a liquid droplet which then gradually crystallizes? To degrade the polymer, the depolymerase enzyme must gain access to its crystalline substrate. Furthermore, susceptibility of the granule to depolymerase action is very readily lost. These are challenging questions if the concept of a crystalline polymer existing within native granules is correct.

However, work by the groups of P. J. Barham (University

of Bristol) and J. K. M. Sanders (11) has necessitated revision of the belief that granules in vivo contain crystalline polymer. Barham and colleagues, using a range of physical techniques, consistently found that the granules were completely amorphous and could be induced to crystallize only on heating or on removal of all water (P. J. Barham, P. Bennett, T. Fawcett, M. J. Hill, J. Stejny, and J. Webb, Abstr. Biol. Eng. Polym. Conf., Cambridge, England, 1989). They have considered two possible explanations for these unexpected observations, namely, that there is a natural plasticizer present in the granules which prevents the PHB molecules from crystallizing or that the polymer in the granules exists in a different form, and they proposed the enolic rather than the usual ketonic form, possibly stabilized enzymatically:



The question raised is therefore whether the polymer is synthesized in the granule in a different form from that subsequently extracted from the cells. This might explain partial solubility in water, leading to accessibility of the polymer to the depolymerase, and would correlate with the observations of Mas et al. (100).

Barnard and Sanders (11) applied ^{13}C NMR spectroscopy to in vivo PHB granules accumulated by the facultative methylotroph *Methylobacterium* sp. strain AM1 and also concluded that the bulk of the PHB was in a very mobile state. Their conclusion was based on the fact that highly resolved natural abundance signals were associated with the particulate (native granule) fraction of the cells, whereas ^{13}C NMR signals in solids are massively broadened and do not yield a typical high-resolution spectrum. They went on to show that PHB and 3HB-co-3HV copolymer from *A. eutrophus* displayed similar behavior (12); variable temperature and relaxation measurements indicated that the bulk of the polymer within the granule is effectively an elastomer well above its glass transition point at all stages of growth (S. R. Amor, G. N. Barnard, and J. K. M. Sanders, Abstr. Biol. Eng. Polym. Conf., Cambridge, England, 1989). This metastable, mobile form is in the enzymically active state for depolymerization, and evidence was adduced that the lability of granule, which leads to loss of susceptibility to depolymerase, is due to polymer solidification within the granule. They suggested that water is probably involved in polymer mobility, although it is possibly not the only factor implicated in this phenomenon, and they highlighted the continuing problem of location of the synthase and depolymerase; e.g., are they distributed throughout the mobile mass or, as in the traditional view, concentrated at the granule surface?

Kawaguchi and Doi (86) have examined by X-ray diffraction the structure of native PHB granules of *A. eutrophus* in both wet cells and isolated granules and concluded that in each case the polymer was in an amorphous state. Treatment of granules with alkaline hypochlorite, NaOH, aqueous acetone, or lipase initiated crystallization of PHB, leading these investigators to propose that removal of a lipid component initiates crystallization. Thin-layer chromatography of the acetone extracts disclosed the presence of two lipid components.

BIOSYNTHESIS, DEGRADATION, AND REGULATION OF PHA ACCUMULATION

Polymer Synthesis from Carbohydrates

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; EC 2.3.1.9), acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase; EC 1.1.1.36) and poly(3-hydroxybutyrate) synthase.

$$2\text{acetyl-CoA} \rightleftarrows \text{acetoacetyl-CoA} \xrightarrow[\text{CoASH}]{} \text{3-hydroxybutyryl-CoA} \rightarrow \text{PHB} + \text{CoASH}$$

In *Rhodospirillum rubrum*, two stereospecific enoyl-CoA hydratases are also involved (111); these enzymes catalyze the conversion of L-(+)-3-hydroxybutyryl-CoA via crotonyl-CoA to D-(−)-3-hydroxybutyryl-CoA, which is polymerized to yield PHB. Since the cyclic pathway of PHB synthesis and degradation was established in 1973 for *Azotobacter beijerinckii* and *A. eutrophus* (118, 157), one modification has been noted. In the floc-forming bacterium *Zoogloea ramigera*, acetoacetate produced in polymer degradation is esterified with CoA by a CoA-synthetase reaction with ATP (173):

$$\text{acetoacetate} + \text{CoASH} + \text{ATP} \rightarrow \text{acetoacetyl-CoA} + \text{AMP} + \text{PP}_i$$

instead of the acetoacetate:succinate CoA transferase reaction characteristic of other organisms. A similar mechanism has been found in *Methylosinus trichosporium* OB3b (A. M. Williams, Ph.D. thesis, Cranfield Institute of Technology, Cranfield, England, 1988).

Enzymology of PHB Biosynthesis

The individual steps of PHB biosynthesis have been studied in detail for few bacteria, and further research, particularly relating to biosynthesis and control of PHB formation from noncarbohydrate carbon sources, is required. Little is known of the pathways and control involved in the synthesis of bacterial polyesters other than PHB, the current examples of which contain two or more different hydroxyacid repeating units (see above).

3-Ketothiolase. 3-Ketothiolase has been purified from various PHB-synthesizing bacteria including *Azotobacter beijerinckii* (157), *Hydrogenomonas eutropha* (now *A. eutrophus*) (70, 118), and *Z. ramigera* (116). *Z. ramigera* (173) and *Bradyrhizobium japonicum* (170) contain a second 3-ketothiolase, of different substrate specificity from the principal enzyme.

3-Ketothiolase was shown to be the enzyme controlling PHB biosynthesis in *A. eutrophus*, with CoA as the key effector metabolite (118). In our own laboratory, we have extended these studies with a glucose-utilizing variant of the organism used by Oeding and Schlegel (118) and have demonstrated the presence of two distinct enzymes possessing different substrate specificities (70). One enzyme was active with only C_4 and C_5 3-ketoacyl-CoAs, but the other was active with the range (C_4 to C_{10}) of substrates tested. It is uncertain whether one or both 3-ketothiolases are involved in PHA accumulation by this organism; in vitro studies with purified enzyme preparations demonstrate that either enzyme can function in PHB synthesis (71), and, since only C_4 and C_5 3-hydroxyacids can be incorporated into PHA by *A. eutrophus* (Holmes et al., European patent,

1985), either enzyme could be involved. Nevertheless, it seems probable that the 3-ketothiolase that is active with longer substrates is a degradative enzyme involved in fatty acid metabolism, as previously suggested (173).

The biosynthetic 3-ketothiolase of *Z. ramigera* is a homotetrameric enzyme which has been purified to homogeneity by Tomita et al. (173) and subjected to mechanistic investigations to analyze the C—C bond-forming sequence by Davis et al. (31). Amino acid composition, N-terminal sequence, susceptibility to stoichiometric inactivation by iodoacetamide, and isolation of an active-site cysteine-containing nonapeptide were established, and, by using substrate analogs, the ability of the thiolase to catalyze proton abstraction from the C_2 methyl group of the acetyl portion of substrate in a transition state separate from C—C bond formation was demonstrated. Haloacetyl-CoA analogs were used in an effort to identify active-site residues (30), and, in conjunction with studies on the isolation of the gene coding for the thiolase (127), a catalytically essential cysteine residue was located at position 89. The structural gene of the *Z. ramigera* thiolase is 1,173 nucleotides long and codes for a polypeptide of 391 amino acids.

Sinskey's group has extended its work to the 3-ketothiolase of *A. eutrophus* (101) and, similarly, identified a highly active cysteine residue at position 89. Further mechanistic studies suggest that there are three cysteine residues and possibly a histidine residue in or near the active site of the *Z. ramigera* enzyme, the rate-limiting step for which in the thiolytic direction is the enzymatic deacylation half-reaction and, in the condensation direction, the acetoacetyl-CoA carbon-carbon bond-forming step. Crystallographic analysis of the enzyme is being undertaken.

Acetoacyl-CoA reductase. Acetoacetyl-CoA reductase has been detected in a number of PHB-synthesizing bacteria including *Azotobacter beijerinckii* (142), *Rhodopseudomonas spheroides*, *Rhodomicrobium vannielii* (16), and *Streptomyces coelicolor* (123), although the presence of isoenzymes in these organisms was not reported. Two acetoacyl-CoA reductases, possessing different substrate and coenzyme specificities, have been found in *Z. ramigera* (143) and *A. eutrophus* (71). The NADH reductase of *Z. ramigera* is active with a range of L-(+)-3-hydroxyacyl-CoAs (160), whereas the NADPH reductase is active with only C_4 and C_5 D-(−)-3-hydroxyacyl-CoAs (59). Experiments with purified enzymes suggested that in this organism, acetoacetyl-CoA is reduced to D-(−)-3-hydroxyacyl-CoA, catalyzed by the NADPH reductase, during PHB synthesis (143, 160). The NADPH reductase of *A. eutrophus* is active with C_4 to C_6 D-(−)-3-hydroxyacyl-CoAs and has no activity with L-(+) substrates; in addition, the reduction of acetoacetyl-CoA yields only D-(−)-3-hydroxybutyryl-CoA. In contrast, the NADH reductase of *A. eutrophus* produces only L-(+)-3-hydroxybutyryl-CoA from acetoacetyl-CoA and is active with both D-(−) and L-(+) substrates in the reverse reaction. Only the NADPH reductase was found to participate in PHB synthesis from acetyl-CoA in reconstituted systems containing purified 3-ketothiolase, acetoacyl-CoA reductase, and PHB synthase (71, 72); this is because the PHB synthase of *A. eutrophus* is specific for D-(−) substrates (73). It is conceivable that L-(+)-3-hydroxyacyl-CoAs produced during β-oxidation of fatty acids could be converted to D-(−)-3-hydroxyacyl-CoAs (via acetoacetyl-CoA) by the sequential reactions catalyzed by the NADH and NADPH reductases, thus enabling their use in PHB biosynthesis. In *Rhodospirillum rubrum*, both D-(−)- and L-(+)-enoyl-CoA hydratases are present (111), enabling

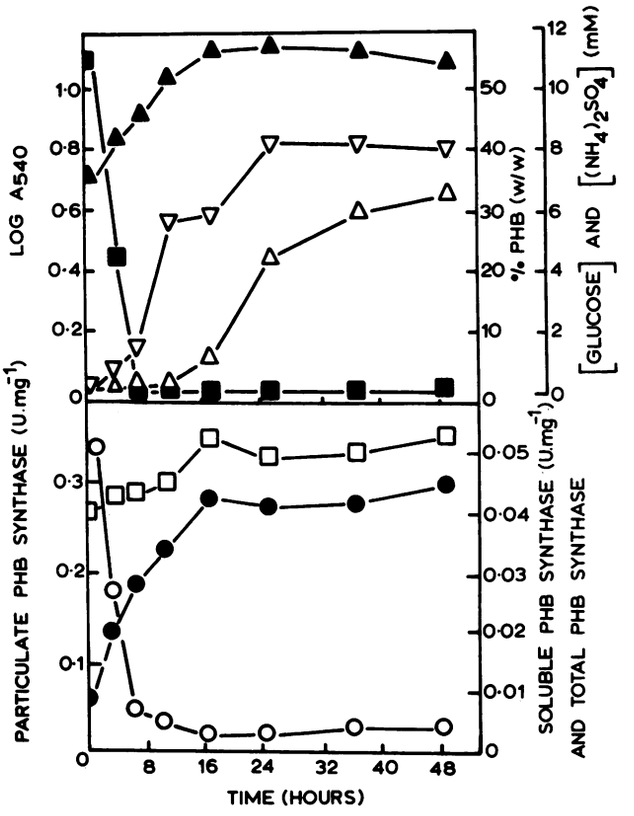


FIG. 1. Effect on PHB synthase solubility of transition from carbon-limited to nitrogen-limited growth. *A. eutrophus* was grown in continuous culture (dilution rate = 0.1 h^{-1}) initially under carbon limitation, and then at zero time a nitrogen-limiting medium was introduced. Symbols: ■, $[(NH_4)_2SO_4]$; ▲, log A_{540}; ▽, %PHB (wt/wt); △, [glucose]; □, total PHB synthase; ○, soluble PHB synthase; ●, particulate PHB synthase. Reproduced from reference 73 with permission.

L-(+)-3-hydroxyacyl-CoA produced via the acetoacetyl-CoA reductase to be used in PHB synthesis from acetic acid. In *A. eutrophus*, such racemization of 3-hydroxyacyl-CoAs is not possible because only one stereospecific [L-(+)] enoyl-CoA hydratase is present (71).

PHA synthase. The association of the polymerizing enzyme with PHB granules has been known for many years. Granule-associated synthase has been found in *R. rubrum* (105), *B. megaterium* (66), *Azotobacter beijerinckii* (141, 142), and *Z. ramigera* (61, 173). The PHB synthase activity of *Z. ramigera* has been observed in two forms, each attributed to the same protein whose location (soluble or granule-bound) was dependent on prevailing growth conditions (61, 173). Organisms with high polymer contents possess granule-bound activity, whereas those with low contents possess a soluble form of the enzyme; both forms of enzyme have been partially purified but proved increasingly unstable as purification proceeded. The particulate activity could not be solubilized; it did not display conventional Michaelis-Menten kinetics, and the apparent K_m for 3-hydroxybutyryl-CoA was about 2.1×10^{-4} M. An estimated Hill coefficient of 1.3 implied some positive cooperativity and at least two binding sites for 3-hydroxybutyryl-CoA. In contrast, the soluble form of the synthase displayed a linear Lineweaver-Burk plot with a K_m of 5.3×10^{-5} M (61). Our studies show the presence of both soluble and particulate

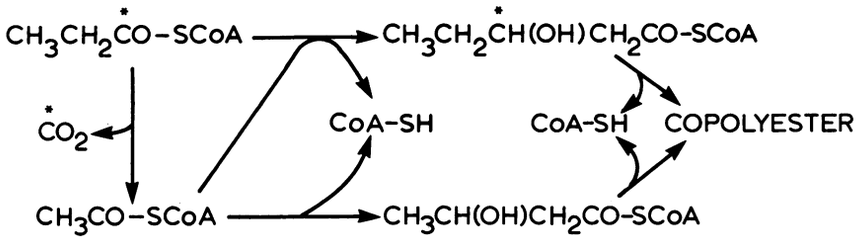


FIG. 2. Biosynthesis of 3HB-co-3HV copolymers labeled solely in the 3HV monomer units from [1-^{13}C]propionate, in *A. eutrophus*. Modified and reproduced from reference 43 with permission.

synthases in *A. eutrophus* (73). During growth under carbon limitation, the synthase exists mainly in the soluble form (Fig. 1). Upon transition to nitrogen limitation, PHB accumulation occurs and granule-associated PHB synthase appears; the rapid disappearance of soluble synthase under these conditions is consistent with its association with PHB granules.

The PHB synthase of *A. eutrophus* is specific for D-(−) enantiomers, and when tested with 3-hydroxyacyl-CoAs, it was active only with C_4 and C_5 substrates (73), consistent with the observation (Holmes et al., European patent, 1985) that only C_4 and C_5 3-hydroxyacid monomer units are incorporated into the polymer by this organism. The incorporation of 4HB (44, 47) and 5HV (49) into PHAs by *A. eutrophus* suggests that the synthase is also active with 4-hydroxybutyryl-CoA and 5-hydroxyvaleryl-CoA, respectively, or, alternatively, that an additional synthase may be present.

The mechanism of PHB synthase action remains obscure. The presence of active thiol groups is assumed from inhibitor patterns (66), and Griebel and Merrick (65) proposed a two-stage polymerization reaction involving an acyl-S-enzyme intermediate. A subsequent model suggested by Ballard et al. (7) involves two thiol groups, one locating the incoming 3HB monomer and the other locating the growing polymer chain. Condensation occurs through a four-membered transition state, leaving one of the thiol groups vacant for the next monomer. It is presumed that the chain transfer role performed by the synthase must in some way control the molecular weight of the polymer produced, which is a characteristic of a given organism.

Cloning and Expression of Genes Involved in PHB Biosynthesis

The structural genes for 3-ketothiolase (127) and for NADPH-linked acetoacetyl-CoA reductase (130) from *Z. ramigera* have been cloned and expressed in *Escherichia coli*; in the former case the sequence of the gene was also determined.

Slater et al. (161) and Schubert et al. (149) have independently cloned, and expressed in *E. coli*, the genes involved in the biosynthesis of PHB by *A. eutrophus*. The recombinant strains of *E. coli* were capable of accumulating a substantial amount of PHB as intracellular granules. Analysis of clones indicated that the genes for 3-ketothiolase and the NADPH-linked reductase were present on a 5.2-kbp fragment (161) which must also carry the PHB synthase. Schubert et al. (149) suggested that the PHB synthase, 3-ketothiolase, and NADPH-linked acetoacetyl-CoA reductase are clustered, although it remains to be determined whether they are organized in a single operon. It is interesting that all the PHB-negative mutants of *A. eutrophus* isolated by these workers were devoid of PHB synthase. Peoples and Sinskey (128, 129) have sequenced the structural genes for the three biosynthetic enzymes of *A. eutrophus* and shown that transcription occurs in the sequence polymerase, thiolase, reductase. The hydropathy profile of the synthase did not conform to a typical membrane-spanning protein (129), and the authors suggested that PHB biosynthesis may not require a complex membrane-bound polymerization system.

The ability to transfer PHB biosynthetic genes between unrelated bacteria should enable different PHAs to be produced in organisms that are readily cultivated on a commercial scale. The use of recombinant strains that overproduce the biosynthetic enzymes may also improve the productivity of the process. The first patent (O. Peoples and A. J. Sinskey, Int. patent WO 89/00202, January 1989) relating to the commercial potential of recombinant technology has been published.

Polymer Synthesis from Organic Acids, Alkanes, and Other Substrates

It has been suggested (Holmes et al., European patents, 1983, 1985) that the condensation of propionyl-CoA with acetyl-CoA, catalyzed by 3-ketothiolase, to produce 3-ketopentanoyl-CoA may be responsible for the ability of *A. eutrophus* to accumulate a copolymer of C_4 and C_5 3-hydroxyacids when supplied with glucose plus propionic acid. Alternatively, 3-hydroxyacyl-CoAs could be generated by removal of precursors from general fatty acid metabolism by means of nonspecific enoyl-CoA hydratase and thiokinase activities:

$$R{\cdot}CH{=}CH{\cdot}CO{\cdot}O^- + H_2O \rightarrow R{\cdot}CH(OH){\cdot}CH_2{\cdot}CO{\cdot}O^-$$

$$R{\cdot}CH(OH){\cdot}CH_2{\cdot}CO{\cdot}O^- + CoASH + ATP \rightarrow R{\cdot}CH(OH){\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA + OH^- + AMP + PP_i$$

Doi et al. (39, 43) have used NMR spectroscopy to study the incorporation of [^{13}C]acetate and [^{13}C]propionate into PHB and 3HB-co-3HV copolymer by *A. eutrophus*. The labeling pattern of the polymers produced is consistent with the scheme shown in Fig. 2, in which some of the C_3 units derived from propionate are decarboxylated to C_2 units. Further NMR studies (50) indicated that the ^{13}C-labeled carbonyl carbon of butyrate is incorporated into the carbonyl carbon of PHB without randomization to other carbons. The authors proposed that PHA synthesis from butyric and valeric acids can proceed via a nondegradative pathway (Fig. 3). The direct incorporation of acids into monomer units is of particular interest with regard to the regulation of polymer synthesis. It is plausible that the nondegradative route is of general importance in the biosynthesis of PHA from *n*-alkanoic acids, *n*-alcohols, or *n*-alkanes. Thus, the *n*-alkanoic acid (produced by terminal

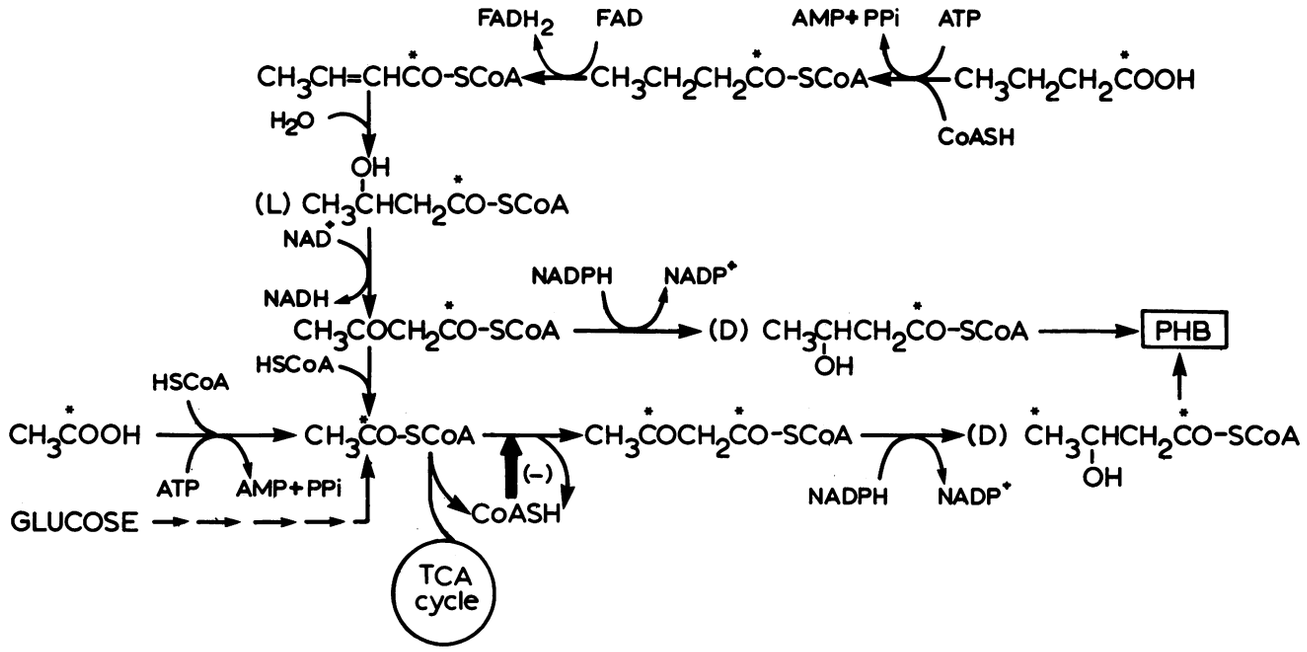


FIG. 3. Pathways of PHB biosynthesis from [1-^{13}C]acetate and [1-^{13}C]butyrate in *A. eutrophus*. Modified and reproduced from reference 50 with permission.

oxidation of the alkane) is converted to the corresponding CoA ester, oxidized to the enoyl-CoA, and hydrated to yield a 3-hydroxyacyl-CoA, the substrate for the PHA synthase.

The accumulation of PHA containing 3-hydroxyacid units with C_{n-2} carbon atoms from *n*-alkanoic acids (19) or *n*-alkanes (91) containing C_n carbons can readily be explained by β-oxidation of the acyl-CoA. Similarly, the accumulation of polymer containing a proportion of C_{n+2} 3-hydroxyacids (19, 91) may result from the condensation of acetyl-CoA with the acyl-CoA derived from the substrate, catalyzed by 3-ketothiolase; alternatively it is possible that chain extension occurs via other reactions, associated with general fatty acid biosynthesis within the bacterium. It is more difficult to explain the presence of the trace amounts of C_{n+1} and C_{n-1} 3-hydroxyacids in PHA produced from several *n*-alkanoic acids, but once again, the reactions of general fatty acid metabolism may be involved. Lageveen et al. (91) postulated that since 1-alkenes could be metabolized either by epoxide formation or by ω-oxidation, only a proportion of unsaturated monomers, produced via the latter pathway, would be incorporated into PHA. This would account for the substantial proportion of saturated monomers found in PHA produced from alkenes.

The accumulation of various copolymers and terpolymers containing C_4 to C_6 3-hydroxyacids by *R. rubrum* has been reported (20). The composition of these polyesters, which were produced from various *n*-alkanoic acids, is entirely different from those produced by *P. oleovorans* from these substrates. Furthermore, the composition of PHA produced by *R. rubrum* shows considerable variation with the concentration of the substrate; the pathways involved and their control are not yet understood. *R. rubrum* has also been reported (67) to accumulate a PHA containing 30 mol% 3-hydroxypentenoate from 4-pentenoic acid. The major monomer unit, 3HV (60 mol%), is presumably derived by saturation of the alkene, but the polymer also contains a proportion (10 mol%) of 3HB.

Regulation of PHA Metabolism

The most detailed studies of the regulation of PHB synthesis from glucose (82, 156, 157) used *Azotobacter beijerinckii*, which accumulates substantial amounts of polymer only under oxygen limitation. The key feature of control in *A. beijerinckii* is the fate of acetyl-CoA, which may be oxidized via the tricarboxylic acid (TCA) cycle or can serve as a substrate for PHB synthesis; the diversion depends on environmental conditions, especially oxygen limitation, when the NADH/NAD ratio increases. Citrate synthase and isocitrate dehydrogenase are inhibited by NADH, and, in consequence, acetyl-CoA no longer enters the TCA cycle at the same rate and instead is converted to acetoacetyl-CoA by 3-ketothiolase, the first enzyme of the PHB biosynthetic pathway, which is inhibited by CoA. There is therefore a greatly decreased flux of carbon through the TCA cycle under these conditions. When the oxygen supply is adequate, the CoA concentration is high, being released as acetyl-CoA enters the TCA cycle, and the 3-ketothiolase is accordingly inhibited, thereby preventing PHB synthesis. Senior and Dawes (156) proposed that PHB served not only as a reserve of carbon and energy but also as a sink of reducing power and could be regarded as a redox regulator within the cell.

The role of oxygen limitation in the accumulation of PHB by azotobacters (155) has been emphasized by recent work of Page and Knosp (125) with a mutant strain of *Azotobacter vinelandii* isolated in genetic transformation experiments. This organism, designated strain UWD, has a deficient respiratory NADH oxidase and consequently is unable to reoxidize NADH via oxygen-respiration; instead, the reductive step of PHB synthesis effects this process. As a consequence, strain UWD accumulates PHB during balanced growth on glucose as the carbon source. This quasi-fermentation process is capable of generating 1 mol each of ATP, NADH, and NADPH per mol of glucose via the Entner-

Doudoroff pathway (26, 156). Additionally, electrons from NADH may be routed to oxygen via the action of nicotinamide nucleotide transhydrogenase and the very active respiratory NADPH oxidase (1, 25). The combination of these reactions may well be responsible for the observation that the mutant and the wild type have virtually indistinguishable growth characteristics, leading to the conclusion that the mutation is a neutral one.

Under oxygen-limited conditions the NADH oxidase of *A. vinelandii* usually becomes an important energy-conserving loop in respiration (69), and thus, in mutant UWD, this deficiency is presumably compensated by the ATP generation which accompanies PHB accumulation. Page and Knosp (125) showed that NAD(P)H-dependent metabolic activities such as nitrogen fixation, growth on acetate, and growth at very high aeration in batch culture also oxidize NADH, thereby decreasing the yield of PHB in the mutant.

Page (124) extended the work by examining the range of carbon sources that would support PHB formation by the mutant. Of particular significance was the discovery that the organism accumulated PHB when grown on complex substrates such as molasses, generally with better yields than when the corresponding refined sugar was used. Clearly, the observation that the *A. vinelandii* strain deficient in NADH oxidase can accumulate substantial amounts of polymer while growing on a complex medium without any overt nutrient limitation is of considerable commercial potential.

Because of the importance of oxygen limitation and the existence of respiratory protection, the *Azotobacter* system is probably not a universally applicable model for the regulation of PHA synthesis. For example, oxygen limitation in the methylotroph *Pseudomonas* sp. strain K (*Protomonas extorquens*) does not lead to PHB accumulation (169). However, a similar mechanism can be invoked for organisms such as *A. eutrophus* and *B. megaterium*, which accumulate the polymer under growth limitations other than oxygen. The cessation of protein synthesis, an endergonic process tightly coupled to ATP generation via the electron transfer chain, would presumably lead to an increase in the intracellular NAD(P)H concentration, with the consequences already discussed for *Azotobacter beijerinckii* (35). Steinbüchel and Schlegel (166) found that under oxygen limitation *A. eutrophus* H16 accumulates PHB at lower rates than with other limitations and that pyruvate is excreted. Schlegel et al. also observed that mutants of H16 defective in PHB synthesis (147) excrete pyruvate when subjected to a limitation in the presence of excess carbon source (lactate, gluconate, or fructose) and that the ratio of the rates for accumulation of PHB by the wild type and for excretion of pyruvate by the mutant was 0.9 to 1.4. They suggest that this behavior stems from the lack of an effective control mechanism for hexose degradation.

PHA synthesis in *A. eutrophus* occurs when growth is restricted by the availability of nitrogen, phosphorus, or oxygen (22; Holmes et al., European patent, 1985). Oeding and Schlegel (118) showed that CoA is the key regulatory metabolite in this organism, its intracellular concentration effecting control of PHB synthesis by inhibition of 3-ketothiolase by the mechanism noted above for *A. beijerinckii*. Both 3-ketothiolase and acetoacetyl-CoA reductase appear to be constitutive enzymes in *A. eutrophus* (70, 71), and PHB synthase is also present, albeit in a soluble form, in the absence of polymer synthesis (73).

Doi et al. (45) have shown that, under nitrogen-free conditions, the simultaneous synthesis and degradation of different PHAs can occur in *A. eutrophus*. Thus, when organisms that had accumulated PHB (to 55% of their biomass from butyric acid as the carbon substrate) were incubated with valeric acid, the gross composition of the accumulated polymer changed as a function of time with the 3HV component, increasing from 0 to 49 mol% over 96 h. There was an initial fall in the total polymer content from 55 to 42% of the biomass after 29 h, followed by a gradual increase to 52%; the number-average molecular weight (M_n) of polyester decreased during the experiment from 5.78×10^5 to 2.69×10^5 and the M_w/M_n ratio rose from 2.0 to 4.2.

In the converse experiment, organisms that had first been allowed to accumulate 3HB-co-3HV polymer (to 50% of the biomass) by incubation with valeric acid displayed a higher M_n (7.16×10^5). On incubation with butyric acid, the 3HB content of total polymer rose from 44 to 81 mol% over 48 h, during which time the polymer content attained a maximum of 66% and then declined to the initial value of 50%; the M_n fell to 5.41×10^5, and the M_w/M_n ratio remained virtually unchanged at 1.7 to 1.8.

These experiments clearly demonstrate that, in the absence of nitrogen, batch cultures of *A. eutrophus* degrade PHB, even though an alternative source of carbon (valeric acid) is present, and synthesize new polymer containing 3HV units. Likewise, copolymer is degraded in the presence of butyric acid, the 3HV component decreasing although the total polymer content of the organism does not change significantly. Regulation of polymer degradation is thus relaxed in these circumstances, while synthesis of new polymer is simultaneously occurring. These experiments do not, of course, provide evidence that the monomer units derived from polymer are reutilized for the synthesis of new polymer, but they do show that polymer synthesis and degradation can occur simultaneously under nitrogen-free conditions. In contrast, when *A. eutrophus* was grown under steady-state nitrogen-limiting conditions in a chemostat, no evidence for turnover of PHB in glucose-grown cells was obtained (73).

The regulation of PHB biosynthesis from substrates that are not metabolized via acetyl-CoA is of interest because the key regulatory enzyme of PHB synthesis from glucose, 3-ketothiolase, is not involved. Doi et al. (50) added $(NH_4)_2SO_4$ to PHB-accumulating suspensions of *A. eutrophus* supplied with glucose or butyric acid and found that production of PHB from the latter substrate continued at relatively high concentrations [3 to 10 g of $(NH_4)_2SO_4$ $liter^{-1}$], at which polymer synthesis from glucose was completely inhibited. The accumulation of substantial amounts of PHA by *P. oleovorans* from *n*-alkanoic acids and *n*-alkanes under apparently unrestricted growth conditions (68, 91) suggests that effective inhibition of polymer synthesis during exponential growth may not occur in this organism.

For noncarbohydrate substrates, PHA accumulation could be controlled simply by competition for metabolites. Even in the absence of direct control of PHA synthesis, inhibition of growth by nutrient restriction will still favor polymer accumulation, as in the case of PHB synthesis from glucose. Further research on the general control mechanisms for PHA accumulation is clearly relevant to the commercial production of these polymers.

PHB metabolism in the obligate methylotroph *Methylosinus trichosporium* OB3b, which is capable of growth on methane and methanol only, is controlled in a manner different from that in *A. eutrophus* and *Azotobacter beijerinckii*, being insensitive to feedback inhibition by intermediates of the TCA cycle. Both synthesis and degradation of PHB are regulated at the level of PHB cycle intermediates

and through the redox state of the cell (D. J. Best, personal communication; Williams, Ph.D. thesis).

Intracellular PHB Degradation

Relatively little has been reported in the literature concerning the intracellular degradation of PHB since the subject was last reviewed in detail by Dawes and Senior (35). At that time, two different types of depolymerase systems had been recognized, in *Rhodospirillum rubrum* and *B. megaterium*. Native granules from *R. rubrum* are self hydrolyzing (J. M. Merrick, F. P. Delafield, and M. Doudoroff, Fed. Proc. 21:228, 1962), whereas those from *B. megaterium* are quite stable, although a soluble extract from *R. rubrum* was active in the degradation of native granules from *B. megaterium*; purified polymer or denatured granules did not serve as substrates (106). A soluble activator protein was isolated from *R. rubrum* extracts which, in the presence of depolymerase, activated PHB hydrolysis, yielding D-(−)-3HB as the major product with an appreciable amount of dimeric ester (107). Native PHB granules isolated from *Azotobacter beijerinckii* are also self hydrolyzing and produce acid at a high rate (P. J. Senior and E. A. Dawes, unpublished work).

A soluble PHB depolymerase isolated from *B. megaterium* yielded a mixture of dimer and monomer as hydrolysis products (62), whereas the soluble depolymerase from *Alcaligenes* (*Hydrogenomonas*) spp. gave D-(−)-3HB as the sole product (78). A more detailed study of the *B. megaterium* system (66) disclosed that depolymerization required a heat-labile factor associated with the granules together with three soluble components, namely, a heat-stable protein activator, PHB depolymerase, and a hydrolase. The concerted action of the first three factors produced principally D-(−)-3HB with about 15 to 20% dimer; the hydrolase converted the dimer to monomer. Treatments such as freezing and thawing, repeated centrifuging, or extensive proteolytic attack rendered granules partially or completely inactive as a substrate for hydrolysis, presumably by damaging the bounding membrane. The conclusion drawn was that although the depolymerase is not granule bound, there is some protein component associated with the granule that inhibits depolymerase activity. Extraction of native granules with 10 mM NaOH removes PHB synthase together with this inhibitor, rendering the granules susceptible to hydrolysis by the depolymerase (65). However, although it is presumed that this interplay between inhibition and activation controls PHB degradation, the detailed mechanism awaits elucidation (104).

BIODEGRADATION

One of the commercially attractive features of PHAs is their degradation in the natural environment, effected principally by the enzymic activities of microorganisms. Various investigations have been directed to the study of environmental factors that influence biodegradation, the enzymology of the process, and the importance of polymer composition. Some aspects of this work will now be discussed.

Extracellular PHB-Depolymerizing Enzymes

Certain bacteria are capable of growth with PHB as the sole carbon source (reviewed in reference 35). Extracellular enzymes capable of depolymerizing PHB and oligomers derived from the polymer have been found in a few organisms, but research has concentrated on the depolymerase excreted by *Alcaligenes faecalis*.

Tanio et al. (171) purified the single extracellular depolymerase of *A. faecalis* to electrophoretic homogeneity. The purified enzyme had a molecular weight of approximately 50,000 and a K_m of 13.3 mg ml^{-1} for PHB. The enzyme was also capable of degrading oligomers, but these were relatively poor substrates: the K_m for the trimer was 5.4 mM, and the dimer was not a substrate for the enzyme. Depolymerization of PHB yielded dimer with a trace of monomer. Quantitation of the D-(−)-3HB liberated and the decrease in ester bonds during the hydrolysis of oligomers suggested that dimer units are released one at a time from the free hydroxyl terminus; however, more detailed studies (158) with radioactive oligomers indicated that the depolymerase is an endo-type hydrolase with a hydrophobic site separate from the substrate-cleaving site.

In addition to PHB depolymerase, *A. faecalis* possesses a quite distinct extracellular oligomer hydrolase, which has also been purified to electrophoretic homogeneity (159). The hydrolase had a high affinity for oligomeric esters, having apparent K_m values of 32.8 and 1.3 μM for the dimer and dodecamer, respectively, but was inactive with polymeric material. The oligomer hydrolase appears to act by sequential removal of D-(−)-3HB units from the carboxyl terminus.

Treatment of purified PHB depolymerase from *A. faecalis* with trypsin (60) yielded a product with a molecular weight of 44,000, compared with 50,000 for the native enzyme; the modified enzyme was inactive with (hydrophobic) PHB but retained its activity with water-soluble oligomers. Furthermore, the modified enzyme appeared to be less polar, suggesting that the native enzyme possesses a hydrophobic region which can be removed by trypsin and which is remote from the catalytic site of the enzyme.

Subsequently, the structural gene for PHB depolymerase from *A. faecalis* was cloned into *E. coli* and sequenced by Saito et al. (144). The enzyme was expressed at a high level in *E. coli*; the excreted depolymerase represented approximately 10% of the total activity, with the majority of the enzyme localized in the periplasmic space. Sequence analysis showed that the depolymerase consists of a 488-amino-acid precursor with a signal peptide of 27 amino acids. In contrast to the single protein present in *A. faecalis*, isoenzymes of PHB depolymerase have been found in *Pseudomonas lemoignei* (96, 113). Four enzymes could be separated by chromatography on CM-Sepharose and showed some differences in their properties and sensitivity to inhibitors. No major difference was found in the substrate specificities of the depolymerase: each isoenzyme was inactive with the dimeric ester of D-(−)-3HB but active with larger oligomeric esters and also hydrolyzed PHB.

Effect of Polymer Composition on Biodegradation

Comparisons have been made by Doi and colleagues of the rates of degradation of polyester films (0.07 mm thick) in buffer solutions, soil, and activated sludge. The general finding was that copolymers containing 4HB monomer units degraded more rapidly under all conditions tested than either PHB or 3HB-co-3HV copolymers; furthermore, the inclusion of 3HV with 3HB had no significant effect compared with the degradation rate of the homopolymer PHB. In aerated activated sludge at 30°C, a 3HB-co-9% 4HB film was completely decomposed in 14 days (89); a 3HB-co-17% 4HB film was also decomposed in 2 weeks in soil at 30°C, but PHB took more than 10 weeks (38).

Temperature affected rates of degradation in 0.01 M phosphate buffer (pH 7.4) (37). At 37°C no polymer erosion

occurred over 180 days; however, after induction periods of about 20 days for 3HB-co-4HB samples and some 80 days for PHB and 3HB-co-3HV, there was molecular weight loss, which was highest for the 4HB-containing copolymers. At 70°C the process was accelerated and for 3HB-co-9% 4HB both erosion and molecular weight loss occurred without an appreciable induction period and the crystallinity of the film increased from 49 to 59% over 115 days, probably owing to crystallization of chain fragments in the amorphous regions.

Doi and colleagues concluded that hydrolytic degradation occurs by homogeneous erosion in two stages: (i) random hydrolytic chain scission of the ester groups, leading to a decrease in molecular weight, and (ii) erosion and weight loss when the M_n has reached about 13,000. The rate of chain scission is increased by and is proportional to the presence of 4HB monomer units.

Biodegradability of PHB and Other PHAs In Vivo

The initial optimism for the use of PHB in vivo as a biodegradable material for sutures, microcapsules, bone plates, and gauzes has not yet been fully realized. Two representative examples of the burgeoning literature are considered here. Evidence for the biodegradability of PHB in mice was presented by Korsatko et al. (87, 88), who observed weight losses of tablets of PHB ($M_w = 2 \times 10^6$) containing the drug 7-hydroxethyltheophylline, which was rapidly released after subcutaneous implantation. There was, however, some contribution to the overall weight loss by mechanical abrasion at the surface of the tablets. They observed that PHB of molecular weight greater than 10^5 was undesirable for long-term medication dosage. Miller and Williams (108) carried out a study with monofilaments of PHB and of copolymers of 3HB and 3HV containing, respectively, 8 and 17 mol% of 3HV, implanted subcutaneously in rats. At intervals over a period of 180 days, animals were sacrificed and the implants were removed and subjected to a variety of tests in comparison with in vitro control monofilaments that had been incubated for comparable periods at 37°C in sterile phosphate-buffered saline. The conclusion reached was that, in vivo, PHB biodegradation occurred only when the PHB had been predegraded by 10 Mrad of γ-irradiation prior to implantation. The copolymers did not display any increase in biodegradation, and, in vitro, if they had been incubated in the buffered saline at 70°C, their rate of hydrolysis was lower than that of PHB. The susceptibility of PHB to biodegradation is thus dependent upon the material characteristics of the polymer as well as the environmental conditions. Holmes (80) has suggested that observed variations in the rate of degradation of PHB might be correlated with changes in the activity of nonspecific esterases associated with the body's immune response.

PRODUCTION OF PHAs

Factors Affecting Molecular Weight of PHAs

The molecular weight of PHB is a function of the organism, the method of isolation, and possibly the prevailing environmental conditions (7). The fact that extraction with neutral solvents yields polymer of higher molecular weight than does sodium hypochlorite has been known for many years (35). Although Berger et al. (14) have optimized conditions for hypochlorite digestion of *A. eutrophus*, the M_w of the recovered PHB (95% purity) was approximately half that of the polymer present in the original biomass (about 6×10^5 versus 1.2×10^6). Pretreatment of the biomass with a surfactant (sodium dodecyl sulfate or Triton X-100) prior to hypochlorite digestion yielded polymer of higher purity (98 to 99%) and higher M_w (7.3×10^5 to 7.9×10^5); the reverse sequence of treatment was not as effective (133).

Different bacteria consistently produce polymers of different molecular weights (7). Azotobacters, for example, accumulate PHB in the range 8×10^5 to 2×10^6, and *A. eutrophus* accumulates PHB of 6×10^5 to more than 1×10^6, whereas the methylotroph *Pseudomonas* sp. strain AM1 produces polymer of only 5×10^4 to 6×10^4 and *Methylobacterium* sp. strain B3-Bp, also grown on methanol, gives much higher values of 2.5×10^5 to 3×10^5. The reasons for this behavior are not currently understood, so it is not yet possible to manipulate the process to secure polymer of a given molecular weight significantly different from that produced naturally by the organism.

The problem of genetic control and the extent to which environmental manipulation may influence the polymer size are topics that, to date, have not been fully addressed. One such study is that of Suzuki et al. (168), who examined the effects of temperature, pH, the molar ratio of methanol and ammonia, and the concentration of methanol on the molecular weight of PHB produced by *Protomonas extorquens* in fed-batch culture. They found that the average molecular weight of polymer accumulated could be controlled from ca. 8×10^5, at a methanol concentration of 0.05 g liter^{-1}, to less than 0.5×10^5 at 32 g of methanol liter^{-1}, but the methanol concentration was the only parameter investigated that had a significant effect. However, the molecular weight of the PHA produced by *P. oleovorans* decreased with increasing chain length of the *n*-alkanoic acid substrate provided (19). Thus, M_w for heptanoate was 3.7×10^5, M_w for octanoate was 2.1×10^5, M_w for nonanoate was 1.9×10^5, and M_w for decanoate was 9.2×10^4, whereas the M_w/M_n ratio for all was in the range of 2.5 to 2.0. Interestingly, the observed M_w values bore an inverse relationship to the efficiency of PHA production from these substrates.

There is evidence that the molecular weight of the polymer may decrease throughout the fermentation; e.g., during PHB accumulation by glucose-fed, phosphate-limited *A. eutrophus*, the polymer content of the organism increased from 5% to about 75% over a period of 72 h, while the M_w decreased from more than 2×10^6 to 6×10^5 (7). These workers obtained similar results irrespective of the nutrient limitation and the fermentation conditions (pH, temperature, essential nutrient concentrations, etc.) and concluded that the decrease was a general phenomenon. They reported that 3HB-co-3HV copolymers produced by *A. eutrophus* have very similar molecular weights irrespective of composition.

Growth rate also affects the M_n of PHB accumulated by *A. eutrophus*. For example, nitrogen-limited chemostat cultures grown at dilution rates of 0.2 and 0.06 h^{-1} contained some 31 and 58.5% polymer respectively. The former culture yielded polymer with M_n of 9.6×10^5, M_w of 1.84×10^6 and M_w/M_n of 1.91, whereas the latter yielded polymer with M_n of 1.21×10^6, M_w of 1.83×10^6, and M_w/M_n of 1.51 (A. J. Anderson, G. W. Haywood, E. A. Dawes, A. Webb, and D. Byrom, unpublished results).

Much remains to be learned about the control of the molecular size of the polymer. The soluble monomer substrate is taken from the cytosol by the thiol-dependent synthase located at the granule-bounding membrane, and chain elongation results, with more than 15,000 monomers being incorporated, for example, in *A. eutrophus* before

termination occurs within the hydrophobic granule. Clearly, the PHB synthase of this organism remains a prime focus of attention, and its isolation and purification from granules still constitutes a challenge, although some 50-fold purification of the soluble enzyme from *Z. ramigera* has been reported (61). However, the identification and characterization of the gene for the *A. eutrophus* H16 PHB synthase (129) ought now to pave the way to production of the purified enzyme.

Commercial Aspects of Polymer Production

The technology and economics of PHB production on the industrial scale have been discussed by Byrom (22). Factors involved include the product yield, complexity of the technology and hence the capital cost of the plant, and the ease or difficulty of product separation. Consequently, selection of the organism and substrate can critically influence costs. The yield values for PHB and cell biomass from a given substrate can be determined by experiment or by calculation based on the known metabolic pathways involved (26). This gives the approximate substrate cost per tonne of PHB produced, although it may not be the determining factor. Thus, ICI evaluated three organisms as principal contenders for the industrial production of PHB, namely, an *Azotobacter* sp., a *Methylobacterium* sp., and *A. eutrophus*, which collectively embraced a wide range of substrates. The *Azotobacter* sp., which could grow on glucose or sucrose with high polymer yields, proved unstable and also synthesized carbohydrate, thereby diverting substrate from PHB production. The methylotroph, seemingly attractive on account of ICI's wide experience in methanol fermentation technology, gave only moderate yields of polymer of low molecular weight, which was extracted with difficulty. *A. eutrophus*, grown heterotrophically, became the organism of choice because of high polymer contents of high molecular weight and the availability of various economically acceptable substrates; autotrophic growth (on H_2-CO_2-air mixtures) was rejected on the grounds of high capital cost of plant designed to operate with potentially explosive gas mixtures. This last problem is one which confronts the general applicability and future potential of hydrogen-utilizing bacteria in biotechnology, a topic surveyed by Sebo (151). The kinetics of chemolithotrophic growth and PHB synthesis in *A. eutrophus* H16 have, however, been studied by Sonnleitner et al. (163), and a kinetic model has been developed to describe these parameters (75).

As noted above, Collins (26) has addressed the factors determining the choice of substrate for the commercial production of PHB, considering inter alia methanol for type II (serine pathway) methylotrophs, hydrogen and carbon dioxide for hydrogen-oxidizing organisms such as *A. eutrophus*, and carbohydrates for this bacterium, which employs the Entner-Doudoroff pathway. In this last case, representing the ICI process, the overall equation is

$$PHB_n + \text{glucose} + ADP + P_i + 3NAD \rightarrow PHB_{n+1} + ATP + 3NADH + 2CO_2$$

This metabolic sequence is energy yielding, generating ATP and NADH, but since CO_2 is eliminated the yield cannot exceed 1 mol of monomer incorporated per mol of glucose. About 2.1 g of glucose is required per g of PHB produced, and when a single-stage continuous fermentation is used, energy is also needed for biomass production. Thus, maximum polymer yields are attained only in batch or two-stage processes in which growth precedes PHB accumulation. When allowance is made for non-PHB biomass, a minimum glucose requirement of some 2.5 g/g of PHB is predicted; in practice investigators at ICI have observed values of about 3 g/g of PHB in fed-batch fermentors.

Byrom (22) has outlined the ICI production process for PHB, with fed-batch grown of *A. eutrophus* in a glucose salts medium and with phosphate exhaustion as the growth-limiting factor. At the time of growth cessation (about 60 h), little PHB has been synthesized; glucose is then added to the culture, and during the next 48 h massive accumulation of the polymer occurs, up to 75% of the total dry biomass. The total fermentation time (growth and accumulation phases) is in the region of 110 to 120 h. The equipment used successfully includes a 35,000-liter air-lift fermentor and various sizes of conventional stirred vessels of up to 200,000 liters.

Copolymers of PHB and PHV are produced by *A. eutrophus* in a similar two-stage process with the exception that in the second stage, both glucose and propionic acid are provided. The hydroxyvalerate content of the polymer is controlled by the ratio of glucose to propionate in the feedstock. Because propionate in excess of 0.1% in the culture is toxic and prevents polymer synthesis, the propionate feed rate must be carefully controlled. *Pseudomonas cepacia*, which produces a PHA similar to that of *A. eutrophus*, is more resistant to the toxic effects of propionic acid but is less efficient in converting substrate into PHA (134).

Recovery of polymer from the biomass is a vital stage of the process. Large-scale solvent extraction, although giving high recovery, is an expensive technique and involves large volumes of solvent and high capital investment in the solvent recovery plant. ICI has used a process involving hot-methanol reflux to remove lipids and phospholipids followed by extraction of PHB with chloroform or methylene chloride. The solution is filtered hot to remove cell debris and cooled to precipitate PHB, which is then vacuum dried (J. F. Stageman, European patent 124 309, July 1983). To reduce costs an alternative, nonsolvent process has been developed by ICI (22, 80). Cells disintegrated by heat shock are treated with a series of enzymic and detergent digestive processes to solubilize the non-PHB components. The PHB is washed, flocculated, and recovered as a white powder, which is converted to polymer chips in a process involving blending, melt extrusion, and comminution.

FUNCTIONS OF PHB AND OTHER PHAs

PHB as a Carbon and Energy Reserve Material

The role of PHB as a carbon and energy reserve material in bacteria has been previously reviewed by Dawes and Senior (35); subsequent reviews by Dawes (32–34), Merrick (103), and Preiss (132) provide detailed references, and the main features only are summarized here. One of the major advantages to accrue to an organism from the synthesis of these high-molecular-weight materials, contained in some 8 to 12 granules per cell, is the ability to store large quantities of reduced carbon without significantly affecting the osmotic pressure of the cell. The possession of PHB frequently, but not universally, retards the degradation of cellular components such as RNA and protein during nutrient starvation. There is not a common pattern of behavior, however, and depending upon the organism, sequential or simultaneous utilization of macromolecules can occur. PHB enhances the survival of some, but not all, of the bacteria investigated and serves as a carbon and energy source for spore formation in some *Bacillus* species, although its accumulation is not a

prerequisite for the sporulation process. Likewise, PHB has been shown to serve as a carbon and energy source for the encystment of azotobacters.

A further, putative function of PHB in azotobacters is to provide an oxidizable substrate which affords respiratory protection to nitrogenase under environmental conditions when appropriate exogenous substrate(s) is not immediately available for oxidation (156). A similar proposal has been advanced for *Rhizobium* sp. strain ORS 571 by Stam et al. (165). These workers investigated the effect of carbon starvation on PHB degradation in this organism (isolated from stem nodules of the tropical legume *Sesbania rostrata*) by using oxygen-limited chemostat cultures of PHB-containing organisms growing on succinate. They found that for these free-living cultures the lack of a carbon source did not necessarily result in PHB breakdown and other factors, such as the oxygen concentration and perhaps the nitrogen source, were important; indeed, PHB degradation never occurred under oxygen-limited conditions irrespective of whether exogenous carbon was present. The observation that higher oxygen concentrations are required for PHB breakdown led them to raise the interesting question of whether this process ever occurs in the nodule (see below). Stam et al. (165) offered two possible roles for PHB degradation in *Rhizobium* sp. strain ORS 571, namely, (i) protecting nitrogenase when the oxygen concentration in the nodule rises and (ii) serving as a carbon and energy source during periods of starvation outside the nodule.

^{31}P NMR was used to demonstrate polyphosphate accumulation, simultaneously with PHB formation, by *A. eutrophus* H16 in nitrogen-free medium, although the absolute content was not stated (38). Such cells, subjected to carbon and phosphate starvation for 72 h, degraded PHB faster aerobically than anaerobically, while the (unspecified) polyphosphate content remained essentially unchanged; 3HB and acetate were excreted in appreciable amounts by aerobic cells. The role of PHB as a carbon and energy reserve is suggested with polyphosphate possibly as a phosphorus reserve.

PHB in Symbiotic Nitrogen Fixation

PHB has been implicated as an energy source in the symbiotic nitrogen fixation process between the bacterial genera *Rhizobium* and *Bradyrhizobium* and leguminous plants (85, 102). These bacteria invade the plant roots, leading to the establishment of root nodules in which the microorganisms exist as bacteroids, i.e., pleomorphic cells that are no longer capable of independent reproduction and that possess an active nitrogenase not present in the free-living bacteria. The heavy demand for energy for nitrogen fixation is met by the metabolism of photosynthetic carbon compounds (principally organic acids) transported to the root nodules. A functional TCA cycle is present (167), although the microaerophilic environment of the nodule may lead to inhibition and/or repression of some of the cycle enzymes such as citrate synthase, NADP-isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase (24, 53, 82, 112).

PHB accumulates up to 50% of the biomass of bacteroids (175), despite the paradox that nitrogen fixation and PHB synthesis both compete for the available reducing equivalents. The pathway of PHB synthesis in *B. japonicum* is similar to that in *Azotobacter beijerinckii* (102), and polymer accumulation is initiated by the onset of microaerophilic conditions, in parallel with an increase in nitrogenase activity. The effect of oxygen limitation on bacteroids has not been investigated in detail, but similarities with the behavior of *A. beijerinckii* are apparent, and it has been suggested therefore that PHB accumulation serves a regulatory role and not all of the available reducing power is apportioned to the operation of nitrogenase (102).

Putative Role of PHAs as a Carbon and Energy Reserve

With the discovery of PHAs other than PHB, it may be reasonably assumed that they, too, are utilized as carbon and energy reserves. For the majority of microorganisms growing in their natural environments, the proportion of PHAs other than PHB is probably quite small relative to PHB (see, for example, reference 55), and their role would therefore be minor. However, when, for example, *P. oleovorans* grows in an environment rich in *n*-octane, it accumulates principally polyhydroxyoctanoate (36), and the assumption is that this material could subsequently be utilized by the organism as a source of carbon and energy. At present, however, experiments to test such assumptions about the putative role of PHAs other than PHB have not been reported.

PHAs as Environmental Markers

The sensitivity of the bacterial content of PHB and its metabolism to the environment in laboratory monocultures prompted Herron et al. (77) to examine estuarine detrital microflora to discover whether the polymer was present and, if so, whether its level reflected the microbial biomass or could be used as an indication of the physiological status of the population. They found that the PHB levels did not correlate, since perturbations (e.g., anoxia) which did not affect biomass in the short term markedly decreased the metabolism of PHB. Nickels et al. (114) proposed the use of PHB and PHA metabolism as a measure of unbalanced growth of estuarine detrital microbiota. By comparing the ratio of phospholipid to PHA synthesis, a means of estimating disturbance to the sediment is available. Findlay and White (55) showed that if sediments were disturbed mechanically or by addition of natural chelators, the mass of all major PHA components increased relative to the bacterial biomass, indicative of unbalanced growth under these conditions. In contrast, perturbations caused by the presence of worms in the sediment promoted balanced growth, possibly by increasing local concentrations of essential nutrients and increasing the availability of reduced carbon in the aerobic region of the sediments. The determination in situ of metabolic activity in aquatic environments was selectively reviewed by Findlay and White (56).

PHB in Procaryotic and Eucaryotic Membranes: Transformability and Calcium Signaling

One of the most intriguing developments in PHB biochemistry has been the discovery of PHB in membranes, initially in the plasma membrane of gram-positive and gram-negative bacteria (138) and subsequently in eucaryotic membrane fractions, with the highest concentrations in the mitochondria and microsomes (135). Furthermore, PHB is found in association with inorganic polyphosphate in these locations, and the interesting proposal has been made that since such complexes span the membrane, they may play a role in the regulation of intracellular calcium concentrations and in calcium signaling (135). The details of this work will now be considered.

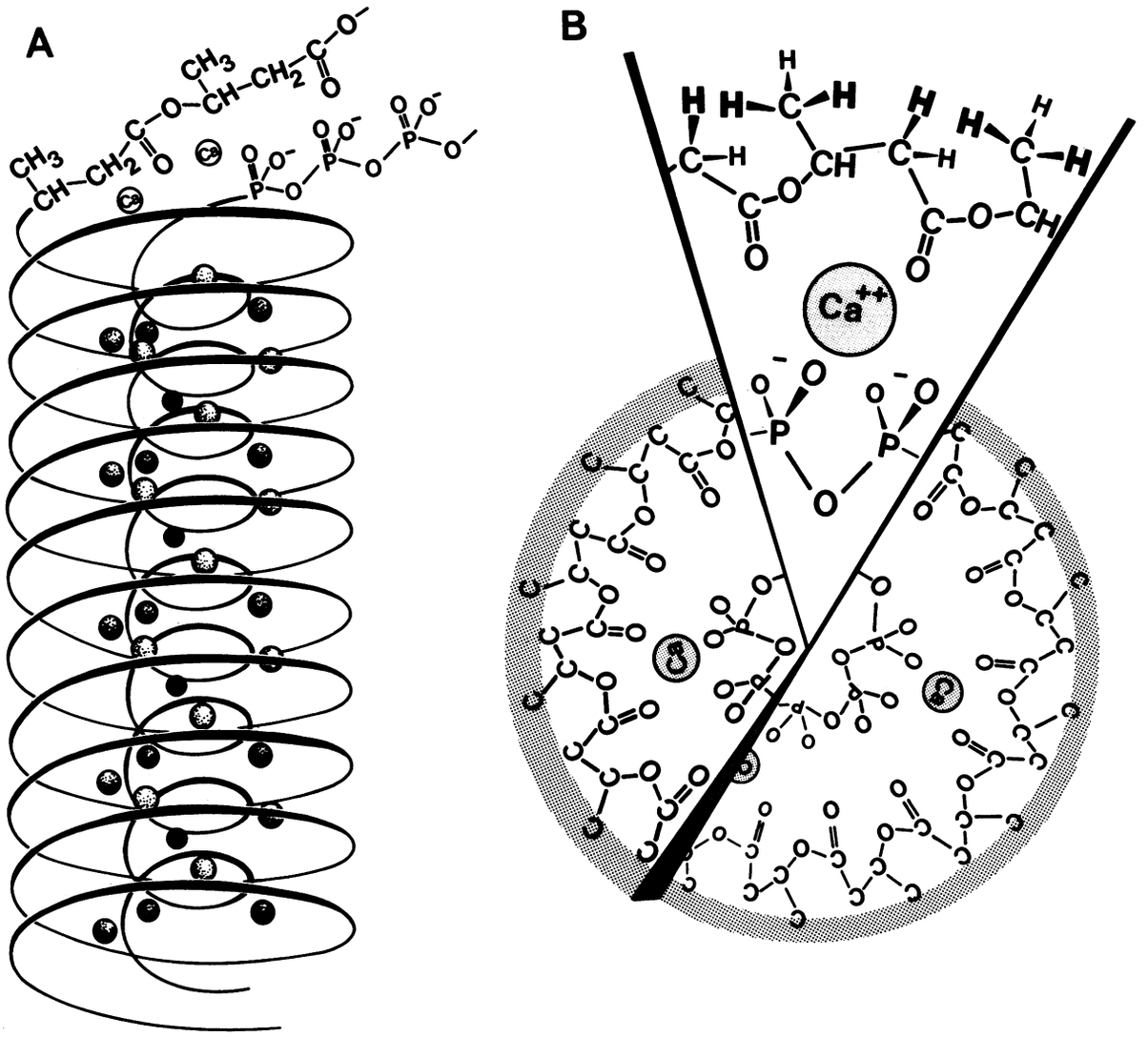


FIG. 4. (A) Proposed structure of a channel spanning the membrane, depicting the relationship between PHB, Ca^{2+}, and polyphosphate. PHB forms an outer helical channel around a core helix of polyphosphate with Ca^{2+} bridging the two polymers. (B) Interior view down the axis of the membrane channel structure. The PHB outer helix has 14 monomer units per turn. The methyl and methylene groups form a lipophilic shell (stippling represents the hydrogens), and the carbonyl ester oxygens form a polar lined cavity. The polyphosphate helix has seven monomer units per turn, with the phosphoryl oxygens facing outwards and the chain oxygens alternately facing in and out. There are 3.5 Ca^{2+} ions per turn; each is coordinated to four phosphoryl oxygens and four ester carbonyl oxygens (two of which are from the turn below and are not shown). Panels A and B reproduced from reference 135 with permission.

Reusch and Sadoff (138) discovered PHB in the plasma membranes of *Azotobacter vinelandii*, *Bacillus subtilis*, and *Haemophilus influenzae*, three organisms that undergo natural transformation but differ structurally and metabolically. Although *A. vinelandii* accumulates significant amounts of PHB in cytoplasmic granules, *B. subtilis* usually synthesizes only small amounts of the polymer, and *H. influenzae* synthesizes none. However, within each species the concentrations of PHB in membranes and cytoplasm correlated with transformability. Fluorescence analysis of the thermotropic lipid phase transitions in *A. vinelandii* and *B. subtilis* indicated that in the membrane the PHB forms an organized gel structure which is very labile. The authors discussed the possible structure and function of this membrane PHB in the light of their findings and proposed that PHB synthesis is a prerequisite for transport of exogenous DNA through the membrane, i.e., the acquisition of competence.

Their studies were then extended to the transformability of *E. coli* (136). The calcium-dependent development of genetic competence in *E. coli* was accompanied by physiological changes which led to de novo synthesis and incorporation of PHB into the plasma membrane. Coincidentally, an irreversible sharp new lipid phase transition appeared at ca. 56°C, indicative of the occurrence of a significant modification of the bilayer structure. No other lipid changes which could account for this new phase transition were detected. It was concluded, therefore, that PHB incorporation into the membranes was responsible for this phenomenon and that the polymer probably played an important role in the transmembrane transport of DNA. PHB synthase was shown to be constitutive in *E. coli* with only low activity during aerobic exponential growth, but when the organism was cooled and oxygen was limited in the presence of Ca^{2+}, a pool of 3HB formed which was converted to the polymer.

Transformation efficiencies correlated with PHB concentrations in the membranes and the intensity of the new phase transition.

The proposal that the PHB forms an organized quasi-crystalline lipid structure in the membrane was investigated by freeze-fracture electron microscopy of membranes from *E. coli* and *A. vinelandii* (137). A definite alteration was observed within the plasma membrane, but not the outer membrane, of both organisms. Small, protein-free intramembraneous plaques, containing shallow particles, studded the whole of the smooth membrane surface. This was observed with both *E. coli*, which does not accumulate intracellular PHB granules, and *A. vinelandii*, which does. In the latter case, it seems that PHB is synthesized first in cytoplasmic granules, which are then presumed to serve as a vehicle for transporting and incorporating PHB into the membrane, as well as for performing their established storage function.

The membrane PHB complex is very labile, dissociating irreversibly in distilled water and even more rapidly in chelating buffers. Thus it was inferred that the complex comprises water-soluble component(s) that may include divalent cation(s), possibly Ca^{2+} in view of the Ca^{2+} dependence of transformation (137). Reusch and Sadoff (139) continued the investigation with *E. coli* to avoid complications arising with organisms that contain cytoplasmic PHB granules. They extracted a PHB complex from plasma membranes of genetically competent cells by a regimen involving sodium hypochlorite digestion; sequential washing with cold methanol, methanol-acetone (1:1), and acetone; and extraction of the residue with dry chloroform. Analysis of the chloroform extracts revealed the presence of PHB and inorganic polyphosphate in molar ratios ranging from 2.6:1 to 2.1:1. Because polyanionic polyphosphate is normally insoluble in chloroform, the extracts were analyzed for accompanying cations; only Ca^{2+} was discovered in significantly larger amounts. The molar ratios PHB/poly(P)/Ca^{2+} in the complex were ca. 1:1:0.5. The chain lengths of PHB and polyphosphate were estimated as 120 to 200 and 130 to 170 monomer units, respectively. The structural integrity of the complex was such that it could be incorporated into liposomes.

A structure was proposed for the complex which would satisfy the criteria for a transmembrane channel, namely, that it should possess a lipophilic coat and a lining of highly polarizable groups (Fig. 4). This model is made up of an inner cylinder comprising a helix of polyphosphate with 7 monomer units per turn and an outer PHB helical cylinder with 14 monomer units per turn. Only these particular configurations permit a suitable geometry for accommodating the Ca^{2+} in the complex: each Ca^{2+} is ligated to four phosphoryl oxygens and four ester carbonyl oxygens. The phosphoryl oxygens are from two adjoining metaphosphate units, and the carbonyl oxygens are from two adjacent PHB monomer units of one turn and two adjacent units of the turn directly above or below. In this way the calcium ions, secured by ionic bonds to the core polyphosphate, cross-link the turns of the PHB helix by alternately binding one turn of the helix to the turns directly above and below. Computer and molecular modeling indicated that the putative structure has a diameter of 240 nm and a helical rise of 40 nm, with an average length of 450 nm. Reusch and Sadoff (139) discuss the possible functions of this transmembrane complex in addition to its value as a calcium store, namely, a role in the transport of calcium, phosphate, and DNA. Thus export of Ca^{2+} and phosphate might be effected by elongation of the polyphosphate chain at the cytoplasmic face by polyphosphate kinase-catalyzed transfer of P_i from ATP, in concert with polyphosphatase activity at the periplasmic face. Conversely, Ca^{2+} import might result from changes in membrane potential or an increase in the concentration gradients.

The widespread importance of regulation of calcium transport led Reusch (135) to extend the research to eucaryotic systems, and she examined a variety of plant and animal membranes. Despite the generally held belief that PHB is confined to procaryotes, the complex of PHB-Ca^{2+}-poly(P) was found to be universally present even though storage granules were absent. The intracellular location of the complex in bovine liver was investigated and shown to be the membrane fractions, principally the mitochondria and microsomes, with smaller amounts in the plasma membranes. Estimates of the chain length of eucaryotic PHB gave values (120 to 200 units) similar to those of bacteria, while the associated polyphosphate was rather longer (170 to 220 units) than the polymer in procaryotic membranes (130 to 170 units). Although the intact complex was not unequivocally demonstrated, considerable evidence for its existence was adduced and its role in the regulation of intracellular Ca^{2+} and in Ca^{2+} signal transmission was advanced.

CONCLUDING REMARKS

Research on PHAs in the United States, Japan, and Europe has been encouraged by their potential use as biodegradable alternatives to petrochemical plastics. The commercial use of these materials will, no doubt, intensify research in this field. The first PHA consumer product was launched in April 1990, at the time this review was completed. Wella AG are test marketing, in Germany, their Sanara range of biodegradable shampoos in bottles manufactured from ICI's Biopol, a 3HB-co-3HV copolymer.

Despite the relatively high cost of production of 3HB-co-3HV, current and future restrictions in the use of petrochemical plastics will determine its use. Second-generation PHAs, with different properties and probably produced from a single, inexpensive carbon source, will follow. In the longer term, it may be possible to transfer the necessary genes for PHA synthesis to plants (131), thus avoiding two costly processes, namely, production of carbohydrate as a fermentation substrate and harvest of the microorganisms.

ACKNOWLEDGMENTS

We are grateful to G. W. Haywood and D. Byrom for many valuable discussions and constructive criticism and D. J. Best for communication of unpublished research.

We thank ICI plc for support of our work under their Joint Research Scheme.

LITERATURE CITED

1. **Ackrell, B. A. C., S. K. Erickson, and C. W. Jones.** 1972. The respiratory chain NADPH dehydrogenase of *Azotobacter vinelandii*. Eur. J. Biochem. **26**:387–392.
2. **Akita, S., Y. Einaga, Y. Miyaki, and H. Fujita.** 1976. Solution properties of poly(D-β-hydroxybutyrate). 1. Biosynthesis and characterization. Macromolecules **9**:774–780.
3. **Alper, R., D. G. Lundgren, R. H. Marchessault, and W. A. Cote.** 1963. Properties of poly-β-hydroxybutyrate. I. General considerations concerning the naturally occurring polymer. Biopolymers **1**:545–556.
4. **Anderson, A. J., G. W. Haywood, and E. A. Dawes.** 1990. The biosynthesis and composition of bacterial polyhydroxyalkanoates. Int. J. Biol. Macromol. **12**:102–105.
5. **Asenjo, J. A., and J. S. Suk.** 1985. Kinetics and models for the bioconversion of methane into an intracellular polymer, poly-

β-hydroxybutyrate (PHB). Biotechnol. Bioeng. Symp. **15**:225–234.

6. **Asenjo, J. A., and J. S. Suk.** 1986. Microbial conversion of methane into poly-β-hydroxybutyrate (PHB): growth and intracellular product accumulation in a type II methanotroph. J. Ferment. Technol. **64**:271–278.
7. **Ballard, D. G. H., P. A. Holmes, and P. J. Senior.** 1987. Formation of polymers of β-hydroxybutyric acid in bacterial cells and a comparison of the morphology of growth with the formation of polyethylene in the solid state, p. 293–314. *In* M. Fontanille and A. Guyot (ed.), Recent advances in mechanistic and synthetic aspects of polymerization, vol. 215. Reidel (Kluwer) Publishing Co., Lancaster, U.K.
8. **Ballistreri, A., D. Garozzo, M. Guiffrida, G. Impallomeni, and G. Montaudo.** 1989. Analytical degradation: an approach to the structural analysis of microbial polyesters by different methods. J. Anal. Appl. Pyrolysis **16**:239–253.
9. **Barham, P. J.** 1984. Nucleation behaviour of poly-3-hydroxybutyrate. J. Mater. Sci. **19**:3826–3834.
10. **Barham, P. J., A. Keller, E. L. Otun, and P. A. Holmes.** 1984. Crystallization and morphology of a bacterial thermoplastic: poly-3-hydroxybutyrate. J. Mater. Sci. **19**:2781–2794.
11. **Barnard, G. N., and J. K. M. Sanders.** 1988. Observation of mobile poly(β-hydroxybutyrate) in the storage granules of *Methylobacterium* AM1 by *in vivo* ^{13}C-NMR spectroscopy. FEBS Lett. **231**:16–18.
12. **Barnard, G. N., and J. K. M. Sanders.** 1989. The poly-β-hydroxybutyrate granule *in vivo*. A new insight based on NMR spectroscopy of whole cells. J. Biol. Chem. **264**:3286–3291.
13. **Bauer, H., and A. J. Owen.** 1988. Some structural and mechanical properties of bacterially produced poly-β-hydroxybutyrate-co-β-hydroxyvalerate. Colloid Polym. Sci. **266**:241–247.
14. **Berger, E., B. A. Ramsay, J. A. Ramsay, and C. Chavarie.** 1989. PHB recovery by hypochlorite digestion of non-PHB biomass. Biotechnol. Tech. **3**:227–232.
15. **Bloembergen, S., D. A. Holden, G. K. Hamer, T. L. Bluhm, and R. H. Marchessault.** 1986. Studies of composition and crystallinity of bacterial poly(β-hydroxybutyrate-co-β-hydroxyvalerate). Macromolecules **19**:2865–2871.
16. **Bloomfield, G., G. Sandhu, and N. G. Carr.** 1969. Activation by Hg^{2+} of acetoacetyl-CoA reductase in extracts of *Rhodopseudomonas spheroides* and *Rhodomicrobium vannielii*. FEBS Lett. **5**:246–248.
17. **Bluhm, T. L., G. K. Hamer, R. H. Marchessault, C. A. Fyfe, and R. P. Veregin.** 1986. Isodimorphism in bacterial poly(β-hydroxybutyrate-co-β-hydroxyvalerate). Macromolecules **19**: 2871–2876.
18. **Bluhm, T. L., G. K. Hamer, and P. R. Sundarajan.** 1988. Isodimorphism in poly(β-hydroxybutyrate-co-β-hydroxyvalerate) copolyesters. Polym. Prep. **29**:603–604.
19. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. **54**:1977–1982.
20. **Brandl, H., E. J. Knee, Jr., R. C. Fuller, R. A. Gross, and R. W. Lenz.** 1989. Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly(β-hydroxyalkanoates): potential sources for biodegradable polyesters. Int. J. Biol. Macromol. **11**:49–55.
21. **Braunegg, G., B. Sonnleitner, and R. M. Lafferty.** 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. **6**:29–37.
22. **Byrom, D.** 1987. Polymer synthesis by microorganisms: technology and economics. Trends Biotechnol. **5**:246–250.
23. **Cesaro, A., and M. Scandola.** 1989. Microbial polyesters. Scientific curiosity or industrial reality? Chimicaoggi **7**:81–85.
24. **Chandrasekharan, P. T., and Y. I. Shethna.** 1976. Purification and properties of an $NADP^+$-specific isocitrate dehydrogenase from *Rhizobium meliloti*. Antonie van Leeuwenhoek. J. Microbiol. **42**:471–482.
25. **Chung, A. E.** 1970. Pyridine nucleotide transhydrogenase from *Azotobacter vinelandii*. J. Bacteriol. **102**:438–447.
26. **Collins, S.** 1987. Choice of substrate in polyhydroxybutyrate synthesis. Spec. Publ. Soc. General Microbiol. **21**:161–168.
27. **Comeau, Y., K. J. Hall, and W. K. Oldham.** 1988. Determination of poly-β-hydroxybutyrate and poly-β-hydroxyvalerate in activated sludge by gas-liquid chromatography. Appl. Environ. Microbiol. **54**:2325–2327.
28. **Cornibert, J., and R. H. Marchessault.** 1972. Physical properties of poly-β-hydroxybutyrate. IV. Conformational analysis and crystalline structure. J. Mol. Biol. **71**:735–756.
29. **Cornibert, J., R. H. Marchessault, H. Benoit, and G. Weill.** 1970. Physical properties of poly(β-hydroxybutyrate). III. Folding of helical segments in 2,2,2-trifluoroethanol. Macromolecules **3**:741–746.
30. **Davis, J. T., H.-H. Chen, R. Moore, Y. Nishitani, S. Masamune, A. J. Sinskey, and C. T. Walsh.** 1987. Biosynthetic thiolase from *Zoogloea ramigera*. II. Inactivation with haloacetyl CoA analogs. J. Biol. Chem. **262**:90–96.
31. **Davis, J. T., R. N. Moore, B. Imperali, A. Pratt, K. Kobayashi, S. Masamune, A. J. Sinskey, and C. T. Walsh.** 1987. Biosynthetic thiolase from *Zoogloea ramigera*. I. Preliminary characterization and analysis of proton transfer reaction. J. Biol. Chem. **262**:82–89.
32. **Dawes, E. A.** 1976. Endogenous metabolism and the survival of starved prokaryotes, p. 19–53. *In* T. G. R. Gray and J. R. Postgate (ed.), The survival of vegetative microbes. Cambridge University Press, Cambridge.
33. **Dawes, E. A.** 1985. Starvation, survival and energy reserves, p. 43–79. *In* M. Fletcher and G. D. Floodgate (ed.), Bacteria in their natural environments. Academic Press, Inc. (London), Ltd., London.
34. **Dawes, E. A.** 1989. Growth and survival of bacteria, p. 67–187. *In* J. S. Poindexter and E. R. Leadbetter (ed.), Bacteria in nature, vol. 3. Plenum Publishing Corp., New York.
35. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. **10**:135–266.
36. **De Smet, M. J., G. Eggink, B. Witholt, J. Kingma,and H. Wynberg.** 1983. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Bacteriol. **154**:870–878.
37. **Doi. Y., Y. Kanesawa, Y. Kawaguchi, and M. Kunioka.** 1989. Hydrolytic degradation of microbial poly(hydroxyalkanoates). Makromol. Chem. Rapid Commun. **10**:227–230.
38. **Doi, Y., Y. Kawaguchi, Y. Nakamura, and M. Kunioka.** 1989. Nuclear magnetic resonance studies of poly(3-hydroxybutyrate) and polyphosphate metabolism in *Alcaligenes eutrophus*. Appl. Environ. Microbiol. **55**:2932–2938.
39. **Doi. Y., M. Kunioka, Y. Nakamura, and K. Soga.** 1986. Biosynthesis of polyesters by *Alcaligenes eutrophus*: incorporation of ^{13}C-labelled acetate and propionate. J. Chem. Soc. Chem. Commun., p. 1696–1697.
40. **Doi, Y., M. Kunioka, Y. Nakamura, and K. Soga.** 1986. ^{1}H and ^{13}C NMR analysis of poly(β-hydroxybutyrate) isolated from *Bacillus megaterium*. Macromolecules **19**:1274–1276.
41. **Doi, Y., M. Kunioka, Y. Nakamura, and K. Soga.** 1986. Nuclear magnetic resonance studies on poly(β-hydroxybutyrate) and a copolyester of β-hydroxybutyrate and β-hydroxyvalerate isolated from *Alcaligenes eutrophus* H16. Macromolecules **19**:2860–2864.
42. **Doi, Y., M. Kunioka, Y. Nakamura, and K. Soga.** 1986. Conformational analysis of poly(β-hydroxybutyrate) in *Alcaligenes eutrophus* by solid-state ^{13}C NMR spectroscopy. Makromol. Chem. Rapid Commun. **7**:661–664.
43. **Doi, Y., M. Kunioka, Y. Nakamura, and K. Soga.** 1987. Biosynthesis of copolyesters in *Alcaligenes eutrophus* H16 from ^{13}C-labelled acetate and propionate. Macromolecules **20**:2988–2991.
44. **Doi, Y., M. Kunioka, Y. Nakamura, and K. Soga.** 1988. Nuclear magnetic resonance studies on unusual bacterial copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate. Macromolecules **21**:2722–2727.
45. **Doi, Y., A. Segawa, Y. Kawaguchi, and M. Kunioka.** 1990. Cyclic nature of poly(3-hydroxyalkanoate) metabolism in *Al-*

caligenes eutrophus. FEMS Microbiol. Lett. **67**:165–170.
46. **Doi, Y., A. Segawa, and M. Kunioka.** 1989. Biodegradable poly(3-hydroxybutyrate-co-4-hydroxybutyrate) produced from gamma-butyrolactone and butyric acid by *Alcaligenes eutrophus*. Polym. Commun. **30**:169–171.
47. **Doi, Y., A. Segawa, and M. Kunioka.** 1990. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in *Alcaligenes eutrophus*. Int. J. Biol Macromol. **12**: 106–111.
48. **Doi, Y., A. Tamaki, M., Kunioka, and K. Soga.** 1987. Biosynthesis of an unusual copolyester (10 mol% 3-hydroxybutyrate and 90 mol% 3-hydroxyvalerate units) in *Alcaligenes eutrophus* from pentanoic acid. J. Chem. Soc. Chem. Commun. **1987**:1635–1636.
49. **Doi, Y., A. Tamaki, M. Kunioka, and K. Soga.** 1987. Biosynthesis of terpolyesters of 3-hydroxybutyrate, 3-hydroxyvalerate, and 5-hydroxyvalerate in *Alcaligenes eutrophus* from 5-chloropentanoic and pentanoic acids. Makromol. Chem. Rapid Commun. **8**:631–635.
50. **Doi, Y., A. Tamaki, M. Kunioka, and K. Soga.** 1988. Production of copolyesters of 3-hydroxybutyrate by *Alcaligenes eutrophus* from butyric and pentanoic acids. Appl. Microbiol. Biotechnol. **28**:330–334.
51. **Dunlop, W. F., and A. W. Robards.** 1973. Ultrastructural study of poly-β-hydroxybutyrate granules from *Bacillus cereus*. J. Bacteriol. **114**:1271–1280.
52. **Ellar, D., D. G. Lundgren, K. Okamura, and R. H. Marchessault.** 1968. Morphology of poly-β-hydroxybutyrate granules. J. Mol. Biol. **35**:489–502.
53. **Emerich, D. W., G. E. Anthon, R. R. Hayes, D. B. Karr, R. Liang, G. G. Preston, M. T. Smith, and J. K. Waters.** 1988. Metabolism of Rhizobium-leguminous plant nodules with an emphasis on bacteroid carbon metabolism, p. 539–546. *In* H. Bothe, F. J. Bruinj, and W. E. Newton (ed.), Nitrogen fixation: hundred years after. Gustav Fischer Verlag, New York.
54. **Fernandez-Castillo, R., F. Rodriguez-Valera, J. Gonzalez-Ramos, and F. Ruiz-Berraquero.** 1986. Accumulation of poly(β-hydroxybutyrate) by halobacteria. Appl. Environ. Microbiol. **51**:214–216.
55. **Findlay, R. H., and D. C. White.** 1983. Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. Appl. Environ. Microbiol. **45**:71–78.
56. **Findlay, R. H., and D. C. White.** 1984. *In situ* determination of metabolic activity in aquatic environments. Microbiol. Sci. **1**:90–95.
57. **Fritzsche, K., R. W. Lenz, and R. C. Fuller.** 1990. Production of unsaturated polyesters by *Pseudomonas oleovorans*. Int. J. Biol. Macromol. **12**:85–91.
58. **Fritzsche, K., R. W. Lenz, and R. C. Fuller.** 1990. Bacterial polyesters containing branched poly(β-hydroxyalkanoate) units. Int. J. Biol. Macromol. **12**:92–101.
59. **Fukui, T., M. Ito, T. Saito, and K. Tomita.** 1987. Purification and characterization of NADP-linked acetoacetyl-CoA reductase from *Zoogloea ramigera* I-16-M. Biochim. Biophys. Acta **917**:365–371.
60. **Fukui, T., T. Narikawa, K. Miwa, Y. Shirakura, and K. Tomita.** 1988. Effect of limited tryptic modification of a bacterial poly(β-hydroxybutyrate) depolymerase on its catalytic activity. Biochim. Biophys. Acta **952**:164–171.
61. **Fukui, T., A. Yoshimoto, M. Matsumoto, S. Hosokawa, T. Saito, H. Nishikawa, and K. Tomita.** 1976. Enzymatic synthesis of poly-β-hydroxybutyrate in *Zoogloea ramigera*. Arch. Microbiol. **110**:149–156.
62. **Gavard, R., A. Dahinger, B. Hauttecoeur, and C. Reynaud.** 1967. Degradation of β-hydroxybutyric acid lipid by an enzyme extract of *Bacillus megaterium*. C. R. Acad. Sci. **265**:1557–1559.
63. **Grassie, N., E. J. Murray, and P. A. Holmes.** 1984. The thermal degradation of poly(-(D)-β-hydroxybutyric acid). 1. Identification and quantitative analysis of products. Polym. Degrad. Stabil. **6**:47–61.
64. **Grassie, N., E. J. Murray, and P. A. Holmes.** 1984. The thermal degradation of poly(-(D)-β-hydroxybutyric acid). 3. The reaction mechanism. Polym. Degrad. Stabil. **6**:127–134.
65. **Griebel, R., and J. M. Merrick.** 1971. Metabolism of poly-β-hydroxybutyrate: effect of mild alkaline extraction on native poly-β-hydroxybutyrate granules. J. Bacteriol. **108**:782–789.
66. **Griebel, R., Z. Smith, and J. M. Merrick.** 1968. Metabolism of poly-β-hydroxybutyrate. I. Purification, composition and properties of native poly-β-hydroxybutyrate granules from *Bacillus megaterium*. Biochemistry **7**:3676–3681.
67. **Gross, R. A., H. Brandl, H. W. Ulmer, M. A. Posada, R. C. Fuller, and R. W. Lenz.** 1989. The biosynthesis and characterization of new poly(β-hydroxyalkanoates). Polym. Prepr. **30**: 492–493.
68. **Gross, R. A., C. De Mello, R. W. Lenz, H. Brandl, and R. C. Fuller.** 1989. Biosynthesis and characterization of poly(β-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. Macromolecules **22**:1106–1115.
69. **Haddock, B. A., and C. W. Jones.** 1977. Bacterial respiration. Bacteriol. Rev. **41**:47–99.
70. **Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes.** 1988. Characterization of two 3-ketothiolases in the polyhydroxyalkanoate synthesizing organism *Alcaligenes eutrophus*. FEMS Microbiol. Lett. **52**:91–96.
71. **Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes.** 1988. The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxyalkanoate synthesizing organism *Alcaligenes eutrophus*. FEMS Microbiol. Lett. **52**: 259–264.
72. **Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes.** 1988. Accumulation of polyhydroxyalkanoates by bacteria and the substrate specificities of the biosynthetic enzymes. Biochem. Soc. Trans. **16**:1046–1047.
73. **Haywood, G. W., A. J. Anderson, and E. A. Dawes.** 1989. The importance of PHB-synthase substrate specificity in polyhydroxyalkanoate synthesis by *Alcaligenes eutrophus*. FEMS Microbiol. Lett. **57**:1–6.
74. **Haywood, G. W., A. J. Anderson, and E. A. Dawes.** 1989. A survey of the accumulation of novel polyhydroxyalkanoates by bacteria. Biotechnol. Lett. **11**:471–476.
74a. **Haywood, G. W., A. J. Anderson, D. F. Ewing, and E. A. Dawes.** 1990. Accumulation of a polyhydroxyalkanoate containing primarily 3-hydroxydecanoate from simple carbohydrate substrates by *Pseudomonas* sp. strain NCIMB 40135. Appl. Environ. Microbiol. **56**:3354–3359.
75. **Heinzle, E., and R. M. Lafferty.** 1980. A kinetic model for growth and synthesis of poly-β-hydroxybutyric acid (PHB) from *Alcaligenes eutrophus*. Eur. J. Appl. Microbiol. Biotechnol. **11**:8–16.
76. **Helleur, R. J.** 1988. Pyrolysis-gas chromatography for the rapid characterization of bacterial poly(β-hydroxybutyrate-co-β-hydroxyvalerate). Polym. Prepr. **29**:609–610.
77. **Herron, J. S., J. D. King, and D. C. White.** 1978. Recovery of poly-β-hydroxybutyrate from environmental sludge. Appl. Environ. Microbiol. **35**:251–257.
78. **Hippe, H., and H. G. Schlegel.** 1967. Hydrolyse von PHBS durch intracelluläre Depolymerase von *Hydrogenomonas* H16. Arch. Mikrobiol. **56**:278–299.
79. **Holmes, P. A.** 1985. Applications of PHB—a microbially produced biodegradable thermoplastic. Phys. Technol. **16**:32–36.
80. **Holmes, P. A.** 1988. Biologically produced (R)-3-hydroxyalkanoate polymers and copolymers, p. 1–65. *In* D. C. Bassett (ed.), Developments in crystalline polymers—2. Elsevier Applied Science Publishers, London.
81. **Huisman, G. J., O. de Leeuw, G. Eggink, and B. Witholt.** 1989. Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. Appl. Environ. Microbiol. **55**: 1949–1954.
82. **Jackson, F. A., and E. A. Dawes.** 1976. Regulation of the tricarboxylic acid cycle and poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinckii* grown under nitrogen or oxygen limitation. J. Gen. Microbiol. **97**:303–312.
83. **Jacob, G. S., J. R. Garbow, and J. Schaefer.** 1986. Direct measurement of poly(β-hydroxybutyrate) in a pseudomonad by solid state ^{13}C NMR. J. Biol. Chem. **261**:16785–16787.

84. **Karr, D. B., J. K. Waters, and D. W. Emerich.** 1983. Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exchange high-pressure liquid chromatography and UV detection. Appl. Environ. Microbiol. **46**:1339–1344.
85. **Karr, D. B., J. K. Waters, F. Suzuki, and D. W. Emerich.** 1984. Enzymes of the poly-β-hydroxybutyrate and citric acid cycles of *Rhizobium japonicum* bacteroids. Plant Physiol. **75**:1158–1162.
86. **Kawaguchi, Y., and Y. Doi.** 1990. Structure of native poly(3-hydroxybutyrate) granules characterized by X-ray diffraction. FEMS Microbiol. Lett. **79**:151–156.
87. **Korsatko, W., B. Wabnegg, H. M. Tillian, G. Braunegg, and R. M. Lafferty.** 1983. Poly-D-hydroxybutyric acid—a biologically degradable vehicle to regard release of a drug. Pharm. Ind. **45**:1004–1007.
88. **Korsatko, W., B. Wabnegg, H. M. Tillian, G. Egger, R. Pfragner, and V. Walser.** 1984. Poly D(−)-3-hydroxybutyric acid (poly-HBA)—a biodegradable former for long-term medication dosage. 3. Studies on compatibility of poly-HBA implantation tablets in tissue culture and animals. Pharm. Ind. **46**:952–954.
89. **Kunioka, M., Y. Kawaguchi, and Y. Doi.** 1989. Production of biodegradable copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate by *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. **30**:569–573.
90. **Kunioka, M., Y. Nakamura, and Y. Doi.** 1988. New bacterial copolyesters produced in *Alcaligenes eutrophus* from organic acids. Polym. Commun. **29**:174–176.
90a. **Lafferty, R. M., and E. Heinzle.** 1977. Extraktion eines Thermoplasten aus Bakterien. Chem. Rundschau **30**:14–16.
91. **Lageveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt.** 1988. Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. Appl. Environ. Microbiol. **54**:2924–2932.
92. **Law, J. H., and R. A. Slepecky.** 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. **82**:33–36.
93. **Lemoigne, M.** 1926. Products of dehydration and of polymerization of β-hydroxybutyric acid. Bull. Soc. Chem. Biol. **8**:770–782.
94. **Lundgren, D. G., R. Alper, C. Schnaitman, and R. H. Marchessault.** 1965. Characterization of poly-β-hydroxybutyrate extracted from different bacteria. J. Bacteriol. **89**:245–251.
95. **Lundgren, D. G., R. M. Pfister, and J. M. Merrick.** 1964. Structure of poly(β-hydroxybutyric acid) granules. J. Gen. Microbiol. **34**:441–446.
96. **Lusty, C. J., and M. Doudoroff.** 1966. Poly-β-hydroxybutyrate depolymerase of *Pseudomonas lemoigneii*. Proc. Natl. Acad. Sci. USA **56**:960–965.
97. **Marchessault, R. H., T. L. Bluhm, Y. Deslandes, G. K. Hamer, W. J. Orts, P. J. Sundarajan, M. G. Taylor, S. Bloembergen, and D. A. Holden.** 1988. Poly(β-hydroxyalkanoates): biorefinery polymers in search of applications. Makromol. Chem. Macromol. Symp. **19**:235–254.
98. **Marchessault, R. H., C. J. Monasterios, F. G. Morin, and P. R. Sundarajan.** 1990. Chiral poly(β-hydroxyalkanoates): an adaptable helix influenced by the alkane side-chain. Int. J. Biol. Macromol. **12**:158–165.
99. **Marchessault, R. H., K. Okamura, and C. J. Su.** 1970. Physical properties of poly(β-hydroxybutyrate). II. Conformation aspects in solution. Macromolecules **3**:735–740.
100. **Mas, J., C. Pedrós-Alió, and R. Guerrero.** 1985. Mathematical model for determining the effects of intracytoplasmic inclusions on volume and density of microorganisms. J. Bacteriol. **164**:749–756.
101. **Masamune, S., C. T. Walsh, A. J. Sinskey, and O. P. Peoples.** 1989. Poly-(R)-3-hydroxybutyrate (PHB) biosynthesis: mechanistic studies on the biological Claisen condensation catalysed by β-ketoacyl thiolase. Pure Appl. Chem. **61**:303–312.
102. **McDermott, T. R., S. M. Griffith, C. P. Vance, and P. H. Graham.** 1989. Carbon metabolism in *Bradyrhizobium japonicum* bacteroids. FEMS Microbiol. Rev. **63**:327–340.
103. **Merrick, J. M.** 1978. Metabolism of reserve materials, p. 199–219. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
104. **Merrick, J. M.** 1988. Studies on the enzymic synthesis and degradation of poly-β-hydroxybutyrate. Polym. Prep. **29**:586–587.
105. **Merrick, J. M., and M. Doudoroff.** 1961. Enzymatic synthesis of poly-β-hydroxybutyric acid in bacteria. Nature (London) **189**:890–892.
106. **Merrick, J. M., and M. Doudoroff.** 1964. Depolymerization of poly-β-hydroxybutyrate by an intracellular enzyme system. J. Bacteriol. **88**:60–71.
107. **Merrick, J. M., and C. I. Yu.** 1966. Purification and properties of a D(−)-β-hydroxybutyric dimer hydrolase from *Rhodospirillum rubrum*. Biochemistry **5**:3563–3568.
108. **Miller, N. D., and D. F. Williams.** 1987. On the biodegradation of the poly-β-hydroxybutyrate (PHB) homopolymer and poly-β-hydroxybutyrate-hydroxyvalerate copolymers. Biomaterials **8**:129–137.
109. **Mitomo, H., P. J. Barham, and A. Keller.** 1987. Crystallization and morphology of poly(β-hydroxybutyrate) and its copolymer. Polym. J. **19**:1241–1253.
110. **Morikawa, H., and R. H. Marchessault.** 1981. Pyrolysis of bacterial polyalkanoates. Can. J. Chem. **59**:2306–2313.
111. **Moskowitz, G. J., and J. M. Merrick.** 1969. Metabolism of poly-β-hydroxybutyrate. II. Enzymatic synthesis of D-(−)-β-hydroxybutyryl coenzyme A by an enoyl hydrase from *Rhodospirillum rubrum*. Biochemistry **8**:2748–2755.
112. **Moustafa, E., and C. K. Leong.** 1975. Effect of adenine nucleotides on NAD-dependent isocitrate dehydrogenases in rhizobia and bacteroids of legume root nodules. Biochim. Biophys. Acta **391**:9–14.
113. **Nakayama, K., T. Saito, T. Fukui, Y. Shirakura, and K. Tomita.** 1985. Purification and properties of extracellular poly(3-hydroxybutyrate) depolymerases from *Pseudomonas lemoignei*. Biochim. Biophys. Acta **827**:63–72.
114. **Nickels, J. S., J. D. King, and D. C. White.** 1979. Poly-β-hydroxybutyrate accumulation as a measure of unbalanced growth of the estuarine detrital microbiota. Appl. Environ. Microbiol. **37**:459–465.
115. **Nickerson, K. W.** 1982. Purification of poly-β-hydroxybutyrate by density gradient centrifugation in sodium bromide. Appl. Environ. Microbiol. **43**:1208–1209.
116. **Nishimura, T., T. Saito, and K. Tomita.** 1978. Purification and properties of β-ketothiolase from *Zoogloea ramigera*. Arch. Microbiol. **116**:21–27.
117. **Odham, G., A. Tunlid, G. Westerdahl, and P. Marden.** 1986. Combined determination of poly-β-hydroxyalkanoic and cellular fatty acids in starved marine bacteria and sewage sludge by gas chromatography with flame ionization or mass spectrometry detection. Appl. Environ. Microbiol. **52**:905–910.
118. **Oeding, V., and H. G. Schlegel.** 1973. β-Ketothiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly-β-hydroxybutyrate metabolism. Biochem. J. **134**:239–248.
119. **Okamura, K., and R. H. Marchessault.** 1967. X-ray structure of poly-β-hydroxybutyrate, p. 709–720. *In* G. M. Ramachandran (ed.), Conformation of biopolymers, vol. 2. Academic Press, Inc., New York.
120. **Organ, S. J., and P. J. Barham.** 1988. Electron microscopic investigations of the structure and nucleation of polyalkanoate copolymers and blends. Polym. Prepr. **29**:602.
121. **Ostle, A. G., and J. G. Holt.** 1982. Nile Blue A as a fluorescent stain for poly-β-hydroxybutyrate. Appl. Environ. Microbiol. **44**:238–241.
122. **Owen, A. J.** 1985. Some dynamic mechanical properties of microbially produced poly-β-hydroxybutyrate/β-hydroxyvalerate. Colloid Polym. Sci. **263**:799–803.
123. **Packter, N. M., and S. Flatman.** 1983. Characterization of acetoacetyl-CoA reductase (3-oxoreductase) from *Streptomyces coelicolor*: its possible role in polyhydroxybutyrate biosynthesis. Biochem. Soc. Trans. **11**:598–599.
124. **Page, W. J.** 1989. Production of poly-β-hydroxybutyrate by

Azotobacter vinelandii strain UWD during growth on molasses and other complex carbon sources. Appl. Microbiol. Biotechnol. **31**:329–333.

125. **Page, W. J., and O. Knosp.** 1989. Hyperproduction of poly-β-hydroxybutyrate during exponential growth of *Azotobacter vinelandii* UWD Appl. Environ. Microbiol. **55**:1334–1339.
126. **Pedrós-Alió, C., J. Mas, and R. Guerrero.** 1985. The influence of poly-β-hydroxybutyrate accumulation on cell volume and buoyant density in *Alcaligenes eutrophus*. Arch. Microbiol. **143**:178–184.
127. **Peoples, O. P., S. Masamune, C. T. Walsh, and A. J. Sinskey.** 1987. Biosynthetic thiolase from *Zoogloea ramigera*. III. Isolation and characterization of the structural gene. J. Biol. Chem. **262**:97–102.
128. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. **264**:15293–15297.
129. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-β-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (phbC). J. Biol. Chem. **264**:15298–15303.
130. **Ploux, O., S. Masamune, and C. T. Walsh.** 1988. The NADPH-linked acetoacetyl-CoA reductase from *Zoogloea ramigera*. Characterization and mechanistic studies of the cloned enzyme overproduced in *Escherichia coli*. Eur. J. Biochem. **174**:177–182.
131. **Pool, R.** 1989. In search of the plastic potato. Science **245**: 1187–1189.
132. **Preiss, J.** 1989. Chemistry and metabolism of intracellular reserves, p. 189–258. *In* E. R. Leadbetter and J. S. Poindexter (ed.), Bacteria in nature, vol. 3. Plenum Publishing Corp., New York.
133. **Ramsay, J. A., E. Berger, B. A. Ramsay, and C. Chavarie.** 1990. Recovery of poly-3-hydroxyalkanoic acid granules by a surfactant-hypochlorite treatment. Biotechnol. Tech. **4**:221–226.
134. **Ramsay, B. A., J. A. Ramsay, and D. G. Cooper.** 1989. Production of poly-β-hydroxyalkanoic acid by *Pseudomonas cepacia*. Appl. Environ. Microbiol. **55**:584–589.
135. **Reusch, R. N.** 1989. Poly-β-hydroxybutyrate/calcium polyphosphate complexes in eukaryotic membranes. Proc. Soc. Exp. Biol. Med. **191**:377–381.
136. **Reusch, R. N., T. W. Hiske, and H. L. Sadoff.** 1986. Poly-β-hydroxybutyrate membrane structure and its relationship to genetic transformability in *Escherichia coli*. J. Bacteriol. **168**: 553–562.
137. **Reusch, R. N., T. W. Hiske, H. L. Sadoff, R. Harris, and T. Beveridge.** 1987. Cellular incorporation of poly-β-hydroxybutyrate into plasma membranes of *Escherichia coli* and *Azotobacter vinelandii* alters native membrane structure. Can. J. Microbiol. **33**:435–444.
138. **Reusch, R. N., and H. L. Sadoff.** 1983. D(−)-Poly-β-hydroxybutyrate in membranes of genetically competent bacteria. J. Bacteriol. **156**:778–788.
139. **Reusch, R. N., and H. L. Sadoff.** 1988. Putative structure and functions of a poly-β-hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc. Natl. Acad. Sci. USA **85**:4176–4180.
140. **Riis, V., and W. Mai.** 1988. Gas chromatographic determination of poly-β-hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis. J. Chromatogr. **445**:285–289.
141. **Ritchie, G. A. F., and E. A. Dawes.** 1969. The non-involvement of acyl-carrier protein in poly-β-hydroxybutyrate synthesis in *Azotobacter beijerinckii*. Biochem. J. **112**:803–805.
142. **Ritchie, G. A. F., P. J. Senior, and E. A. Dawes.** 1971. The purification and characterization of acetoacetyl-coenzyme A reductase from *Azotobacter beijerinckii*. Biochem. J. **121**:309–316.
143. **Saito, T., T. Fukui, F. Ikeda, Y. Tanaka, and K. Tomita.** 1977. An NADP-linked acetoacetyl CoA reductase from *Zoogloea ramigera*. Arch. Microbiol. **114**:211–217.
144. **Saito, T., K. Suzuki, J. Yamamoto, T. Fukui, K. Miwa, K. Tomita, S. Nakanishi, S. Odani, J.-I. Suzuki, and K. Ishikawa.** 1989. Cloning, nucleotide sequence, and expression in *Escherichia coli* of the gene for poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. J. Bacteriol. **171**:184–189.
145. **Scandola, M., G. Ceccorulli, and Y. Doi.** 1990. Viscoelastic relaxations and thermal properties of bacterial poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate). Int. J. Biol. Macromol. **12**:112–117.
146. **Scandola, M., M. Pizzoli, G. Ceccorulli, A. Cesaro, S. Paoletti, and L. Navarini.** 1988. Viscoelastic and thermal properties of bacterial poly(D-(−)-β-hydroxybutyrate). Int. J. Biol. Macromol. **10**:373–377.
147. **Schlegel, H. G., R. Lafferty, and I. Krauss.** 1970. The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch. Mikrobiol. **71**:283–294.
148. **Schlegel, H. G., and V. Oeding.** 1971. Selection of mutants not accumulating storage materials. Radiat. Radioisotop. Ind. Microorg. Proc. Symp. **1971**:223–231.
149. **Schubert, P., A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-β-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. **170**:5837–5847.
150. **Scott, D., J. Brannan, and I. J. Higgins.** 1981. The effect of growth conditions on intracytoplasmic membranes and methane mono-oxygenase activities in *Methylosinus trichosporium* OB3b. J. Gen. Microbiol. **125**:63–72.
151. **Sebo, P.** 1986. Hydrogen-oxidizing bacteria in biotechnology. Trends Biotechnol. **4**:321–323.
152. **Seebach, D., and M. F. Zuger.** 1982. Uber die Depolymerisierung von Poly-(R)-3-hydroxy-buttersaureester (PHB). Helv. Chim. Acta. **65**:495–503.
153. **Seebach, D., and M. F. Zuger.** 1985. On the preparation of methyl and ethyl (R)-(−)-3-hydroxy-valerate by depolymerization of a mixed PHB/PHV biopolymer. Tetrahedron Lett. **25**:2747–2750.
154. **Senior, P. J.** 1984. Polyhydroxybutyrate, a speciality polymer of microbial origin, p. 266–271. In A. C. R. Dean, D. C. Ellwood, and C. G. T. Evans (ed.), Continuous culture, vol. 8. Ellis Horwood, Chichester, England.
155. **Senior, P. J., G. A. Beech, G. A. F. Ritchie, and E. A. Dawes.** 1972. The role of oxygen limitation in the formation of poly-β-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*. Biochem. J. **128**:1193–1201.
156. **Senior, P. J., and E. A. Dawes.** 1971. Poly-β-hydroxybutyrate biosynthesis and the regulation of glucose metabolism in *Azotobacter beijerinckii*. Biochem. J. **125**:55–66.
157. **Senior, P. J., and E. A. Dawes.** 1973. The regulation of poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. Biochem. J. **134**:225–238.
158. **Shirakura, Y., T. Fukui, T. Saito, Y. Okamoto, T. Narikawa, K. Koide, K. Tomita, T. Takemasa, and S. Masamune.** 1986. Degradation of poly(3-hydroxybutyrate) by poly(3-hydroxybutyrate) polymerase from *Alcaligenes faecalis* T_1. Biochim. Biophys. Acta **880**:46–53.
159. **Shirakura, Y., T. Fukui, T. Tanio, K. Nakayama, R. Matsuno, and K. Tomita.** 1983. An extracellular D(−)-3-hydroxybutyrate oligomer hydrolase from *Alcaligenes faecalis*. Biochim. Biophys. Acta **748**:331–339.
160. **Shuto, H., T. Fukui, T. Saito, Y. Shirakura, and K. Tomita.** 1981. An NAD-linked acetoacetyl-CoA reductase from *Zoogloea ramigera* I-16-M. Eur. J. Biochem. **118**:53–59.
161. **Slater, S. C., W. H. Voige, and D. E. Dennis.** 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J. Bacteriol. **170**:4431–4436.
162. **Smibert, R. M., and N. R. Krieg.** 1981. General characterization, p. 409–443. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
163. **Sonnleitner, B., E. Heinzle, G. Braunegg, and R. M. Lafferty.** 1979. Formal kinetics of poly-β-hydroxybutyric acid (PHB) production in *Alcaligenes eutrophus* H16 and *Mycoplana ru-*

bra R14 with respect to the dissolved oxygen tension in ammonium-limited batch cultures. Eur. J. Appl. Microbiol. **7**:1–10.

164. **Srienc, F., B. Arnold, and J. E. Bailey.** 1984. Characterization of intracellular accumulation of poly-β-hydroxybutyrate (PHB) in individual cells of *Alcaligenes eutrophus* H16 by flow cytometry. Biotechnol. Bioeng. **26**:982–987.
165. **Stam, H., H. W. van Verseveld, W. de Vries, and A. H. Stouthamer.** 1986. Utilization of poly-β-hydroxybutyrate in free-living cultures of *Rhizobium* ORS571. FEMS Microbiol. Lett. **35**:215–220.
166. **Steinbüchel, A., and H. G. Schlegel.** 1989. Excretion of pyruvate by mutants of *Alcaligenes eutrophus*, which are impaired in the accumulation of poly(β-hydroxybutyric acid) (PHB), under conditions permitting synthesis of PHB. Appl. Microbiol. Biotechnol. **31**:168–175.
167. **Stovall, I., and M. Cole.** 1978. Organic acid metabolism by isolated *Rhizobium japonicum* bacteroids. Plant Physiol. **61**: 787–790.
168. **Suzuki, T., H. Deguchi, T. Yamane, S. Shimizu, and K. Gekko.** 1988. Control of molecular weight of poly-β-hydroxybutyric acid produced in fed-batch culture of *Protomonas extorquens*. Appl. Microbiol. Biotechnol. **27**:487–491.
169. **Suzuki, T., T. Yamane, and S. Shimizu.** 1986. Mass production of poly-β-hydroxybutyric acid by fully automatic fed-batch culture of methylotroph. Appl. Microbiol. Biotechnol. **23**:322–329.
170. **Suzuki, T., W. L. Zahler, and D. W. Emerich.** 1987. Acetoacetyl-CoA thiolase of *Bradyrhizobium japonicum* bacteroids: purification and properties. Arch. Biochem. Biophys. **254**:272–281.
171. **Tanio, T., T. Fukui, T. Saito, K. Tomita, T. Kaiho, and S. Masamune.** 1982. An extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. Eur. J. Biochem. **124**:71–77.

171a. **Timm, A., and A. Steinbüchel.** 1990. Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl. Environ. Microbiol. **56**:3360–3367.

172. **Tombolini, R., and M. P. Nuti.** 1989. Poly(β-hydroxyalkanoate) biosynthesis and accumulation by different *Rhizobium* species. FEMS Microbiol. Lett. **60**:299–304.
173. **Tomita, K., T. Saito, and T. Fukui.** 1983. Bacterial metabolism of poly-β-hydroxybutyrate, p. 353–366. *In* D. L. F. Lennon, F. W. Stratman, and R. N. Zahlten. (ed.), Biochemistry of metabolic processes. Elsevier Science Publishing, Inc., New York.
174. **Wallen, L. L., and W. K. Rohwedder.** 1974. Poly-β-hydroxyalkanoate from activated sludge. Environ. Sci. Technol. **8**:576–579.
175. **Wong, P. P., and H. J. Evans.** 1971. Poly-β-hydroxybutyrate utilization by soybean (Glycine max Merr.) nodules and assessment of its role in maintenance of nitrogenase activity. Plant Physiol. **47**:750–755.
176. **Yokouchi, M., Y. Chatani, H. Tadokoro, and H. Tani.** 1974. Structural studies of polyesters. VII. Molecular and crystal structures of racemic poly(β-ethyl-β-propiolactone). Polym. J. **6**:248–255.