Poly(hydroxyalkanoates): A Fifth Class of Physiologically Important Organic Biopolymers?**

By Hans-Martin Müller and Dieter Seebach *

Along with polyisoprenoids, polypeptides, polysaccharides, and polynucleotides, Nature contains a further group of biopolymers, the poly(hydroxyalkanoates). The commonest member of this group, poly[(*R*)-3-hydroxybutyrate] P(3-HB), had been identified by Lemoigne as early as the 1920s, as a storage substance in the microorganism *Bacillus megaterium* made up of more than 12000 (3-HB) units. However, the widespread distribution and significance of these biopolymers has only become clear recently. The work of Reusch, in particular, has shown that low molecular weight P(3-HB) (100–200 3-HB units) occurs in the cell membranes of prokaryotic and eukaryotic organisms. The function of P(3-HB) in the latter sources is largely unknown; it has been proposed that a complex of P(3-HB) and calcium polyphosphate acts as an ion channel through the membrane. Indeed, it has even been speculated that P(3-HB) plays a role in transport of DNA through the cell wall. In the present article, the following subjects will be discussed: metabolism of P(3-HB) and analogous polyesters in the synthesis and degradation of storage materials; P(3-HB) as a starting material for chiral synthetic building blocks; synthesis of cyclic oligomers (oligolides) of up to ten 3-HB units, and their crystal structure; high molecular weight bio-copolymers of hydroxybutyrate and hydroxyvalerate (BIOPOL) as biologically degradable plastics; nonbiological production of polyhydroxyalkanoates from 3-hydroxy carboxylic acids and the corresponding β-lactones; specific synthesis of linear oligomers with a narrow molecular weight distribution, consisting of about 100 (*R*)-3-hydroxybutyrate units, by using an exponential coupling procedure; structure of the polyesters, and a comparison with other polymers; the experimental results which led to the postulation of a P(3-HB) ion channel through the cell wall; modeling of P(3-HB) helices of various diameters, by using the parameters obtained from the crystal structures of oligolides; formation of a crown ester complex and ion transport experiments with the triolide of 3-HB. The article describes one example of the contributions that synthetic organic chemists can make to important biological problems in an interdisciplinary framework.

1. Introduction: Occurrence and Significance of Polyhydroxyalkanoates in Nature

Polyhydroxy acids (PHAs[1]) are synthesized by microorganisms under conditions of nutrient limitation in the presence of an excess carbon and energy source. Due to their low solubility and their high molecular weight PHAs do not cause an increase in osmotic pressure, and they are therefore ideal storage compounds.[2] In this role they are much more common in microorganisms than are glycogen, polyphosphates, or fats, for example.[3,4] The most prominent PHA is poly[(*R*)-3-hydroxybutyrate] [P(3-HB)], a linear, unbranched homopolymer built up of (*R*)-3-hydroxybutyric acid units. The molecular weight values given in the literature vary considerably, and are dependent on microorganism, cultivation conditions, and the method of isolation. Typically, the values lie between 1×10^5–7.5×10^5 g mol^{-1} ($n = 2500$–9000, polydispersity (M_w/M_n) = 1.7–2.9[5]), but they can be greater than one million g mol^{-1}. The copolymer from (*R*)-3-hydroxybutyrate and (*R*)-3-hydroxyvalerate P(3-HB/3-HV) has achieved a certain economic importance because of its polypropylene-like material properties, and is marketed by ICI under the tradename "BIOPOL". P(3-HB) and P(3-HB/3-HV) are both biodegradable, and their synthesis is based on renewable materials.[6] Great things are expected of them because of these qualities; this is apparent by their regular appearance in the press,[7] review articles which are published ever more frequently,[1b,4,5,8–13] and the increasing number of patents.[14]

P(3-HB) was first described by Lemoigne in 1925,[15] who later isolated and identified the material from *Bacillus megaterium*.[16] Since then, P(3-HB) has been discovered in a large number of different microorganisms,[11] for example in Archaebacteria, in both gram-negative and gram-positive bacteria, and in Cyanobacteria. Apart from a few phototrophic microorganisms, *Clostridium* and *Syntrophomonas* are the only strict anaerobes in which P(3-HB) has been found. Interestingly, enterobacteria (gut bacteria) such as *Escherichia coli* do not normally synthesize P(3-HB) as a storage compound.[11,17,18]

Apart from its occurrence as a storage compound, P(3-HB) is also found—in a low molecular weight form—in bacterial membranes and in the tissues of plants and animals, where it presumably forms part of an ion channel (see Section 7).[19–23] Recently it has also been detected in relatively large amounts in human blood plasma (between 0.6 and 18.2 mg per liter blood), where it is bound mainly to the so-called "low density lipoproteins" (20–30%), and to albumin (70–80%).[24]

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Schulz and Toft have shown that in certain species of spiders from the Linyphia family, (*R*,*R*)-hydroxybutyrate dimers act as pheromones. They lead the male to roll up the web of unfertilized females, possibly in order to demonstrate that the female is now occupied. This behavior is not observed with the webs of already fertilized females.[25] P(3-HB) has also been discussed as a possible energy source for nitrogen fixation in *rhizobia*.[12, 17a, 26]

2. Polyhydroxy Acids as Cellular Storage Materials

Quantitatively, the most important role of poly(3-hydroxyalkanoates) is to store carbon-containing material and reductase equivalents in the cells of prokaryotic microorganisms (up to 90% of cell dry weight!). The biochemistry of the synthesis and degradation of high molecular weight P(3-HB) has therefore been subject to intensive studies. These have demonstrated which other hydroxy acids can be incorporated into the polymer, in natural or synthetic growth media. The enzymes involved in the biosynthesis have been identified, transferred to other microorganisms, and expressed by gene technological methods.

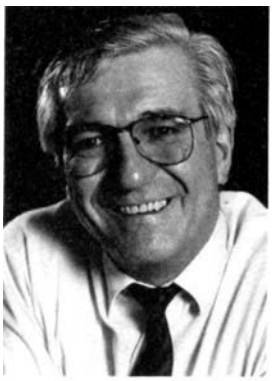
2.1. Intracellular Distribution of Polyhydroxy Acid Esters

PHAs occur in the cytoplasm of the cell in the form of inclusion bodies, so-called granules. Typically, these have a diameter of 100–800 nm, and have been shown to be surrounded by a kind of micelle ("monolayer"), but not by a double layered structure (typical membrane, lipoprotein bilayer).[27] Depending on the bacterium, the PHA-synthase and depolymerase system may also be bound to this envelope. Purified granules from *B. megaterium* consist of 97.7% P(3-HB), 1.8% protein, and 0.5% lipid.[5, 28]

The structure of P(3-HB) within the granules has been subject to debate until recently. All that was previously known was that treatment with solvents, bases or acids, as well as cooling or heating inhibited their enzymatic digestion.[29] More recently Doi et al.[30] by X-ray diffraction and Sanders et al. ([31a], but also[31b]) by high-resolution ^{13}C NMR spectroscopy have shown that the P(3-HB) within the native granules is present in an amorphous form. This is surprising, since isolated P(3-HB) normally reveals between 60 and 70% crystallinity.[32] The reasons for this are not yet clear; Sanders et al. postulate water as a plasticizer, whereas Doi et al. attribute an inhibitory effect on crystallization to an as yet unknown lipid component. Because of its thermodynamically less favorable state, amorphous P(3-HB) is probably more amenable to enzymatic degradation than P(3-HB) with a high content of crystalline domains.

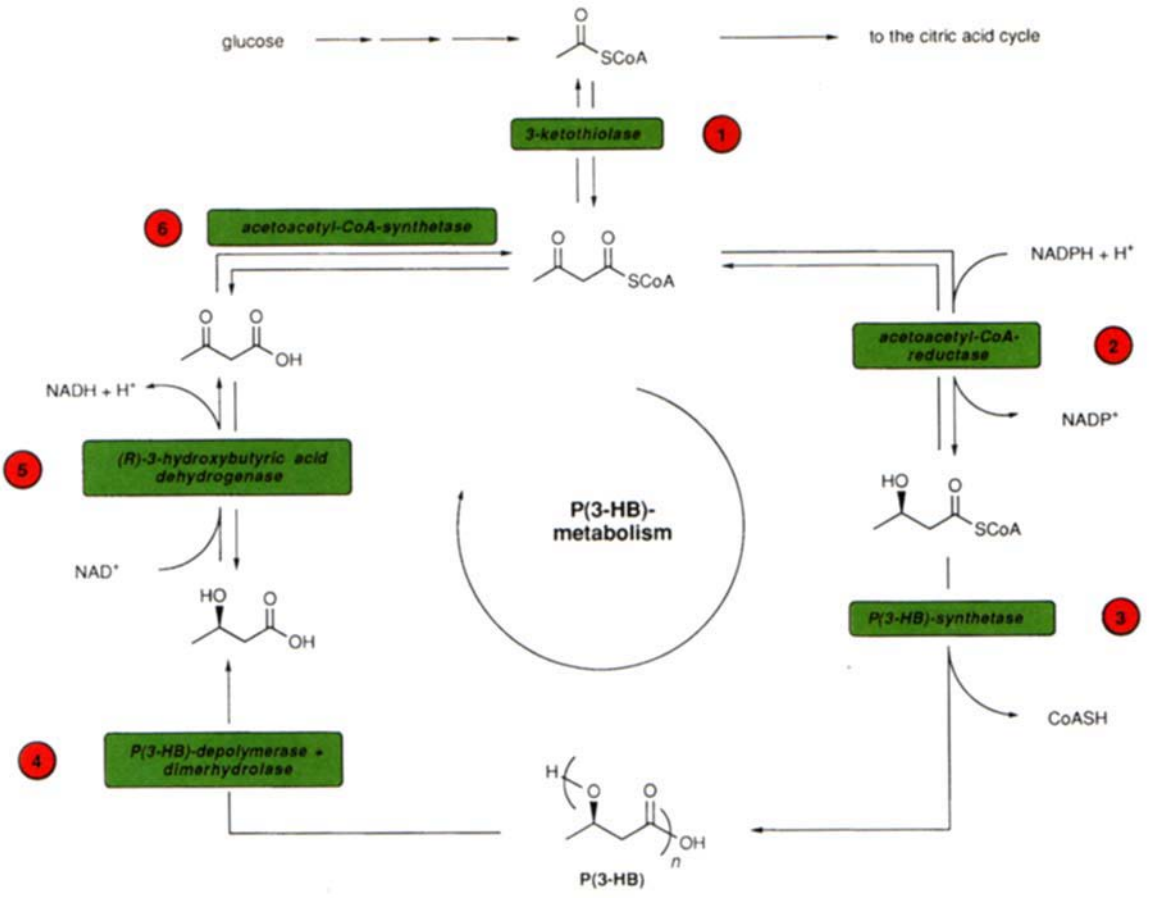
2.2. Poly[(*R*)-3-hydroxybutyrate] Metabolism

Intracellular P(3-HB) metabolism is a cyclic process, involving seven enzymes, as shown in Scheme 1. It has been investigated primarily by Schlegel et al. and Dawes et al. in *Alcaligenes eutrophus*,[33–40] by Dawes et al. in *Azotobacter beijerinckii*,[41–43] by Fukui et al. and Tomita et al. in *Zoologea ramigera*,[44–49] and in somewhat less detail by Merrick et al. and Doudoroff et al. in *Rhodospirillum rubrum* and *Bacillus megaterium*.[29, 50–56] It has also been studied in other microorganisms.[1b, 12]



Dieter Seebach was born in Karlsruhe in 1937, he studied chemistry at the University of Karlsruhe. He received his Ph.D in 1964 with a thesis on small-ring compounds and peroxides (supervisor R. Criegee). After a stay of almost two years at Harvard University as a postdoctoral fellow with E. J. Corey and lecturer of chemistry, he returned to Karlsruhe and his research led to a habilitation on S- and Se-stabilized carbanion and carbene derivatives in 1969. In 1971 he became a full professor at the University of Giessen and since 1977 he has been a full professor at the Eidgenössische Technische Hochschule in Zürich. He has had visiting professorships at the Universities of Madison (Wisconsin), Strasbourg, München (TU), Kaiserslautern, as well as Caltech (Pasadena) and the Max-Planck-Institut in Mülheim. His main areas of research are the development of new synthetic methods, mechanistic studies, structure determinations, and natural product syntheses. Recently his research has been concerned more and more with the topic discussed in this review.

Hans-Martin Müller was born in Herisau/Appenzell Ausserrhoden, Switzerland in 1962. After training as a chemistry laboratory assistant he studied chemistry at the Ingenieurschule in Basel (Diplom 1985). He subsequently studied chemistry at the ETH Zürich (Diplom 1988). He received his doctorate with D. Seebach with a thesis on polyhydroxybutyric acid in 1992 and in the same year joined the company Sandoz.



Scheme 1. The best known and probably most widely distributed P(3-HB) metabolic pathway, which also occurs in *A. eutrophus*, *Z. ramigera*, and *A. beijerinckii*.

A metabolic pathway slightly different from that in Scheme 1 has been proposed for *A. eutrophus* by Doi et al,[57] which uses butyric acid (and probably also the higher carboxylic acids) as substrate. Here, butyric acid is first esterified with coenzyme A (CoA) and then converted stepwise into acetoacetyl-CoA via crotonyl-CoA and (*S*)-3-hydroxybutyryl-CoA. The remainder of the pathway is identical to that shown in Scheme 1. In other bacteria, further metabolic variants have been found (see [58] and Section 2.3.1).

2.2.1. Biosynthesis

The following section will discuss primarily the P(3-HB) biosynthetic pathway observed in *A. eutrophus*, since this microorganism, as the basis of the production of BIPOL by the ICI process, has the greatest commercial importance.[59] The *A. eutrophus* strains NCIB11599[5] and N9A[60] produce more than 90% of their dry weight as P(3-HB), when the C source provided is glucose or fructose, respectively. In contrast to *Pseudomonas oleovorans* and other bacteria, only polyesters with C_3–C_5 monomer units are synthesized, from a variety of substrates.[61]

A. eutrophus possesses two 3-ketothiolases,[36] which catalyze the Claisen reaction of two carboxylic thioesters to the corresponding 3-ketocarboxylate. The mechanism of this reversible reaction has been studied in detail by Masamune et al.[62] Enzyme A acts predominantly on acetoacetyl-CoA, and is somewhat less active with 3-ketopentanoyl-CoA; it is therefore responsible for P(3-HB) metabolism. Enzyme B prefers higher 3-ketoacetyl-CoA substrates (C_4 to C_{10}), and is presumably more concerned with fatty acid metabolism. It is the only enzyme in Scheme 1 which is involved in both the biosynthesis and the biodegradation of polyesters.

For the second step, the reduction of acetoacetyl-CoA, two enzymes are again present in *A. eutrophus*,[37] an NADH- and an NADPH-dependent reductase. The NADH-dependent enzyme can oxidize both (*R*)- and (*S*)-3-hydroxybutyryl-CoA, whereas reduction affords only (*S*)-3-hydroxybutyryl-CoA. The NADPH-reductase is stereoselective in both the oxidation and reduction reactions, and is active only with (*R*)-3-hydroxybutyrate, which is also the exclusive product in the reduction. It is therefore only this reductase that is involved directly in P(3-HB) metabolism.

P(3-HB)-synthase, the last enzyme associated with the biosynthesis, is found in a soluble form and a "granule-associated" form.[39] Under normal growth conditions one finds only the soluble synthase, which is transformed to the granule-associated form under conditions of nitrogen limitation. This enzyme can only polymerize certain hydroxy acids, and therefore governs the composition of the polymer.[40]

2.2.2. Cloning and Expression of the Poly[(R)-3-hydroxybutyrate] Biosynthetic Pathway of Alcaligenes eutrophus

The transfer of an entire biosynthetic pathway into a foreign organism is always advantageous when an industrially

more robust strain is required, when different (cheaper) raw materials are sought, or in order to investigate mechanistic questions.[63] Often the genes coding for the biosynthesis lie close to each other, making transfer to another organism somewhat simpler. When one considers the great expectations placed on PHAs, it is hardly surprising that no fewer than three research groups have cloned and expressed the *A. eutrophus* P(3-HB) biosynthetic genes (steps 1–3, Scheme 1) in *E. coli*.[17b, 64–66] *E. coli* does not normally synthesize PHAs, but in this case can produce about 50% of its dry weight as P(3-HB) (with some strains up to 80%). Here, too, it appears in the form of intracellular granules. The three enzymes responsible for the synthesis are organized in a P(3-HB)-synthesis-operon, which is controlled from one promoter.[66] If the P(3-HB)-synthase gene is transferred alone into *E. coli*, no polyester can be produced. Clearly, *E. coli* is itself not capable of synthesizing (*R*)-3-hydroxybutyryl-CoA.[17b]

In addition to the P(3-HB)-operon, Lubbitz et al.[67] also built in a lysis gene, which is expressed at higher temperatures in the presence of divalent cations. The P(3-HB) granules are thus released into the surrounding medium through openings in the cell wall, and can be separated from the cell debris. This elegant method of isolation is also applicable to *Alcaligenes* species, and has recently been patented.[68]

Other projects are at present probably more visions of the future than reality. Somerville et al.,[69a] for example, plan to insert the P(3-HB)-synthesis genes of *A. eutrophus* into potatoes or other plants, in order to harvest a type of "plastic potato" after expression of the genes. A first realization of this idea has just been reported, but the transgenic plants were only able to produce tiny quantities of P(3-HB). The 3-ketothiolase is present in higher plants, but genes for both the reductase and P(3-HB)-synthase genes must also be introduced here for P(3-HB) production.

Apart from the PHA-structural genes from *A. eutrophus*, those of several other microorganisms, such as *Z. ramigera*[70] and *P. oleovorans*,[71] have also been identified and their nucleotide sequence determined.

2.2.3. Depolymerization

Depolymerization (step 4 in Scheme 1) of the granular, amorphous P(3-HB) to (*R*)-3-hydroxybutyric acid is catalyzed by a depolymerase which degrades P(3-HB) predominantly to dimers. Only in *A. eutrophus* is the direct product (*R*)-3-hydroxybutyric acid.[72] In other microorganisms, the dimers are subsequently cleaved by a dimer hydrolase to the monomers. The depolymerase has been isolated from *A. eutrophus*,[33] *B. megaterium*,[53] and *R. rubrum*.[29] However, its mode of action is still only poorly understood. In contrast to the depolymerase, the mechanism of the dimer hydrolase reaction is quite well known. This enzyme has been isolated from *Z. ramigera*[47] and *R. rubrum*,[52] and hydrolyzes not only the (*R*,*R*) dimers,[73] but, depending on the bacterium, also the (*R*,*S*) dimers (*R. rubrum*, *Z. ramigera*) and the (*S*,*R*) dimers (*Z. ramigera*). The latter are cleaved more slowly, however. The other enantiomers are not saponified by the respective organism. Higher oligomers (pentamers, for example) can also be attacked. As Tomita et al. have shown, these are degraded in *Z. ramigera* in an exo-fashion[74] from the alcohol end; the longer the chain, the greater the reaction rate. Even the corresponding methyl esters are hydrolyzed (except for those of the dimeric or monomeric compound).[47]

The oxidation of (*R*)-3-hydroxybutyric acid to acetylacetate (step 5 in Scheme 1) is catalyzed by a dehydrogenase. The enzymes have been isolated from *A. eutrophus*,[33] *A. beijerinckii*,[43] and *Z. ramigera*,[48] and are very similar with respect to optimum pH values, inhibitors, and the K_m value with (*R*)-3-hydroxybutyric acid. NAD^+ functions as cofactor in all cases.

Two different mechanisms have been found for the esterification of acetoacetate with coenzyme A (step 6 in Scheme 1). In *A. eutrophus* and *A. beijerinckii* the enzyme responsible is a 3-ketoacid-CoA transferase, and the CoA unit is supplied by succinyl-CoA.[43] In *Z. ramigera*, the same step is catalyzed by an acetoacetyl-CoA synthase, and involves ATP.[49]

2.2.4. Regulation of the Poly[(R)-3-hydroxybutyrate] Cycle

The control of PHA metabolism shown in Scheme 1 has been studied most extensively by Dawes et al. in *A. beijerinckii*. It proceeds analogously in *A. eutrophus* and *B. megaterium*, but cannot be extended generally to all bacteria.[12]

3-Ketothiolase is the only enzyme which participates in both the synthesis (Claisen reaction of two acetyl-CoA units) and degradation (retro-Claisen reaction of acetoacetyl-CoA) of P(3-HB), and it plays a central role in the control of the cycle. The Claisen reaction is inhibited by high concentrations of CoASH, while the retro-Claisen reaction is inhibited by acetoacetyl-CoA. The latter inhibition can be overcome by addition of high CoASH concentrations.

Normally, acetyl-CoA is used predominantly in the citric acid cycle, releasing CoASH; that is, the concentration of acetyl-CoA is low, that of CoASH is high, and the 3-ketothiolase is therefore inactive.

Limitation of a nutrient (either nitrogen, oxygen, or phosphorus, depending on bacterium[111]) has the effect of increasing NADH concentration, leading to less efficient degradation of acetyl-CoA by the citric acid cycle. The acetyl-CoA concentration therefore rises, and that of CoASH begins to fall. The inhibition of 3-ketothiolase is thereby overcome, and acetoacetyl-CoA can react with excess NADH according to Scheme 1, to yield P(3-HB). Synthesis of P(3-HB) acts to a certain extent as a sink for acetyl and reduction equivalents.

Degradation is initiated if too little C source is present; the high CoASH levels associated with this situation can override the inhibition of the 3-ketothiolase by acetoacetyl-CoA, and acetyl-CoA units can be released from P(3-HB). The details of regulation of the degradative pathway are still largely unclear, since not enough is known about the P(3-HB)-depolymerase.[75]

For *A. eutrophus*, Doi et al. have shown that under nitrogen limitation P(3-HB) biosynthesis and degradation can take place simultaneously. To what extent the released

monomer units are reused for polymer synthesis has not yet been clarified.[76]

2.3. Microbiologically Accessible Polyesters

A large number of studies have been made with various carbon sources in the growth medium, using different microorganisms capable of producing polyester storage materials. These have been carried out primarily with the aim of synthesizing enantiomerically pure starting materials required for organic synthesis, as discussed below, and in the search for biodegradable materials with a variety of structure-dependent characteristics.

2.3.1. Synthesis of Polyesters Containing no Further Functional Groups with Alcaligenes eutrophus and Pseudomonas oleovorans

A. eutrophus synthesizes only unsubstituted polymers, even when chlorinated or unsaturated acids, for example, 2-chloropropionic acid,[5] 5-chlorovaleric acid,[77] or 4-pentenoic acid[78] are used as substrate. Polyesters with C_4/C_5 hydroxy acid units are produced from almost all C sources, and C_3 structural units have till now been discovered in only a few cases (Table 1, no. 4); C_6 units have never been found.

Table 1. Preparation of polyesters with structural units 3-HP (3-hydroxypropionate), 3-HB (3-hydroxybutyrate), 4-HB (4-hydroxybutyrate), 3-HV (3-hydroxyvalerate), and 5-HV (5-hydroxyvalerate) from various carbon sources by *Alcaligenes eutrophus*.

No.	Substrate	3-HP	3-HB	4-HB	3-HV	5-HV
1	glucose	–	+	–	–	–
2	glucose + propionate	–	+	–	+	–
3	γ-butyrolactone [5, 79, 80] [a]	–	+	+	–	–
4	3-hydroxypropionate or 1,5-pentanediol [5]	+	+	–	–	–
5	5-chlorovalerate [77]	–	+	–	+	+

[a] In addition 4-hydroxybutyrate, 1,4-butanediol, 1,6-hexanediol, or 4-chlorobutyrate can also be used.

Glucose and propionic acid are the substrates which ICI employ for the production of P(3-HB) and P(3-HB/3-HV), and the limiting nutrient is phosphate in each case.[81] By changing the glucose/propionate ratio, the proportion of 3-HV can be varied between 0–20%. These polymers are available commercially under the tradename "BIOPOL".[82] The P(3-HV) homopolymers appear not to be microbiologically accessible; the highest 3-HV content found was about 90%, using valeric acid as C source.[57]

By using [D_3]acetate as substrate in H_2O or D_2O, P(3-HB) with varying degrees of deuteration can be synthesized.[83] Higher levels of deuteration can be achieved in *Rhodobacter sphaeroides*, which affords a maximum of 16% [D_6]3-HB with acetate in D_2O/H_2O (92:8).[84] Under the same conditions, [D_3]acetate leads to synthesis of 6% [D_6]3-HB, 73% [D_5]3-HB, and 16% [D_4]3-HB units.[85]

By use of suitably labeled C sources, ^{13}C- and ^{14}C-P(3-HB) can also be synthesized,[1b, 8] from which either crotonic acid or hydroxybutyrate derivatives of (*R*) configuration can be obtained.

Doi, Kunioka et al.[79, 80] discovered in 1988 that with certain substrates a copolymer of 3-HB and 4-hydroxybutyrate (4-HB) is synthesized (Table 1, no. 3). Depending on the composition of the C source, a 4-HB content between 9% and 40% can be achieved,[86] with the polymer constituting up to 30% of the dry cell mass. Both biodegradability[86] and material properties[87] are different from those of P(3-HB) or P(3-HB/3-HV).

The last two polymers in Table 1 have been relatively little studied, but help to demonstrate the variety of microbiologically accessible PHAs.

Witholt et al.[88] discovered in 1983 that *Pseudomonas oleovorans* also produces intracellular PHA granules, by using *n*-octane as substrate. However, they did not find P(3-HB), but exclusively poly-(*R*)-3-hydroxyoctanoic acid. Several groups subsequently investigated the potential of *P. oleovorans* and other pseudomonads to synthesize unusual PHAs.[40, 89, 90] *P. oleovorans* can metabolize straight-chain aliphatic hydrocarbons, their alcohols and carboxylic acids as substrates,[91] but cannot utilize glucose. In general, statistical copolymers of C_6 to C_{12} (*R*)-3-hydroxy acid units are formed. Units with a chain length smaller than C_6 or longer than C_{12} are only found in small quantities (Table 2).

Table 2. Composition of the PHAs from *Pseudomonas oleovorans* with carboxylic acids as carbon sources (from [89b]). The homologous series starting from 3-*hy*droxycaproate to 3-*hydroxydod*ecanoate is denoted by 3-HC–3-HDD.

Substrate	PHA [a] [%]	Repeating unit in the polyester [%] 3-HC	3-HH	3-HO	3-HN	3-HD	3-HUD	3-HDD
(*R*)-3-hydroxybutyrate	1.2	–	–	22	–	57	–	21
caproate	3.3	95	–	5	–	–	–	–
heptanoate	2.3	–	100	–	–	–	–	–
octanoate	8.7	8	–	91	–	1	–	–
nonanoate	9.1	–	35	–	65	–	–	–
decanoate	12.5	8	–	75	–	17	–	–
undecanoate	9.8	–	28	–	59	–	13	–
dodecanoate	6.6	6	–	57	–	32	–	5
pentadecanoate	5.3	–	32	–	47	8	13	–
heptadecanoate	–	–	–	–	–	–	–	–

[a] Given as percentage of the cell dry weight. The isolated PHA quantities are also only given in ref. [90b]. Evidently, the highest PHA quantites (0.7 $g L^{-1}$) are obtained with nonanoate as substrate.

With C_{2n+1} acids, polyesters with C_7, C_9, and C_{11} hydroxy acid units are produced, though the C_9 units predominate. C_{2n} acids result in analogous polymers built up of the even-numbered C_6, C_8, C_{10}, and C_{12} hydroxy acid units; the C_8 acid playing the major part. Polymers with relatively uniform side chain lengths are also formed from the hydrocarbons hexane, heptane, and octane.[40, 89a]

The longer the side chains of the hydroxy acids, the more elastic and the less crystalline are the polymers produced.[92a] On the other hand, the higher PHAs are no longer biodegradable, as has been shown for poly-(*R*)-3-hydroxyoctanoate or poly-(*R*)-3-hydroxynonanoate![1b, 5, 11]

Presumably, PHA biosynthesis developed from P(3-HB) metabolism in pseudomonads when various strains developed the ability to utilize substrates with more than four C atoms. The direct polymerization of such substrates is en-

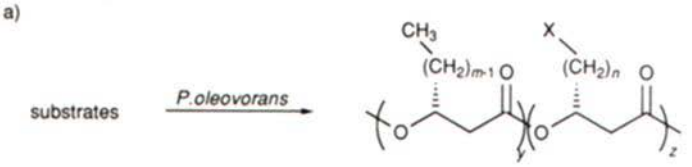
ergetically more sensible than first degrading them to the C_4 acids.[89a] Alkanes, however, can only be utilized by microorganisms possessing the so-called OCT-plasmid. This plasmid encodes the genetic information for those enzymes responsible for transforming alkanes to the corresponding alcohols.[93]

2.3.2. *Production of Polyesters with Further Functional Groups by Microorganisms*

With all the polyhydroxy acids described so far, subsequent modifications in the structure of the polymer are not possible; however, functionalized polymers are accessible by so-called polymer-analogous reactions. Certain bacteria can synthesize polyesters from functionalized substrates without loss of the functionality during construction, as does *A. eutrophus*. Here, too, the synthesis of homopolymers is the exception rather than the rule, since the biosynthesis of polyesters allows the incorporation of 3-hydroxy acid units with side chains shortened by C_2 or C_4 units. If, for instance, 3-hydroxy-7-octenoate is treated under analogous conditions to those given in Scheme 2b, a polymer is obtained which contains 23% 3-hydroxyhexanoic acid units.[94]

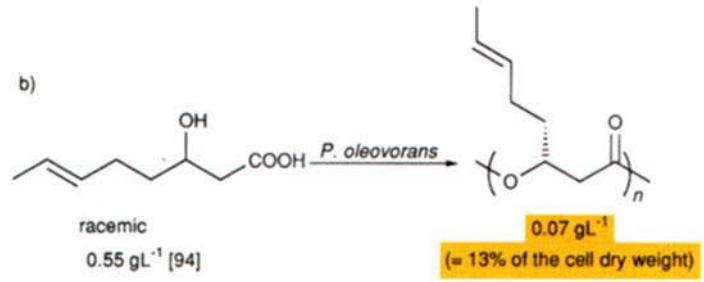
The first polyester with an additional functional group was isolated from *Nocardia* in 1964, though the enol ester structure of this polymer was only determined by IR spectroscopic studies.[98] In general, the functional groups may not lie too close to the main chain. Thus, for instance, no cell growth is observed with the substrates 6-bromohexanoic acid,[83] 3-phenylpropionic acid,[97] and 3-hydroxy-3-phenylpropionic acid.[97] The only substrate which can be converted to a homopolymer is 5-phenylvaleric acid (when 6-octenoic acid is used, the polymer shown in Scheme 2b contains only 80% of the given structural unit, whereas the remainder is composed of a mixture of other units.[94]). Surprisingly, a mixture of nonanoic and phenylvaleric acid yields the two corresponding homopolymers, and no copolymer![99] The yields are unsatisfactory in all cases.

a)

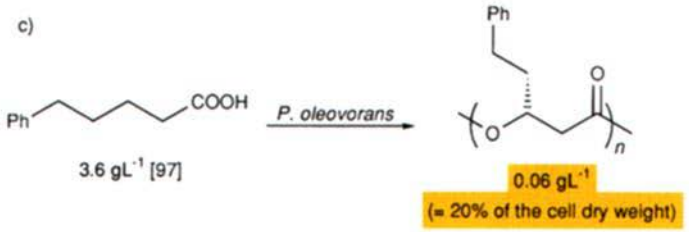


Substrates	X	m	n	y [%]	z [%]	Cell dry weight [gL^{-1}]	PHA content [gL^{-1}]
nonane (3 mLL^{-1})/1-fluoro-nonane (2 mLL^{-1})	F [95]	9	9	80	20	0.37	0.07
octane (33 gL^{-1})/1-chloro-octane (40 gL^{-1})	Cl [96]	8	8	30	60	2.6	0.13
nonanoic acid (1 equiv)/8-bromo-octanoic acid (1 equiv)	Br [83]	9	8	75	25	0.55	0.07

b)



c)



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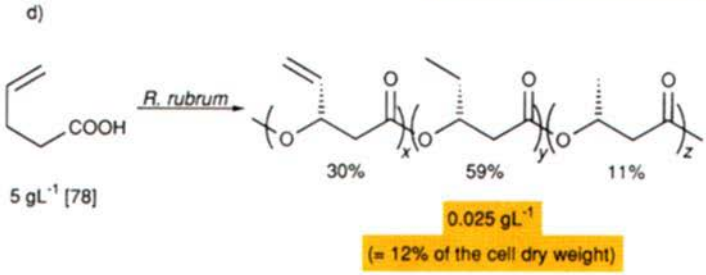

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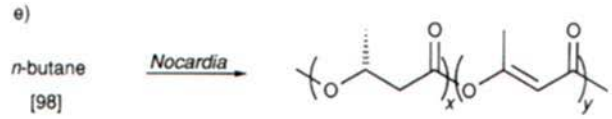


Scheme 2. Microbially accessible polyesters with functional groups in the side chain.

3. Polyhydroxy Acids as Starting Materials for Chiral Compounds of Low Molecular Weight

Ten years ago, this subject brought our research group into contact with P(3-HB) for the first time, since we saw P(3-HB) as a welcome source of C_4 synthetic units with (*R*) configuration. We began to search for suitable procedures for the preparative degradation of P(3-HB) to the monomers, and were soon interested in the directed synthesis of oligomers from the monomers thus isolated.

3.1. Degradation of Poly[(R)-3-hydroxybutyrate] and the Chemistry of the Monomer

Because of their stereoregularity, microbially synthesized polyesters are extremely attractive sources of chiral building blocks. The investigations carried out for this reason were all limited to the polymers P(3-HB) or P(3-HB/3-HV), since these two were the only two polyhydroxy esters available in large quantities at the time. Depending on the aims of the study, either the oligomer mixtures or the monomers could be of interest. Various mixtures of oligomers can be formed by partial hydrolysis or alcoholysis, for example, or by ester pyrolysis at temperatures $>175\,^{\circ}C$, where the average molecular weight is determined by the length of the heating period.[100–103] At the alcohol end, the pyrolysis products all carry a crotonyl group, as is to be expected for mechanistic reasons. Product mixtures of the same type have also been obtained by Brändli et al.[104] by treating P(3-HB) or P(3-

HB/3-HV) in the presence of an excess of lithium diisopropylamide/LiCl at low temperatures. Even after repeated treatment of the same sample under the same conditions, one sees only a slight shift in the distribution maxima (assumed to be bimodal) to lower molecular weights (Fig. 1 b). Similarly, the yields of isolated oligomers change very little (ca. 75% for each degradation step). The mechanism of the degradation is not yet satisfactorily understood, but probably does not go through the poly-enolates originally postulated,[104] since no alkylation products could be identified with a variety of electrophiles (Fig. 1 a).

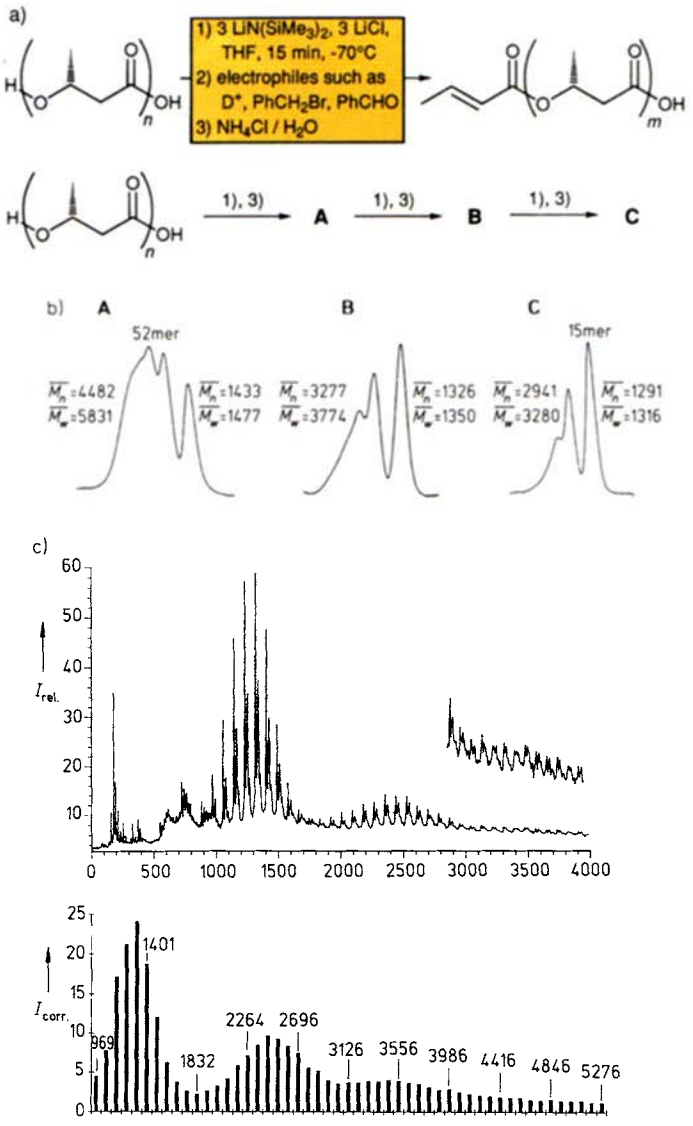
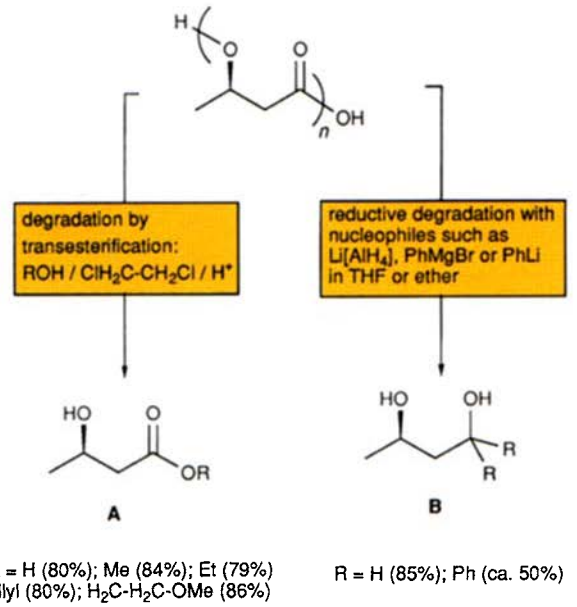


Fig. 1. Degradation of P(3-HB) ($M_n = 7.5 \times 10^5$) with $LiN(SiMe_3)_2$ in tetrahydrofuran at low temperature. Since the products arising from base treatment cannot be alkylated with electrophiles, polyenolates cannot be present as intermediates [104]. b) Gel permeation chromatograms of oligomer mixtures obtained by one (**A**), two (**B**), or three (**C**) repetitions of the reaction sequence shown in a). The two maxima are displaced only slightly towards lower molecular masses. The signal at high molecular weight corresponds to a 52mer for **A**, and to a 36mer for **C**, while the low molecular weight peak is a 17mer for **A** and a 15mer for **C**. The chromatograms shown were obtained by fractionation of a mixture on Shodex columns K-802, K-802.5, and K-803, by using chloroform as eluent. c) Top: matrix-supported laser desorption ionization spectrum (LDI) of the triple degradation product **C**. The insert is increased fivefold: the corresponding bar spectrum obtained when the [M + H] and [M + Na] peaks are counted together, and the area rather than the relative peak height is measured. Each m/z is given on the x axis. We thank Dr. K. Börnsen and Dr. M. Schär (Ciba-Geigy, Basel) for recording the LDI-spectra for us [105,106].

The work of Züger et al.[107] and Vanlautem et al.[108] has also made available the monomeric (*R*)-3-hydroxybutyric acid and its corresponding ester (**A** in Scheme 3), by acid-catalyzed saponification or transesterification of P(3-HB).[109] The reaction conditions were later optimized by Breitschuh et al.,[110] so that the monomers can now be obtained by a simple procedure in about 80% yield. Similarly, reduction of P(3-HB) with lithium aluminum hydride or its treatment with organometallic reagents leads directly to the 1,3-butanediols with (*R*) configuration of type **B** (Scheme 3).

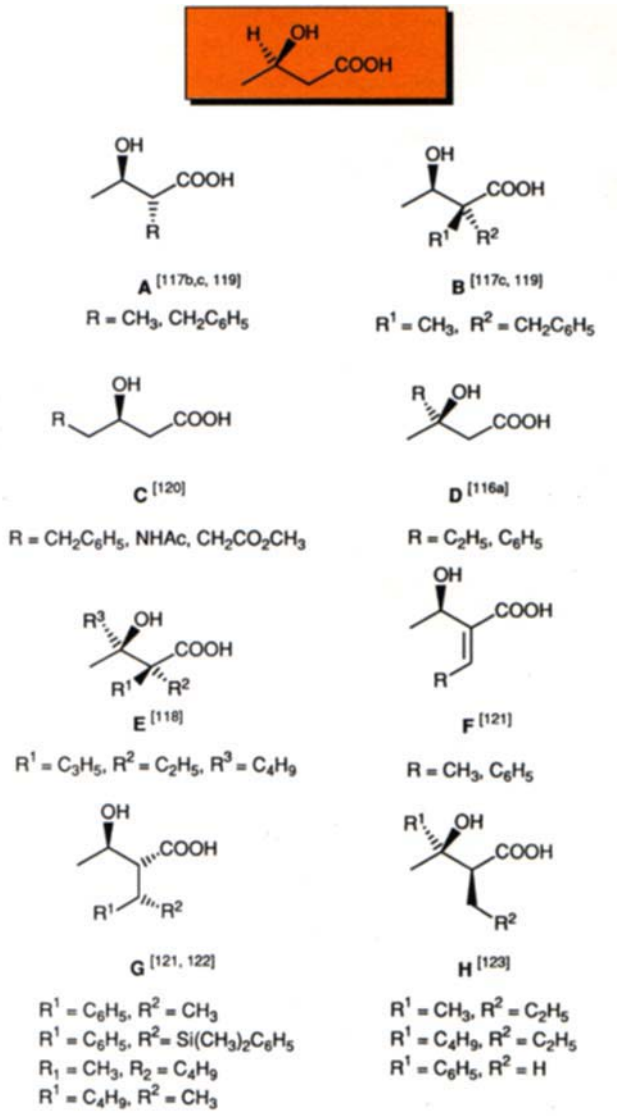


Scheme 3. Depolymerization of P(3-HB) by transesterification or by reduction of the ester carbonyl groups.

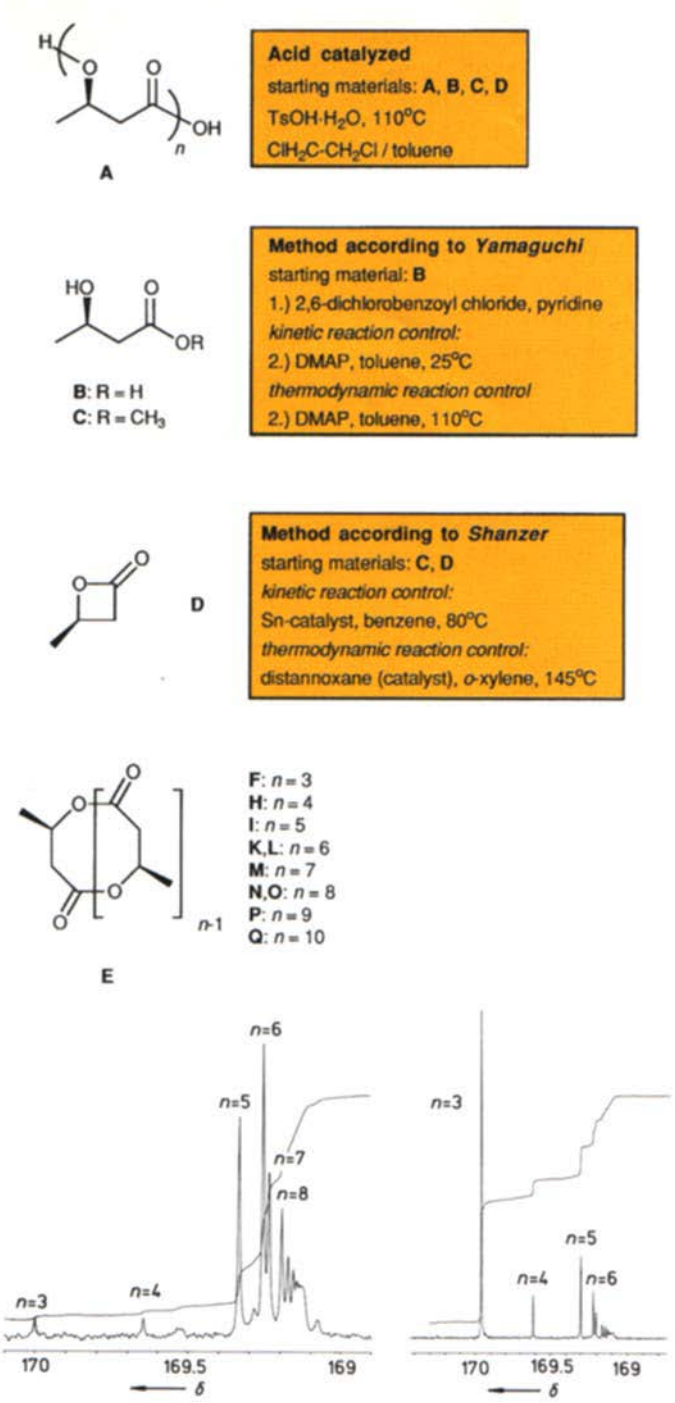
Derivatives as accessible as this can certainly be interpreted as an extension of the "pool of chiral building blocks".[111a] Natural product syntheses beginning from (*R*)-hydroxybutyric acid are being published continually,[111b] as shown by recent syntheses of gloeosporon,[112] grahamimycin A_1,[113] and (*R*)-lasiodiplodin.[114]

By now, any of the hydrogen atoms in (*R*)-3-hydroxybutyric acid can be replaced by other groups (product types **A**–**H** in Scheme 4).[115] The most notable developments in this area are the chiral acetoacetic ester derivative (*R*)-*tert*-butyl-6-methyl-2*H*,4*H*-1,3-dioxin-4-one[116] (this cycle yields compounds of type **D** and in principle also of type **C**, with regeneration of the stereogenic center[117]), and the diastereoselective synthesis of 2,5,5,6,6-pentaalkyl-substituted dioxanones[118] (see **E**; this corresponds formally to the product of an enantioselective aldol reaction of a α-branched carboxylic acid with a ketone).

The alcohol function of 3-hydroxybutyric acid can be substituted nucleophilically by other groups with inversion of configuration, either via the corresponding tosylate or via the β-butyrolactone[124, 125] (the former compound requires weakly basic nucleophiles such as $LiAlD_4$,[126] $Bu_4N^+NO_3^-$,[127] or NaN_3;[128a] the lactone can be opened with S-nucleophiles such as HS^- or $tBuS^-$,[129] for example, or with N-nucleophiles like benzylamine or NaN_3[129a]). Diastereoselective opening of dioxanones from HB by S_N2



Scheme 4. Products of the transformation of (*R*)-3-hydroxybutyric acid while retaining the acid and alcohol functions.



Scheme 5. Top: Synthesis of macrocycles of type E according to the methods of Yamaguchi, (via the mixed anhydride with 2,6-dichlorobenzoyl chloride) Shanzer, (by using the tin catalyst 1,1,6,6-tetrabutyl-1,6-distanna-2,5,7,10-tetraoxacyclodecane [136] (= template)), or by acid catalysis with toluenesulfonic acid monohydrate ($TsOH \cdot H_2O$). DMAP: *N*,*N*-dimethylaminopyridine. Bottom: ^{13}C NMR spectra of the carbonyl region of oligolide mixtures obtained under the conditions specified in a) by Yamaguchi lactonization (left) of (*R*)-3-hydroxybutyric acid (kinetic control) and acid catalyzed (right) by degradation of P(3-HB) (thermodynamic control). For completeness, it should be mentioned that the β-butyrolactone **D** of (*R*) configuration was never used as starting material, but rather its enantiomer, which is also formed by known methods from (*R*)-3-hydroxybutyric acid [124]. The (*R*) form was preferred here only for graphic reasons. Measurement conditions for the ^{13}C NMR spectra (100 MHz): Inverse-gated experiment with a relaxation delay of 3 s, an acquisition time of 3.2 s, and a pulse angle of 45°.

reaction at the acetal C atom is a greatly improved variant of the Johnson method with regard to selectivity and ease of execution in greater quantities as well as in the presence of groups sensitive to oxidation, in which hydroxybutyric acid is converted into crotonic acid, and thus sacrificed (*immolative* enantioselective reaction sequence).[117b, c, 128]

3.2. Synthesis of Oligolides from 3-Hydroxybutyric Acid, and Structural Studies

Chiral macrolides of type **E** (Scheme 5) can be obtained directly from P(3-HB) **A** either by acid-catalyzed transesterification or from the monomeric building blocks **B**–**D**, by the methods of Yamaguchi,[130–133] Shanzer.[132–134] or again by acid catalysis.[135] Depending on the reaction conditions, the oligolides are obtained in various proportions, and can be separated from each other by careful chromatography.[133] Under thermodynamic control, triolide **F** (in Scheme 5) predominates; thus, acid-catalyzed transesterification of P(3-HB) affords this really quite complex compound in about 50% yield by simple distillation of the product mixture.[135]

Single crystals of the oligolides **F**–**O** in Scheme 5 were grown, and their structures were determined. For both the

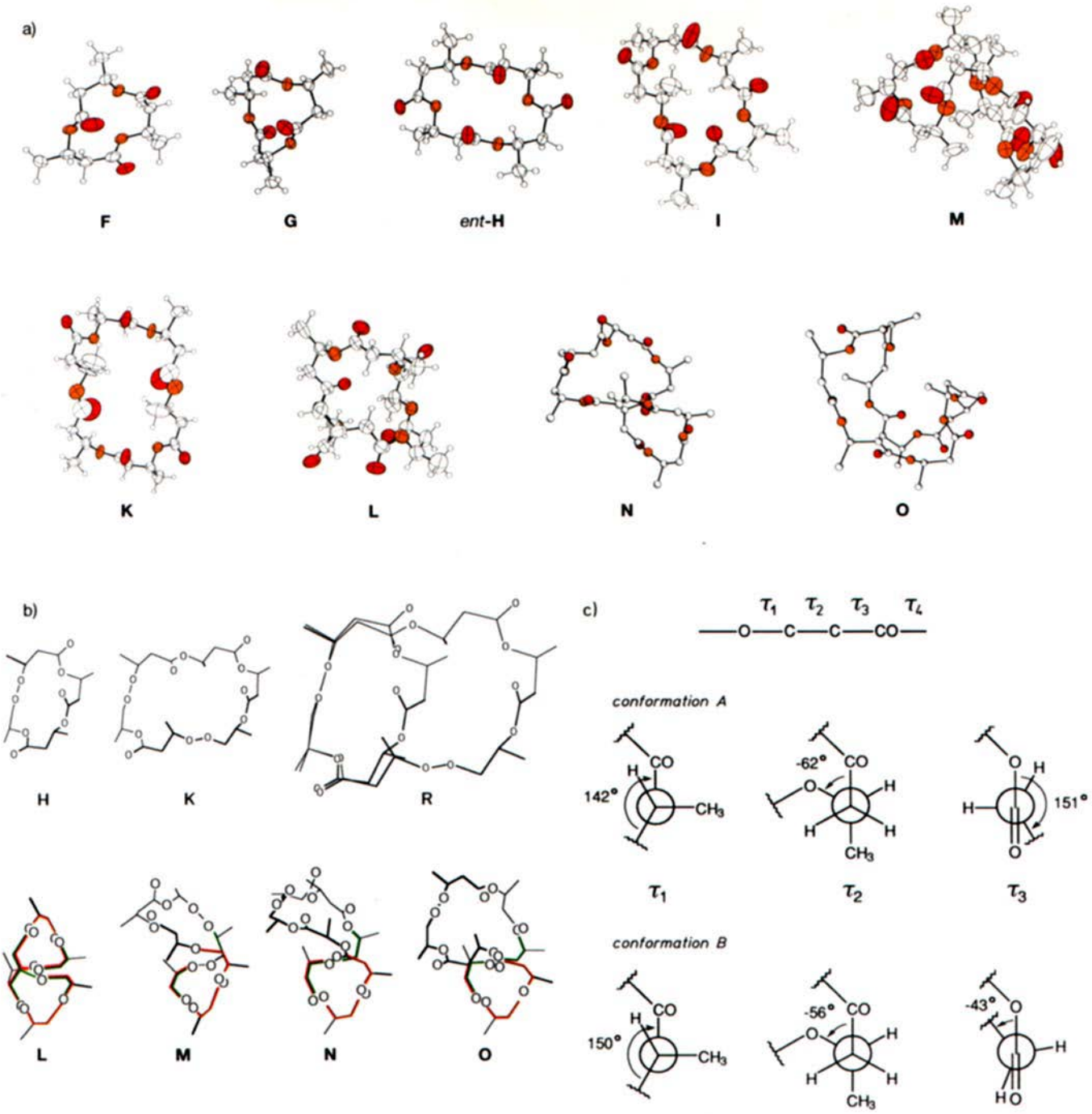


Fig. 2. a) Structures of the oligolides **F–O**, all in the all-*R*-form with the exception of *ent*-**H**, which is depicted in the all-*S*-configuration (**F**–**M** as ORTEP diagrams, and **N**,**O** as PLUTO plots). The *rac-epi* triolide **G** (shown here in the (*R*,*R*,*S*) form) is obtained, for example, by acid-catalyzed lactonization of *rac*-β-butyrolactone in boiling toluene/1,2-dichloroethane together with the racemic triolide *rac*-**F** in a 3:1 ratio. Red spheres are oxygen atoms of the CO functional groups, orange are OR oxygens on the ester groups. For synthesis and characterization of the oligolides see [131–135]. b) Structural similarities between the macrolides. MacMoMo views of the all-(*R*)-tetrolide **H** and of the C_2 symmetrical hexolide **K** and its superposition **R**. The higher macrolides **L–O** all contain as common structural fragments a Δ-shaped portion (red) and an S-shaped moiety (green). In the hexolide L they even appear twice [133]. c) Mean torsion angles τ_1–τ_3 and Newman projections of the bonds of the (*R*)-3-hydroxybutyric acid units in conformations **A** (the Δ-shaped building block) and conformation **B** (the S-shaped fragment) of the higher macrolides **L–O** [133,135b]. The torsion angles are given according to the IUPAC-IUB definition [138]; a zero-value represents a synperiplanar arrangement for the main chain.

hexolide ($n = 6$)[132] and the octolide ($n = 8$), two conformers were found (**K**, **L**, and **N**, **O**, respectively, see Fig. 2a). The structures show remarkable similarities (Fig. 2b). Comparison of the two C_2 symmetric conformations of the tetrolide **H** and the hexolide **K** show that the latter can be constructed from **H** by halving and introducing two hydroxybutyric acid units. The larger macrolide structures **L–O** are all constructed of folded rings and contain an S-shaped (Fig. 2b, green), and a Δ-shaped region (Fig. 2b, red) as common structural elements. A study of the interdependence of the torsion angles τ_1, τ_2, and τ_3 reveals two preferred configurations of the (*R*)-hydroxybutyric acid units in these cycles (Fig. 2c). Conformation **A** (which forms the Δ-shaped structural element) occurs about three times more frequently than **B** (which forms part of the S-shaped fragment).[137]

The potential of polyesters as precursors of chiral building blocks has certainly not yet been exhausted, as can be seen from the continuing publication of syntheses of further compounds from them.[139]

4. Polyhydroxy Acids as Biologically Degradable Plastics

Plastics make up about seven percent by weight and over thirty percent by volume of the total garbage produced today in the USA and Japan.[140] This can be explained in part by the fact that plastics are more and more replacing glass, paper, or metal as packaging materials. In principle, there are three possibilities for the disposal of plastics: incineration, recycling, and degradation in the environment. Each of these will presumably find future applications.

Several countries, such as Italy for example, have introduced legislation which requires all plastic packaging materials to be degradable by 1991.[141] The growth rate in the market for degradable polymers is expected to increase by 75% per year, representing a turnover in 1992 of about 400000 tons of polymer and approximately US$ 340 million. By then, some 15% of plastic refuse will be biodegradable, compared with 1% in 1987.[141, 142]

In principle, a macromolecule can be degraded by chemical, biological, or physical methods.[143] Cleavage by light is the most important degradative pathway for C–C main chain polymers, and is accelerated by the introduction of carbonyl groups[144] (which can be brought about by copolymerization of an olefin with carbon monoxide, for example[145]), or by photoactive additives such as benzophenone[146] (degradation by Norrish type I and II cleavage). Oxidative chain fission can be achieved by addition of hydrogen peroxide or suitable transition metals, and is important particularly with unsaturated polymers.[147]

The production of polymer mixtures (blends) of largely undegradable and biologically degradable materials (usually starch) is also widespread. In this way, the advantages of petrochemically derived plastics can be combined with those of biopolymers. During degradation, the biologically utilizable part of the mixture is dissolved, and the remainder must then be dealt with by nonbiological systems.

4.1. Requirements for Biodegradable Polymers

In contrast to chemical processes, microbial degradation processes are to a certain extent autocatalytic, since the substrate being degraded leads to an increase in the organism population. The bacteria concerned profit from the degradation; that is, the fragments produced must also be able to be utilized. Many monomers of synthetic polyesters and polyamides are in principle microbially degradable, but since the corresponding microorganisms do not have suitable depolymerases available, degradation is impossible. The same is true if the polymerase is present, but the resulting fragments cannot be broken down or metabolized. The most important type of cleavage is the hydrolysis of ester, amide, and glycosidic bonds. The corresponding hydrolases require only water as a reaction partner, and are therefore more common in the extracellular environment, since no complex cosubstrates are needed. Polyesters with easily degradable monomers such as glycolate, lactate, malate, caprolactone, and 3-hydroxy acid derivatives therefore have a good chance of being biodegraded.[149] Pure C-chain polymers are less suitable, though their degradation has been demonstrated.[150]

4.2. Extracellular Polyhydroxy Acid Degradation

A remarkable property of many microbially synthesized PHAs is their biodegradability in the environment, which proceeds more rapidly under anaerobic conditions than aerobically. For *A. eutrophus*, Doi et al.[5, 76] found that under anaerobic conditions the main intracellular metabolites are acetate and (*R*)-3-hydroxybutyrate. The conversion of acetyl-CoA to acetate is presumably coupled to the phosphorylation of ADP to ATP in this case. Under aerobic conditions, acetyl-CoA is broken down to CO_2 and H_2O by the citric acid cycle, releasing 12 ATP equivalents and allowing a far more economic use of the polyester.

Many bacteria and fungi possess extracellular depolymerases,[151] with which they can degrade PHAs and if necessary utilize them as a sole C source. Enzymes of this nature have been found in *Alcaligenes faecalis*,[152–155] *Pseudomonas lemoignei*,[156–160] *Pseudomonas delafieldii*,[161] *Penicillium simplicissimum*,[162] and *Eupenicillium sp.*,[162] though the enzymes of the first two species have been by far the best studied.

A. faecalis has two extracellular esterases, a P(3-HB) depolymerase and an oligomer hydrolase. The depolymerase degrades both P(3-HB) and water soluble oligomers. However, it displays significantly higher activity towards the longer oligomers, and attacks in an endo fashion at the second ester bond from the hydroxy terminus.[152] An octolide (i.e. a ring constructed of eight (*R*)-3-hydroxybutyrate units) is attacked in a similar fashion, but the open-chain trimer protected on the alcohol function with *tert*-butyldimethylsilyl groups is not degraded.[154]

The oligomer hydrolase attacks only water-soluble oligomers, but with approximately ten times higher activity than the depolymerase. Longer polymer chains are more rapidly degraded than the dimers. Of the four possible stereoisomers of 3-hydroxybutyrate dimers, the (*R*,*R*) form and the (*R*,*S*) form are hydrolyzed, the latter rather more slowly.[73] The monomeric methyl ester is also attacked, while the corresponding ethyl and benzyl esters are practically inert. Degradation occurs in an exo manner from the acid end.[153]

The gram-negative organism *P. lemoignei* also possesses two enzymes which are involved in extracellular degradation, but differs from *A. faecalis* in that only the depolymerase is exported from the cell. P(3-HB) is broken down outside the cell to a trimer/dimer/monomer mixture which is then transported through the cell wall and is only fully saponified to the monomer by a dimer hydrolase within the cell. Here too, the depolymerase attacks in an endo fashion at the second or third ester bond from the hydroxy terminus, though in this case both oligomers and P(3-HB) can be hydrolyzed.[160] In contrast to *A. faecalis*, the dimer hydrolase degrades the (*S*,*R*) dimer as well as the (*R*,*R*) form, while

their enantiomers are not saponified. (*R*)-3-(butanoyloxy)butyric acid is also attacked.[157]

Despite numerous investigations, many questions remain unanswered. It is not clear, for instance, how the depolymerase can attack both the water soluble oligomers and the hydrophobic P(3-HB), which in contrast to the intracellular case (see Section 2.2.3) is present here in a crystalline form! From a chemical point of view, it would also be interesting to investigate the influence of various end groups on the degradability; synthetically produced (all-*S*)-P(3-HB) is no longer biodegradable, and neither is a stereoblock-P(3-HB) built up of (*R*) and (*S*) residues.[111]

5. Synthesis of Poly[(*R*)-3-hydroxybutyrate] and Derivatives

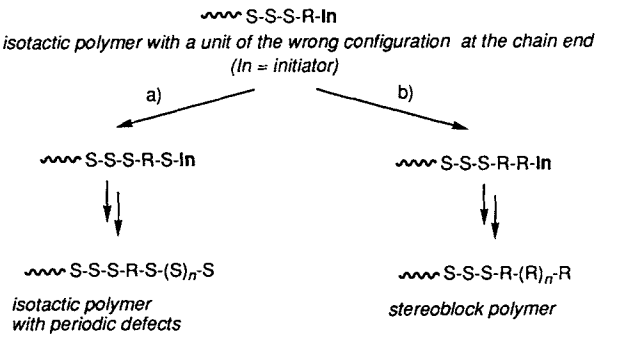
P(3-HB) was produced by synthetic means far earlier than by biotechnological methods. Processes of this sort are becoming ever more attractive, due to the facile methods now available for the synthesis of (*R*)- and (*S*)-hydroxy acids. In principle, two types of monomers are available for the polymerization, the 3-hydroxy acids and the corresponding β-butyrolactones. Because of their tendency to crotonize, the acids have never been used, however, since polyesters of high average molecular weight can only be obtained by polycondensation of very pure starting materials.[163] All the studies published to date have therefore started from β-butyrolactones, and have polymerized these with simultaneous ring-opening. β-Propiolactone can be polymerized easily with either anionic or cationic initiators, and the α-monosubstituted and α,α-disubstituted β-lactones react cleanly. Substituents in the β-position reduce the reactivity dramatically, so that conversion is often incomplete even after extended reaction periods.[164]

In all the studies published up till now, P(3-HB) was synthesized by one of the following procedures: a) from racemic β-butyrolactone and an achiral initiator, b) from racemic β-butyrolactone and a chiral initiator, or c) from enantiomerically pure β-butyrolactone and an achiral initiator.

5.1. Polymerization of Racemic β-Butyrolactone with an Achiral Initiator

Biologically degradable (all-*R*)-polymers cannot, of course, be obtained in this manner. However, suitable reaction conditions were thus developed with the readily available racemic β-butyrolactone,[165] and some of these could also be used for polymerization of enantiomerically pure lactone.

The step determining stereoselectivity in the ring-opening polymerization is the insertion of a monomer into the end position of the polymer-initiator complex.[166] In principle, this can lead to isotactic (*-RRR-/-SSS-*), syndiotactic (*-SRSRSR-*), or atactic (*-SSRSRRS-*) polyesters. β-Butyrolactone, however, yielded only isotactic and atactic P(3-HB). The result can be either a stereoblock polymer or an isotactic chain with periodic defects, depending on whether the polymer chain end or the initiator determines selectivity; atactic polymers are obtained when neither is dominant (Scheme 6).



Scheme 6. Stereoselectivity of monomer insertion into the terminal initiator-polymer bond. *R,S*: (*R*) configuration or (*S*) configuration unit in the polymer. In case a) the initiator is responsible for the selectivity, in b) the terminal monomer unit.

The tacticity was determined in earlier times by fiber X-ray diffraction,[167, 168] but today is much simpler to measure from the ^{13}C and ^{1}H NMR spectra. Isotactic and stereoblock-P(3-HB) cannot yet be distinguished either by X-ray diffraction analysis or by high-field NMR spectroscopy.[169] For this reason, whenever isotactic polyesters are mentioned in the following sections, stereoblock polymers may also be present.

Atactic and isotactic fractions may be separated by fractional crystallization from acetone or methanol, where the atactic polymer remains in solution. Initiators which have been applied with more or less success are summarized in Table 3. Classical Brønsted or Lewis acids such as CF_3SO_3H,[170] $BF_3 \cdot OEt_2$,[171, 172] $Et_3O^+BF_4^-$,[164] Et_2AlCl,[171] or nucleophiles like *n*BuLi,[171] pyridine,[173] NEt_3,[171, 173] PPh_3,[173] NaOMe,[170] and tetraethylammonium benzoate[170] afford oily mixtures of oligomers, at best.

In 1971, Agostini et al.[174] described in detail for the first time a synthesis of P(3-HB) from β-butyrolactone and $AlEt_3 \cdot H_2O$; Inoue et al. had briefly mentioned such a reaction in 1961, as part of a communication on polymerization of β-butyrolactone with $ZnEt_2/O_2$.[172] Tani et al.[170, 171] later improved the reaction conditions for the Al-initiated ring-opening polymerization, which was subsequently adopted by several other research groups. The long reaction times and the generally unsatisfactory conversions (Table 3) are conspicuous; the yield of isotactic P(3-HB) reaches 50% at best. The molecular weights achieved are comparable with those for microbiologically produced P(3-HB), depending on the catalyst used, but the molecular weight distribution is much broader. Exceptions are the two last systems (Table 3, nos. 8 and 9), in which relatively narrow molecular weight distributions are obtained. In no. 9, the actual initiator is potassium crotonate which, like potassium acetate or potassium benzoate, can be applied in the presence of [18]crown-6[177] or dibenzo[18]crown-6[173] instead of potassium naphthalide as a starter.

5.2. Polymerization of Racemic β-Butyrolactone with a Chiral Initiator

Polymerization of a racemic monomer with a suitable chiral initiator ought in the ideal case to yield only one enantiomer of the product, while the other enantiomer remains in

Table 3. Polymerization of *rac*-β-butyrolactone with achiral iniators.

No.	Initiator	Reaction conditions T [°C]	t [d]	solvent	Yield polymer [%]	of the isotactic fraction [%]	$M_n \times 10^{-3}$ (of the isotactic fraction)	M_w/M_n [d]	Ref.
1	$AlEt_3:H_2O = 1:1$ [a]	53	4	without	52	–	–	–	[174]
2	$AlEt_3:H_2O$:epichlorohydrin = 1:0.8:1 [a]	60	10	toluene	52	37	–	–	[171]
3	$AlEt_3:H_2O = 1:1$ [a]	60	7	toluene	–	28	21.4 [d]	5.6	[175]
4	EAO [b]	60	7	toluene	78	52	400 [d]	–	[170]
5	EAO [b]	60	14	toluene	51	20	110 [d]	8.0	[169]
6	$Zn(OEt)_2 \cdot Al(OiPr)_3$	40	14	toluene	45	–	–	–	[164]
7	Bu_3SnOMe	50	2	without	–	–	ca. 6 [e]	–	[173]
8	TPPAlCl [c]	25	33	CH_2Cl_2	60	–	4.4 [f]	1.05	[176]
9	potassium naphthalide + cryptand [2.2.2]	25	8	THF	95	0	11 [f]	1.29	[177]

[a] The initiator is synthesized from $AlEt_3$ and H_2O before the addition of the monomer in toluene. [b] The *ethyl*aluminum *o*xide (EAO) initiator was synthesized as in [a], then the solvent was removed, and the residue dried at 180 °C/10^{-3} Torr, and this was then redissolved in toluene. [c] Initiator from 5,10,15,20-tetraphenylporphin and Et_2AlCl. [d] Determined with gel permeation chromatography by comparison with a polystyrene standard. [e] Estimated from the ^1H NMR spectrum. [f] Determined with vapor pressure osmometry. The monomer/initiator ratio for no. 8 is 100 and for no. 9 150.

the reaction mixture (the yield of polymer can therefore only be 50%, at most). LeBorgne and Spassky et al. have polymerized oxiranes and thiiranes with considerable success, by using as initiator a complex of (*R*)-3,3-dimethyl-1,2-butanediol and $ZnEt_2$.[178] However, with β-butyrolactone the reaction is far less selective (Table 4).

Table 4. Polymerization of *rac*-β-butyrolactone with the chiral initiator *A* (complex from (*R*)-3,3-dimethyl-1,2-butanediol and $ZnEt_2$) and *B* (*N*,*N*′-disalicylidene-(1*R*,2*R*)-1,2-cyclohexanediaminatocobalt(II)-$AlEt_3$ complex).

Catalyst	Reaction conditions T [°C]	t [d]	solvent	Conversion [%]	Isotactic fraction [%]	$[\alpha]_D$ of the polymer [a]	$M_n \times 10^{-3}$	M_w/M_n
A [179]	25	0.25	toluene	84	27	−0.6 ($CHCl_3$) [b]	ca. 20	–
B [180]	30	14	benzene	41	–	−46.1 (benzene) [c]	–	9.4

[a] Optical rotation of microbial all-(*R*)-P(3-HB): $[\alpha]_D = -1.8$ ($CHCl_3$). [b] $[\alpha]_D$ of the unreacted monomer: −12.8 ($CHCl_3$); (*S*)-β-butyrolactone: $[\alpha]_D = -34.8$ ($CHCl_3$). [c] $[\alpha]_D$ of the unconverted monomer: −1.65 (benzene); (*S*)-β-butyrolactone: $[\alpha]_D = -50.8$ (benzene).

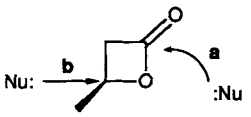
The selectivities achieved by this strategy are definitely not yet satisfactory. In the second case, the compound obtained is presumably a strongly atactic polyester or an isotactic P(3-HB) of low molecular mass, since (all-*R*)-P(3-HB) oligomers of size >1000 g mol^{-1} are almost insoluble in benzene.

5.3. Polymerization of Enantiomerically Pure β-Butyrolactone with an Achiral Initiator

Enantiomerically pure β-butyrolactone can be synthesized either by cyclization of 3-bromobutyric acid[124d] or 3-mesyloxybutyric acid,[124e] or by pyrolysis of the cyclic orthoester of 3-hydroxybutyric acid and triethyl orthoacetate,[124a,b] though with a maximal yield of 35%. Four-membered ring lactones can be opened by nucleophiles either with retention (path **a** in Table 5) or with inversion (path **b** in Table 5)[181] of configuration, and hence P(3-HB) constructed by this procedure does not necessarily consist entirely of units with identical configuration (i.e. 100% isotactic) (Table 5).

Once again, it was Agostini et al.[124d] who were the first to polymerize (*R*)-β-butyrolactone with $AlEt_3 \cdot H_2O$ to give P(3-HB) of preferentially (*R*) configuration, opening the lactone with retention of configuration. Lenz et al.[182] later found inversion, exactly the opposite reaction mechanism, in the same system (no. 2 in Table 5). They explained the change in mechanism with a different method of producing the aluminum complex.[183] In all cases, no stereoregular P(3-HB) was obtained, probably because of the polymeric nature of the initiators used, which can contain various active centers (see [183], but also [178a]). On the basis of the optical rotation

Table 5. Polymerization of chiral β-butyrolactone with achiral initiators.



No.	Initiator	Lactone configuration	Polymer	$[\alpha]\lambda_i$ in $CHCl_3$ [a]	$M_n \times 10^{-3}$	M_w/M_n	Ref.
1	$AlEt_3 \cdot H_2O$ (1:1)	(*R*)	(*R*)-P(3-HB)	+4.0 [b]	–	–	[124d]
2	$AlEt_3 \cdot H_2O$ (1:1)	(*S*)	(*R*)-P(3-HB)	+6.9	30	8.0	[182]
3	$ZnEt_2 \cdot H_2O$	(*S*)	(*S*)-P(3-HB)	−7.0	14	1.5	[182]
4	EAO [c]	(*S*)	(*S*)-P(3-HB)	−5.8	24	7.8	[182]

[a] For λ_i all-(*R*)-P(3-HB) has an optical rotation of +7.4 (the wavelength (λ = 589 nm) given in ref.[182] (no. 2–4) is probably incorrect). [b] $[\alpha]_{365}$ of all-(*R*)-P(3-HB): +10.2 ($CHCl_3$). (*R*)-β-butyrolactone with an optical purity of 73% was used as monomer. [c] EAO see footnote [b] in Table 3.

of the polymers obtained and the reproducibility of the experiments, the Zn system seems to be the best one. Recently Doi et al. used this system to show that in the polymerization of enantiomeric β-butyrolactone mixtures, the (*R*) and (*S*) units are statistically distributed in the polyester.[184]

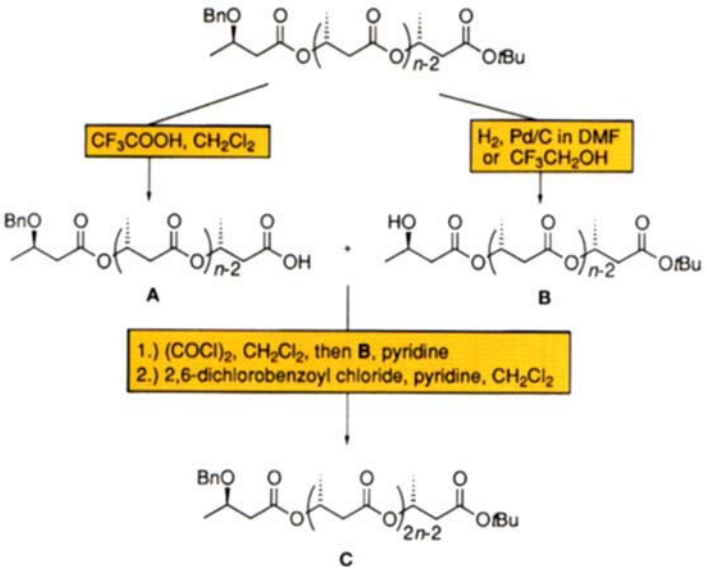
With the ethylaluminum oxide (EAO) initiator, the attainable molecular weights decrease as the size of the residue on the β-butyrolactone increases. In this way, Tani et al. were able to polymerize γ-mono, di-, and trichloro-substituted β-lactones in 1977,[170] which by now are also accessible in the enantiomerically pure form by the Wynberg method.[185] A further functionalized polymer was synthesized by Lenz et al. from racemic or from (*R*)-β-alkoxycarbonyl-β-propiolactone. The resulting malic acid polyesters are biodegradable.[186]

5.4. Synthesis of Defined Oligomers and Polymers from (*R*)-3-Hydroxybutyric Acid

Oligomers of uniform molecular mass have also been produced by directed synthesis. Such compounds were of interest in the past for a variety of reasons; they were used to obtain more detailed information about the intra- and extracellular degradation mechanism for P(3-HB),[152, 154, 187] or for synthesis of the corresponding straight-chain oligolide precursors.[131] In all previous syntheses, chain extension proceeded by stepwise addition of monomer units. Our aim was to study the structure and function of the presumed P(3-HB)-containing ion channel, already mentioned above, which required polymers with a length of 100–150 monomer units. For the synthesis of such large molecules, a stepwise technique is, of course, no longer practical. Polymerization of enantiomerically pure β-butyrolactone would probably lead to P(3-HB) samples with a relatively narrow size distribution (Table 3, nos 8 and 9), but from the results described above, these would not be likely to possess uniform configuration. A possible alternative was the segment condensation strategy, in which the two protecting groups on an unasymmetrically protected oligomer are selectively removed. The deprotected portions are then coupled to afford a molecule of "twice the size". The tetramers used as starting material in Scheme 7 were synthesized by one of the procedures described earlier,[131] though with some modifications. However, the coupling step via the acid chloride, and the benzyl ether and *tert*-butylester protecting groups were retained.

Characterization of the purified products becomes more and more difficult with increasing chain length, since because of their structural similarity, they display more or less identical analytical properties. Luckily, great progress has been made recently in the area of mass spectrometry, with the so-called soft ionisation methods.[189] In the case of P(3-HB), however, not all of these methods were equally useful, and larger molecules could finally only be characterized by means of matrix-supported laser desorption ionization (LDI) mass spectrometry (Fig. 3a,b).

For the fully protected 32mer **M**, the mass spectra each show three signals (as isotope distributions) with approximately the same *m/z* values and relative intensities (Fig. 3a). Such peaks were not observed with the smaller oligomers. They do not come about by fragmentation, but represent the



n	*n*mer acid **A**/ *n*mer alcohol **B**	2*n*	2*n*mer **C**	Yield [%] after the acid chloride coupling
4	**D/E**	8	**F**	>95
8	**G/H**	16	**I**	90
16	**K/L**	32	**M**	60
32	**N/O**	64	**P**	45
64/32	**Q/R**	96	**S** (96mer)	45

Scheme 7. Segment condensation algorithm for the synthesis of defined oligomers of (*R*)-3-hydroxybutyric acid. An *n*mer acid **A** is coupled to an *n*mer alcohol **B**, giving rise to a 2*n*mer product **C** of "twice the size". The yield after the acid chloride coupling step decreases with increasing size of the compounds. To improve the total yield for the larger molecules (**M**, **P**, and **S**), we "recoupled" with 2,6-dichlorobenzoyl chloride. This method allows activation of an acid in the presence of an alcohol [188].

molecular composition of the sample. A more detailed study of the reaction sequence showed that under the deprotection conditions specified in Scheme 7, terminal subunits may be cleaved off. For this reason, the average degree of polymerization (X_n) of the polymer mixture obtained was only 93, though this was distributed over a narrower range than would have been the case under ideal polymerization conditions[190] (Fig. 3b, see also reference to our own work in [106] and ref.[135b]).

These synthetic P(3-HB) oligomers represent a series of compounds, which can be used as standards to determine the molecular masses of polymers with similar constitution and configuration, by using gel permeation chromatography. For us, this was of special interest with respect to the ion channel already mentioned. Comparing molecular weights with those obtained after standardization with polystyrene or polyisoprene showed that the molecular weight values derived by using the latter standards can be appreciably higher (Fig. 3c).

5.5. Possible Future Applications of Polyhydroxyalkanoates

Up till now, the polyesters P(3-HB) and P(3-HB/3-HV) are the only PHAs that have been produced on a large scale

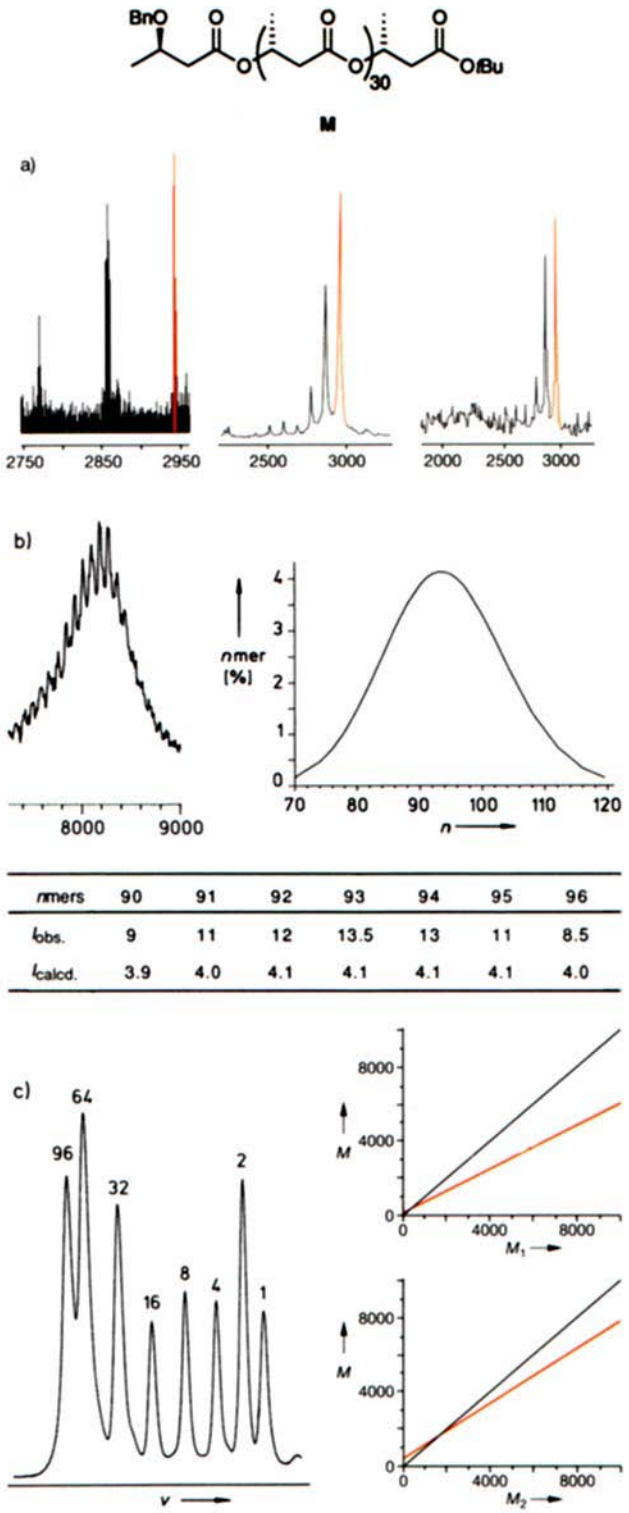


Fig. 3. a) Secondary ionization MS (in a 3-nitrobenzyl alcohol matrix, left), Plasma desorption ionization (PDI)-MS (center), and laser desorption ionization (LDI)-MS (in a 2,6-dihydroxy benzoic acid matrix, right) of the fully-protected 32mer (**M** in Scheme 7). We thank Prof. Dr. M. Przbylski and K. Schneider, University of Constance, for recording the PDI spectrum, and Dr K. Börnsen and Dr. M. Schär (Ciba-Geigy, Basel) for the LDI-spectra. b) Left: LDI mass spectrum of the fully-protected "96mer" (**S** in Scheme 7). The commonest compound in the distribution (right) is no longer the 96mer but the 93mer. In the distribution diagram the x axis is the degree of polymerization n; and the y axis is the share of $n$mer in %. Table: Comparison of the measured distribution of the "96mer" with that calculated for ideal polymerization conditions with a polymer length of 93 ($M_w/M_n = 1.01$) [163]. c) Gel permeation chromatogram of the fully protected P(3-HB) compounds (left). v = current. Comparison with the molecular masses determined after standardization using polystyrene or polyisoprene (right). M = real molecular mass; M_1 = apparent molecular mass with polystyrene; M_2 = apparent molecular mass with polyisoprene.

by using biotechnology. From 2.5 kg glucose, about 1 kg P(3-HB) can be synthesized in this way.[191a] In the middle of the nineties, the annual production of BIOPOL will be increased from about 300 tons to 5000–10000 tons.[191b] The production costs at present constitute about 50 DM per kilogram; the future aim is a kilo-price of 5–10 DM.[141]

By employing phototrophic microorganisms, it is even possible to utilize sunlight as energy source. Thus, *Rhodobacter sphaeroides* synthesizes up to 70% of its dry weight as P(3-HB).[192] Microbiological production of PHAs with a great variety of compositions and material properties is therefore possible, even if at present the yields are unsatisfactory, and the starting materials are oil-derived. How far these disadvantages can be countered by the use of gene-technologically modified organisms remains to be determined.

Synthetic methods are at present not well enough developed to compete with biotechnological production, at least of BIOPOL. On the other hand, a broad range of (*R*)- and (*S*)-hydroxy acids is now readily available, by enantioselective hydrogenation of the corresponding 3-keto esters.[193] We can therefore expect that in future synthetic processes will be developed which will be a match for microbial methods. With such a procedure, a variety of polymers would probably be accessible without great changes of the reaction conditions. In addition, workup of these reactions would be likely to prove far more simple than for biotechnological production methods, since it would be possible to work at high concentrations and in organic solvents.

With respect to molecular weight, melting region, crystallinity, flexibility, and tensile strength, BIOPOL and polypropylene are quite similar, though the former has disadvantages in its low tensile strength, poor solvent resistance,[194] limited resistance to acids, bases, and heat, and last but not least, its high price. It can be moulded thermoplastically, and processed to sheets and fibers,[195] but till now has been used primarily for the production of shampoo bottles (Fig. 4). Other applications for such polyesters can be found in agriculture (e.g. as cold frame sheeting, seed capsules, or formulations for the controlled release of pesticides or herbicides[1b]), or in human and veterinary medicine, because of their biocompatibility. Thus, numerous patents

Fig. 4. Shampoo bottles made of BIOPOL, after 0, 3, and 9 months in compost. The slower degradation of the printed areas is clearly visible. Doi et al. [92b] have shown that degradation occurs from the surface, and proceeds more quickly the larger the surface area. Nonenzymatic hydrolysis is several orders of magnitude slower than enzymatic depolymerization.

have already been registered, in which such materials are used for surgical implants to support organ and tissue healing processes,[196] as supports for "slow-release" systems,[10, 197] for the production of powders,[198] or as a component of membranes.[199] A process for elimination of nitrate from drinking water using P(3-HB) has also been developed.[200]

Recently attempts have been made to extend the field of application of P(3-HB) and P(3-HB/3-HV) by preparing degradable block copolymers with polystyrene and polyethers,[201] or "blends" with polyethylene, polystyrene, polyvinyl chloride, or polyethylene oxide.[202] Films of P(3-HB) act as a gas barrier in a similar way to polyvinyl chloride or polyethylene terephthalate.[202a] Because of these properties, P(3-HB) could well play an important role as a packaging material.

Judging by the increasing number of publications, the area is in the midst of great developments. How well PHAs can assert themselves against other biopolymers remains to be seen.

6. What is Known About the Structure of Polyesters?

The structures of many polyesters have been determined from fiber X-ray diffraction experiments.[203] In contrast to polyamides, polyesters cannot form hydrogen bonds, and so the force field is composed only of torsion, nonbonding, and electrostatic energy terms.[204] Figure 5 presents the structures that are known for aliphatic polyesters of hydroxy acids and their amino acid analogues, as far as the latter are known. Depending on the method of workup, solids with differing conformations are often isolated; for poly-β-propionolactone and polyglycine not all of these structures have been determined.

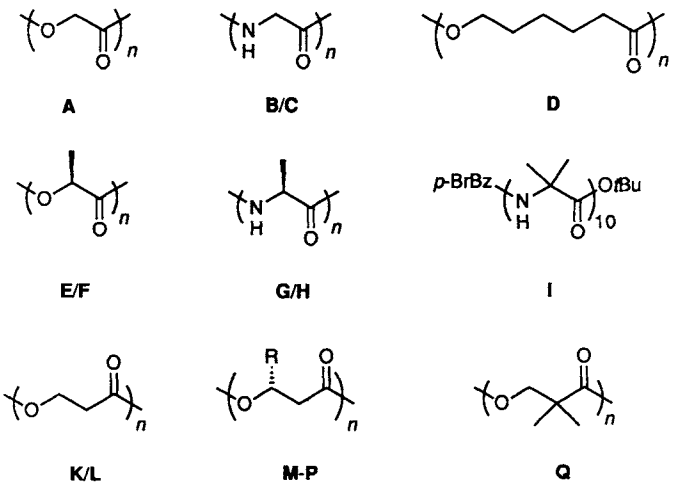
Prediction of the secondary structure of peptides and proteins is well established.[221] It has turned out that certain rules can also be derived for polyesters:

- polymers of the type -[O-(CH_2)n-CO-]- assume a staggered conformation (zigzag chain), at least for $n = 1$, 2, and 5 (**A**, **D**, and **L**, respectively in Fig. 5).[203]
- substituted poly-β-propiolactones always form a 2_1-helix (**M**–**Q** in Fig. 5). The pitch (axial spacing) of the helix decreases with increasing substituent size in the β-substituted poly-β-propionolactone series (**N**, **O**, and **P** in Fig. 5). According to Marchessault et al. a lower limit of 4.5 Å exists, below which changes in the helix geometry must occur.[217]

In the following sections, a few selected structures will be presented in more detail:

6.1. Structural Comparison of Homopolymers of α-Hydroxy Acids and α-Amino Acids

For polylactides, two conformations were found, a 10_3- and a 3_1-helix. These have practically the same energy content, and the latter is strongly reminiscent of the 3_1-helix of polyglycine (Table in Fig. 6), which forms no hydrogen bonds in this modification.[207] The α-helix of polyalanine



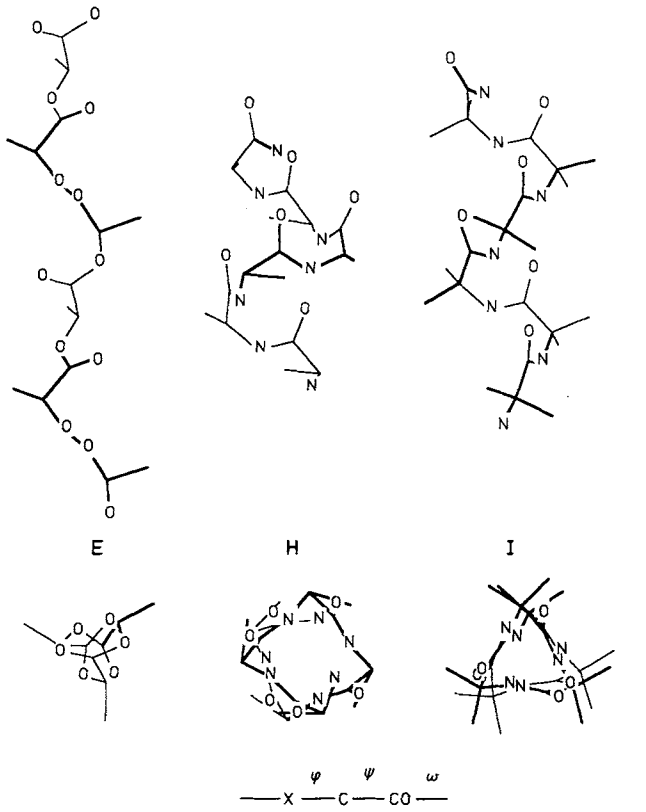
Name		Conformation [a]	Identity period [Å]	Ref.
A	polyglycolide	staggered	7.02	[205]
B	polyglycine I	?	–	[206]
C	polyglycine II	3_1-helix	9.3	[207]
D	polycaprolactone	staggered	17.05	[208]
E	β-poly-(*S*)-lactide	3_1-helix	9.00	[209b]
F	α-poly-(*S*)-lactide	10_3-helix	28.8	[209]
G	α-poly-(*S*)-alanine	β-staggered sheet	6.9	[210]
H	β-poly-(*S*)-alanine	4^7_{13}-helix [b]	70.3	[211]
I	deca-α-aminoisobutyrate	16_5-helix [c]	31.5	[212]
K	poly-β-propiolactone I	?	–	[213]
L	poly-β-propiolactone II	staggered	4.77	[214]
M	polydiketene (R: =CH_2)	2_1-helix	7.75	[215]
N	p(3-HB) (R: -CH_3)	2_1-helix	5.96	[167, 204,216]
O	p(3-HV) (R: -C_2H_5)	2_1-helix	5.56	[168]
P	p(3-HO) (R: -C_5H_{11})	2_1-helix	4.55	[217]
Q	poly-α,α-dimethylpropiolactone	2_1-helix	6.03	[218]

[a] On the basis of the crystallographic designation of screw axes the helix symmetry is expressed in the form n_r, which means that within an identity period n structural units form r rotations [219, 220]. This classification differs from the usual nomenclature for proteins; there in a n_r helix n denotes the number of amino acids per rotation and r the atom number of the ring formed from a hydrogen bond and its associated main chain segment [138]. [b] Corresponds to an α-helix. [c] Corresponds to a 3_{10}-helix.

Fig. 5. The known solid structures of polyesters of bifunctional monomers known to date, and those of the corresponding poly-(α-amino acid)amides, as far as these have been studied.

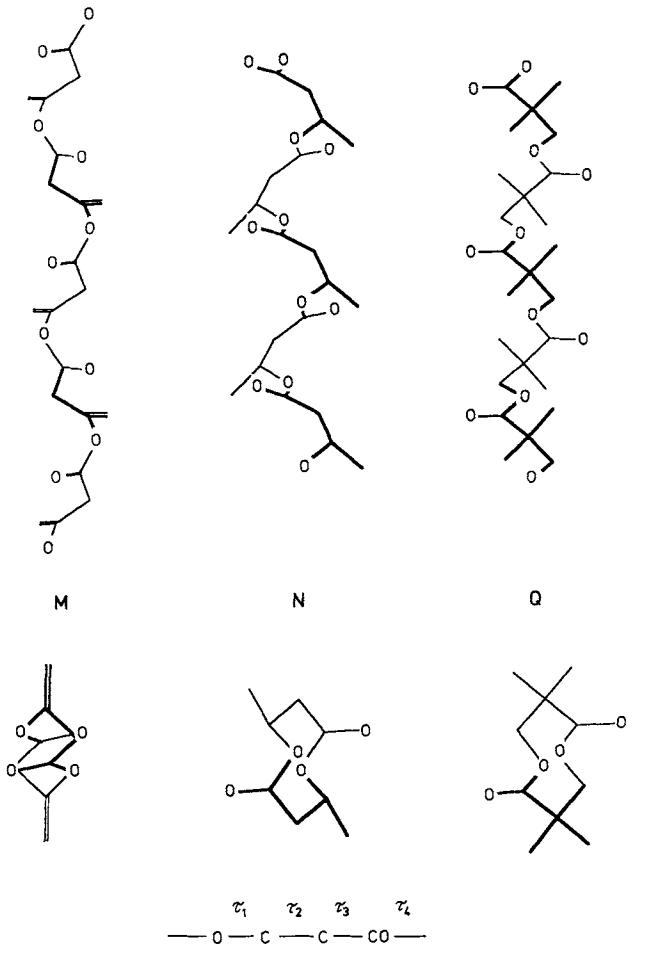
(**H** in Figs. 5 and 6), whose structure is stabilized by intramolecular hydrogen bonds, is not found with polyesters.

α-Aminoisobutyric acid (Aib) is a non-proteinogenic amino acid which is nonetheless found in many natural peptides (e.g. in peptaibols).[222] Single crystals have been grown from the fully protected oligomers containing up to ten Aib units.[212, 223] Because of the substitution pattern of Aib, the preferred secondary structure of such oligomers is not an α-helix but a 3_{10}-helix with a somewhat smaller internal diameter, which with the decamer **I** (Figs. 5 and 6) is almost ideal in form. The structure is also stabilized by intramolecular hydrogen bonds, and therefore has no analogue in the polyester series. Surprisingly, oligomers of α,α-diethylglycine (Deg) and α,α-dipropylglycine (Dpg) have staggered conformations, stabilized by intramolecular hydrogen bonds between the amide H atoms and the adjacent carbonyl groups, which are obviously energetically more favorable than a 3_{10}-helix.[223]



Polymer	Pitch [Å]	φ [°]	ψ [°]	ω [°]
poly-(*S*)-lactide : 3_1-helix **E**	9.0	-74	+144	180
polyglycine : 3_1-helix **C**	9.3	+/-80	-/+150	180
poly-(*S*)-alanine **H**	5.4	-66	-44	+179
p-BrBz-(Aib)$_{10}$-O*t*Bu **I**	6.3	+/-54	+/-31	+/-176

Fig. 6. MacMoMo views and a comparison of the 3_1-helix torsion angles of poly-(*S*)-lactide **E**, the α-helix of poly-(*S*)-alanine **H** and the 3_{10}-helix of deca-α-aminoisobutyrate **I**. The torsion angles are defined as for Figure 2c; the letters **E**, **H**, and **I** refer to the compounds in Figure 5.



Polymer	Pitch [Å]	τ_1 [°]	τ_2 [°]	τ_3 [°]	τ_4 [°]
poly-α,α-dimethyl-β-propiolactone **Q**	6.03	+/-178	+/-41	+/-61	-/+164
polydiketene **M**	7.75	+/-84	+/-74	+/-147	+/-177
poly-(*R*)-3-hydroxybutyrate **N**	5.96	+162	-52	-42	-175
poly-(*R*)-3-hydroxyvalerate **O**	5.56	+136	-60	-21	179
poly-(*R*)-3-hydroxyoctanoate **P**	4.55	+111	-68	+15	-161

Fig. 7. MacMoMo views, and a comparison of the 2_1-helix torsion angles of polydiketene **M**, poly-(*R*)-3-hydroxybutyrate **N**, and poly-α,α-dimethyl-β-propiolactone **Q**. In each case, five structural units are shown. The torsion angles are defined as for Figure 2c; the letters **M**, **N**, and **Q** refer to the compounds in Figure 5. The values for P(3-HB) are taken from ref.[170].

6.2. Structural Comparison of Homopolymers of 3-Hydroxy Acids: Modeling Studies with the Structural Parameters from Oligolides of (*R*)-3-Hydroxybutyric Acid

The same left-handed 2_1-helix with an identity period of 5.9 Å has been derived independently by three different groups[167, 204, 216] from the diffraction pattern of P(3-HB) fibers, by using different algorithms.[224a] Depending on the method, slight variations in the torsion angles were found, but they still lie quite close to each other ($\tau_1 = +142°$ to $+152°$, $\tau_2 = -52°$ to $-59°$, $\tau_3 = -31°$ to $-42°$, $\tau_4 = -173°$ to $-180°$; Fig. 7).

So far, it has not been possible to grow suitable crystals of P(3-HB) or of linear (3-HB) oligomers,[224b] and so the P(3-HB)-helix has never been directly "seen". The only exact structural parameters available at present are those of (3-HB) oligolides (see above, Fig. 2). Thus, from the S-shaped partial structure (Fig. 2b, green) a left-handed helix with approximately two units per turn (2_1-helix) may be constructed, while the Δ-shaped structural element (Fig. 2a, red) can be used to make a right-handed helix with about three units per turn (3_1-helix) and a correspondingly larger internal diameter (Fig. 8).[133, 135b]

In the 2_1-helix the ester carbonyl plane is almost perpendicular to the helix axis. For the 3_1-helix, in contrast, it lies almost parallel to the axis. The latter structural type has not yet been found in solid P(3-HB), though this does not preclude its presence in solution. The structure in solution was and is the subject of numerous studies, with somewhat controversial results.[225]

Under tension, the identity periods of the polyesters change, presumably because either the helix or the amorphous regions of the polymer assume a staggered conformation (zigzag chain).[167, 204b] Recently Marchessault et al. have calculated the conformation of such a zigzag structure for P(3-HB), by energy minimization with an MM2 force field.[226] The chain is not ideally staggered, and the torsion angles are $\tau_1 = +113°$, $\tau_2 = -169°$, $\tau_3 = -113°$, and

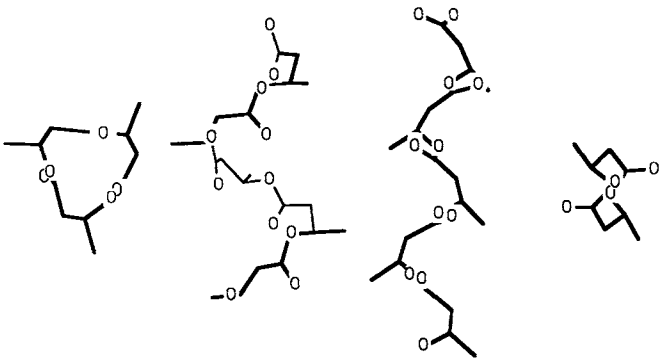


Fig. 8. MacMoMo views of the 2_1-helix (right) constructed from the S-shaped structural moiety (Fig. 2b, green), and the 3_1-helix (left) derived from the Δ-shaped spiral (Figure. 2b, red). In the middle, the two projections along the helical axes are shown (in each case only one turn of the helix is depicted).

$\tau_4 = +172°$ (identity period: 4.60 Å, for ideal staggering it would be 4.74 Å).

By using X-ray diffraction and ^{13}C NMR spectroscopy, it has been shown that the copolymer P(3-HB/3-HV) crystallizes in a P(3-HB)-helix up to 40% HV content, and in a P(3-HV)-helix above 55% HV (isodimorphism).[5, 227] Poly-β-isopropyl-β-propiolactone probably also crystallizes in a 2_1-helix, though all that is known about it is the identity period of 6.49 Å.[228] Unfortunately, at present it is not possible to compare the structure of the PHAs with the corresponding β-amino acid homopolymers, since there appear to be no structures known for the latter.[229]

7. Poly-(*R*)-3-hydroxybutyric Acid–Calcium Polyphosphate [P(3-HB)Ca-polyP$_i$] Complex: A Natural, Non-Proteinogenic Ion Channel?

Every cell has its own "microclimate". In order to preserve the special conditions required inside each cell, it surrounds itself with membranes. Biological membranes are essentially lipid bilayers containing interspersed proteins, usually about 75 Å thick. The hydrophobic interior is usually about 25 Å in size, but depending on the number of double bonds in the lipid chains can be up to 35 Å in breadth.[230] Because of their structure, lipid bilayers are practically impermeable for ions and most polar molecules. Nonetheless, these must somehow be able to pass through the membrane, since they play an important part in many physiological processes. Two transfer modes exist, carriers and channel mechanisms, whereby the latter is the more efficient and widespread of the two. Ion channels are mostly composed of proteins, with the channels themselves formed by ringlike conformations of several amphiphilic helices. The channel may consist of a single protein or of an aggregate of similar or identical subunits.[231] In addition, channel-forming peptides are also known, such as gramicidin,[232] for example, or alamethicin.[233]

Because of their great physiological importance, ion channels are at present the subject of intensive research; their importance was demonstrated in 1991 by the presentation of the Nobel Prize for medicine to Neher and Sakmann.

7.1. Findings which Led to the Postulation of an PHB-Containing Ion Channel

In 1983, Reusch et al. observed abrupt changes between 50 and 60 °C in the fluorescence intensity of *N*-phenyl-1-naphthylamine in the membranes of a variety of quite unrelated, genetically competent[234] gram-negative and gram-positive bacteria (*Azotobacter vinelandii*, *Bacillus subtilis*, and *Haemophilus influenzae*).[235] In each case, P(3-HB) was detected in the cell membranes, in significantly higher concentrations than were found in the noncompetent state. On the basis of these observations, the authors concluded that the characteristic changes in the fluorescence intensity must be somehow connected with P(3-HB).[19] Further experiments with *E. coli* showed that in the competent state, considerable quantities of P(3-HB) can be isolated (see Table 6).[235] The jump in fluorescence intensity at 50–60 °C was more intense if the competence protocol, which had been arrived at by "trial and error", was adhered to rigorously. A transition of this sort was not observed when biosynthesis of P(3-HB) was blocked with acetaldehyde, or if P(3-HB) was incorporated into liposomes. Clearly, the genetic transformability is connected with P(3-HB), but the P(3-HB) is not present by itself, but in the form of a quite unstable complex.[20] The authors later showed by freeze-fracture electron microscopy that in the plasma membranes of competent *A. vinelandii* and *E. coli* bacteria, islands with a visibly different structure are present.[21] Reusch and Sadoff were later able to isolate a labile complex from genetically competent *E. coli* bacteria, whose constituents were identified as P(3-HB), polyphosphate,[236] and calcium ions, and postulated a complex structure (see Figs. 9 and 10).[22, 23] In addition, they were able to show that the complex can be incorporated in vitro into liposomes, again using fluorescence spectroscopy.[22] Recently Reusch has also detected the complex more or less convincingly in plant and animal tissues (above all in the mitochondria and microsomes, to a lesser extent in the plasma membranes), though at significantly lower levels than in competent bacteria (see Table 6).[23]

Table 6. Occurrence of the complexes identified as [P(3-HB)Ca-polyP$_i$] in selected prokaryotes and eukaryotes [22,23].

Source	P(3-HB)$_{total}$ [μg g^{-1}]	Complex components [a] P(3-HB) [ng g^{-1}]	PolyP$_i$ [ng g^{-1}]	Ca^{2+} [ng g^{-1}]
E. coli log. phase	0.26	–	–	–
E. coli competent	156	9600	4200	2000
spinach leaves	6.5	435	232	126
cow heart	9.2	662	353	178
cow liver	0.86	163	88	47

[a] The given numbers are based on the wet weight.

7.2. Composition and Possible Structure of the PHB-Containing Complex

According to Reusch, in the complex isolated from *E. coli*, 3-HB, phosphate, and Ca^{2+} occur in a ratio of 1:1:0.5. In contrast, the eukaryotic complex contains a longer polyP$_i$ chain (see Table 7). On the basis of molecular modeling and

Table 7. Chain lengths of the P(3-HB) and polyphosphate chains in organisms. (Determined from the gel permeation chromatogram by comparison of the retention times with standards) [22,23].

Complex	Chain length in monomer units	
	P(3-HB)	PolyP$_i$ [a]
prokaryotic complex	120–200 [b]	130–170
eukaryotic complex	120–200 [c]	170–220

[a] Gel: Showdex B804; standard: polyethylene glycol. [b] Gel: Altex m-Spherogel; standard: polyisoprene. [c] Gel: Altex m-Spherogel; standard: *erroneously given as polystyrene* [23]! According to a personal communication from Reusch, however, they too used polyisoprene.

energy minimization considerations (with the force field program Charmm), Reusch et al. postulate the exolipophilic-endopolarophilic structure shown in Figure 9.

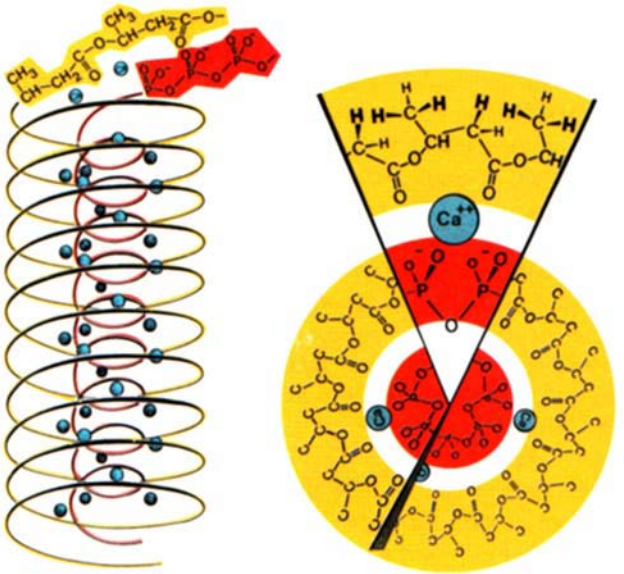


Fig. 9. Structure of a [P(3-HB)Ca-PolyP$_i$] complex suggested by Reusch [23].

The Ca^{2+} coordination geometry was decisive for the postulated spatial configuration. The external, right-handed P(3-HB)-helix must contain 14 units per turn, and the internal, also right-handed polyP$_i$-helix 7 units per turn, in order to bring the carbonyl and phosphate oxygen atoms into a suitable geometry for complexation with the Ca^{2+} ions (Fig. 10).[237] The Ca^{2+} ions lie vertically one above the other within the P(3-HB) cylinder. On the outside face, the methyl and methylene groups of the hydroxybutyric acid units also lie one above the other.

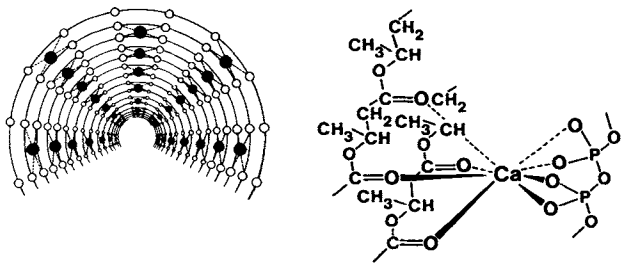


Fig. 10. Coordination geometry of the calcium in the postulated [P(3-HB)Ca-PolyP$_i$] complex [22].

A diameter of 24 Å,[238] and a length of 45 Å have been calculated, with an identity period of 4 Å, representing about 150 3-HB units and 80 P$_i$ units. To traverse the hydrophobic part of a membrane, 45 Å ought to be more than sufficient. However, in the projection shown in Figure 9, the ester bonds of the P(3-HB)-helix appear to assume at best the less favorable *E* conformation, which is about 3 kcal mol^{-1} less stable than the *Z* form.[239]

According to Reusch, the complex is quite labile in vitro, and extremely water-sensitive, probably due to the absence of stabilization of the postulated exolipophilic-endopolarophilic structure by the hydrophobic lipid bilayer.[240]

7.3. Possible Functions of the Ion Channel: DNA Transport?

The presumed exolipophilic–endopolarophilic structure is the ideal arrangement for an ion channel. Reusch assumes that such a channel would, above all, transport Ca^{2+} ions and polyphosphates across the plasma membrane. The inner, Ca-polyP$_i$-helix twists like a screw through the outer P(3-HB)-helix, and the Ca^{2+} ions are progressively transferred from one carbonyl group to the next. The transport of the neutral Ca-polyP$_i$ salt requires less energy than that of Ca^{2+} and polyP$_i$ ions separately. The interdependence of genetic transformability and complex concentration led Reusch to postulate DNA transport as well. However, for this to take place, the polyP$_i$ would first need to be removed from the P(3-HB)-helix. The outer P(3-HB)-helix remains intact as long as sufficient glucose is available as an energy source. According to Reusch and Sadoff, the internal diameter is sufficient to permit transport of single-stranded DNA. The structural requirements used to calculate this remain open—must the DNA be transported as a linear strand, or are secondary structures allowed? The secondary structures of single-stranded polynucleotides can display a considerable diameter. Thus, for example, the helix of polyadenosine has a diameter of 19 Å, only slightly less than that of double-stranded DNA (A-form: ca. 21 Å , B-form: ca. 22 Å, and Z-form: ca. 19 Å).[220] In addition, in transformation experiments, plasmids (cyclic, double-stranded DNA) are usually used, which in their supercoiled form have a far larger diameter! The role of calcium in genetic transformation, which is emphasized repeatedly by the authors, must also be somewhat modified, since transformation was obviously carried out according to the Hanahan protocol, where calcium plays only a subsidiary role![241] It is also unclear how such a channel could be opened and closed.

Nonetheless, this possible DNA-transport system through cell membranes is very intriguing, also with a view to future development of antisense medicaments.[242] The transport of charged oligonucleotides through cell membranes is still largely not understood.[242a, 243]

8. Complexation and Transport Experiments with Metal Ions and Polyesters

In the [P(3-HB)Ca-polyP$_i$] structure postulated by Reusch et al. the calcium ions are coordinated both to four

polyphosphate oxygen atoms and to four ester carbonyl oxygen atoms (Fig. 10). Complexes in which several ester carbonyl groups of the same acyclic molecule are coordinated to one cation are not known, to our knowledge. On the other hand, relatively many complexes exist between various cations and cyclic ligands, in which the ester carbonyl oxygen atoms cooperate with other functional groups. In nonactin, these are the ether oxygen atoms of the tetrahydrofuran rings.[244] Interestingly, these alkali metal complexes are very similar to the folded structure of the octolide of (*R*)-3-hydroxybutyric acid **O** (in Fig. 2), which in contrast to nonactin is not, however, active as an antibiotic (Fig. 11).[245] In the case of the depsipeptides, such as valinomycin, it is only the ester carbonyl groups which take part in complexation, but the structure is additionally stabilized by hydrogen bonds between the amide groups.[244]

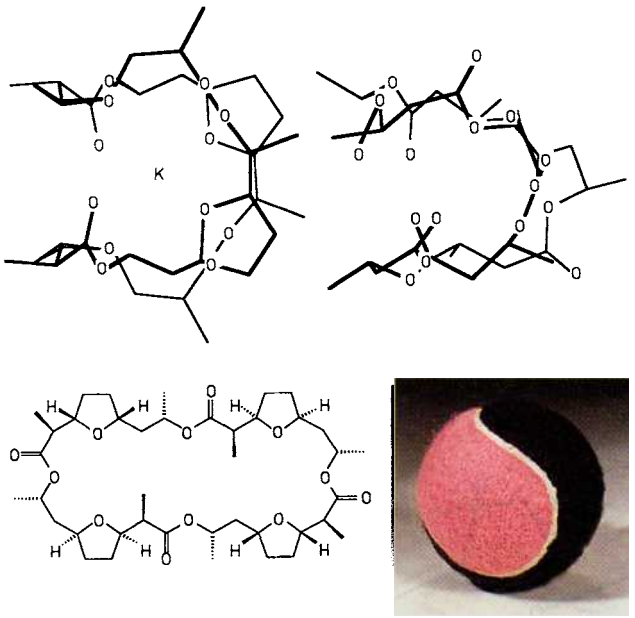


Fig. 11. MacMoMo views of the potassium complex of nonactin (top left) and the octolide structure **O** (top right) from Figure 2, and of the formula for nonactin (bottom left). Both 32-membered rings resemble the seam of a tennis ball in their structure (bottom right). The K^+ ion in the nonactin complex is surrounded by the four O atoms of the tetrahydrofuran rings and the four ester carbonyl O atoms in approximately cubic geometry. In the uncomplexed form nonactin has a different conformation to that in the potassium complex, and no longer resembles the octolide [244].

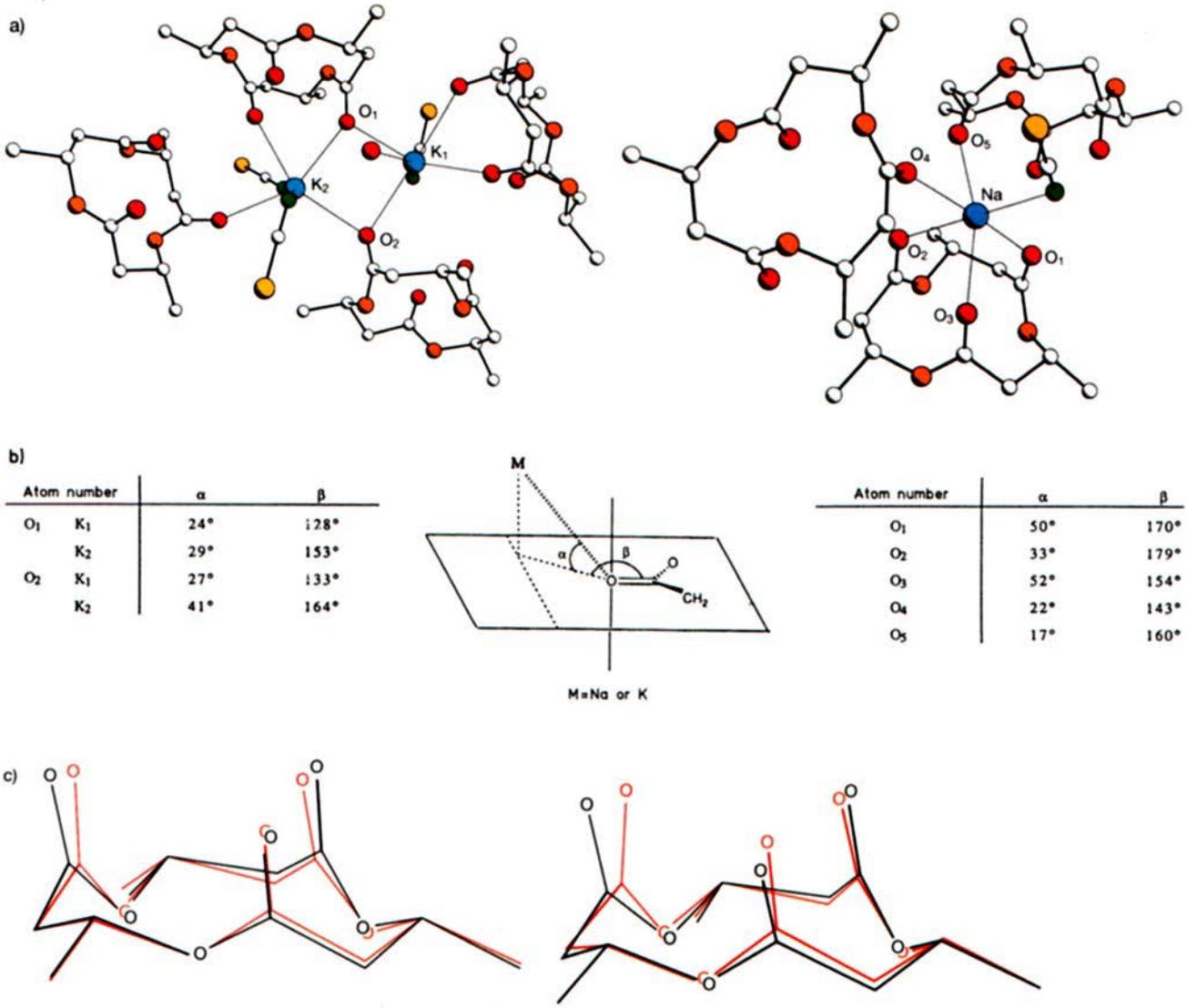
With the synthesized linear and cyclic P(3-HB) compounds in hand, we were in a position to investigate the complexing ability of polyesters of (*R*)-3-hydroxybutyric acid in more detail. All attempts at crystallization of such complexes with the open-chain molecules and larger cycles were unsuccessful, but promising results were obtained with the triolide **F** (in Fig. 2). Its structure is almost ideal for complexation, since the carbonyl groups are all practically parallel to each other, in the same hemisphere. Solutions of the triolide with sodium or potassium rhodanide in acetonitrile led to precipitation of solids whose structures could be determined.[135] For KSCN, crystals were only formed after the solution had been left open to air moisture for a little time: this proved that a little water was required for crystal formation. Both complexes are polymeric, and only in exceptional cases, for the sodium complex, were three ester carbonyl groups of one triolide molecule chelated to the same cation. Both the sodium and the potassium ions showed no particular preference for one coordination geometry with the ester carbonyl oxygen atoms (Fig. 12). Schreiber et al. made the same observation for alkali metal complexes with other carbonyl ligands.[246]

On the basis of ^{1}H NMR studies, and measurements of the dipole moment, the triolide appears to have a similar structure in solution to that in the crystal. In methanol, an interaction was observed with the alkali metal ions, which disappeared progressively, however, with gradual dilution. P(3-HB) also seems to undergo a certain degree of complexation with lanthanides in solution. Delsarte and Weill have suggested a structure for P(3-HB) in solution,[225c] by using the geometric data which they obtained from the McConnell–Robertson relationship of the pseudocontact complex of P(3-HB) with $Eu(fod)_3$ (fod = 1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octadienato).

Okada et al.[247] and Burke et al.[248] have studied the transport of alkali metal picrates through an organic medium, by using cyclic polyesters which also contained additional acetal or ether oxygen atoms. Analogous experiments with triolide **F** showed increasing transport efficiency as the radius of the alkali metal ion increased (Fig. 13).[104b] These results can be explained readily by the increasing lipophilicity of the ions. The lack of ion selectivity could be connected to the rigidity of the triolide skeleton, whose structure remains almost unchanged even in the complexes with NaSCN and KSCN (Fig. 13). Similarly, high molecular weight P(3-HB) and linear oligomers of about 35 (3-HB) units also transported Cs^+ ions through a CH_2Cl_2 phase.[104b]

9. Conclusions and Outlook

After the discovery of P(3-HB) by Lemoigne about seventy years ago, interest in this biopolymer was at first very limited. The existence of this compound was known only to a few specialists. Because of the accessibility of polymers of this type from regenerable carbon sources, by using fermentation, an explosive development took place in this field at the time of the oil crisis about twenty years ago. Recently this has been strengthened by the fact that the poly(hydroxyalkanoates) are biodegradable and biocompatible polymers. Review articles and books have been written, and conferences held, whose participants from industry, academia, and state institutions were a more colorful interdisciplinary mixture than even the more experienced of us had ever seen. In a very recent international symposium on bacterial polyhydroxyalkanoates (ISBP '92, 1–5. June 1992) in Göttingen, the speakers and audience comprised biologists, microbiologists, geneticists, botanists, chemists, physicists, polymer scientists and polymer engineers, biotechnologists, pharmaceutical and medical scientists, many of whom came from institutes for raw materials and agrotechnology, food sciences,[249] groups concerned with waste disposal, housing and water management,[250] catalyst and surface research, even construction and building![251] Interest was concentrated almost exclusively (35 of 37 lectures) on high molecular



Atom number		α	β
O_1	K_1	24°	128°
	K_2	29°	153°
O_2	K_1	27°	133°
	K_2	41°	164°

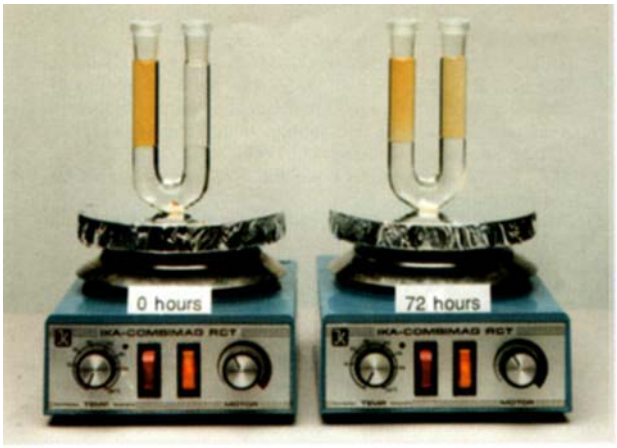
Atom number	α	β
O_1	50°	170°
O_2	33°	179°
O_3	52°	154°
O_4	22°	143°
O_5	17°	160°

Fig. 12. a) PLUTO diagram of sections of the crystal structures of the triolide complex with KSCN (left) and with NaSCN (right). All potassium and sodium ions are hexacoordinated with the ester carbonyl O atoms of the triolides and the N atoms of the rhodanide ions as ligands. In the potassium complex, an additional water molecule is complexed to K_1. Red spheres are oxygen atoms of the CO functional groups, orange are OR oxygen atoms of the ester groups, white are carbon atoms, yellow are sulfur atoms, and green are nitrogen atoms. b) Coordination geometries of the potassium and sodium ions in the two complexes with selected ester carbonyl groups. For comparison: In the C_2 symmetrical nonactin–potassium complex, the angles are about α 61° and 67°, β 154° and 150°. c) Superposition of the free triolide (black) on the threefold coordinated triolide (red) in the Na complex (right), and on the twofold coordinated triolide (red) in the K complex (left).

weight polyhydroxyalkanoates, the bacterial storage materials made up of over 10000 (3-HB) units (referred to in the following as s-PHA or s-P(3-HB)[252]): their genesis, production, structure, material properties, applications, and biological degradation.[253]

Besides high molecular weight P(3-HB) in granules in microorganisms, the work of Reusch et al. has shown that short-chain P(3-HB), consisting of 100–200 (3-HB) units is also present in prokaryotic and eukaryotic organisms (referred to in the following as c-P(3-HB)[254]). It is always found in a complexed form, for example, in the cell membranes with Ca ions and probably a polyphosphate [19–23] and in human blood plasma with albumin and the so-called "low-density lipoproteins" (LDL).[24] In *E. coli*, which normally does not produce s-P(3-HB) (see also ref.[18]), the c-P(3-HB)-containing complex is only found in the inner cell membrane in the genetically competent state of the microbe, while in eukaryotes the complex is concentrated primarily in the mitochondria (the "microorganisms of the eukaryotic cell") and the microsomes; these facts suggest that c-P(3-HB) has important effects and functions. The results of our studies on the structure and properties of linear and cyclic (3-HB) oligomers do not contradict the hypothesis that c-P(3-HB) could be a component of an ion channel through cell membranes. It is notable that it should occur specifically as part of a complex with Ca polyphosphate, especially in view of a proposal made by Lipmann in 1965; he suggested that the first organisms on our planet may have used not ATP as their energy carrier, but inorganic polyphosphate or pyrophosphate (Polyphosphate – "a metabolic fossil"!).[255]

If one disregards inorganic polyphosphate [251, 257] and the aromatic amino acid-derived plant polymer lignin, which is non-uniform and difficult to classify,[258, 259] there are four classes of physiologically important biopolymers ("biomacromolecules"): polyisoprenoids, polynucleotides,



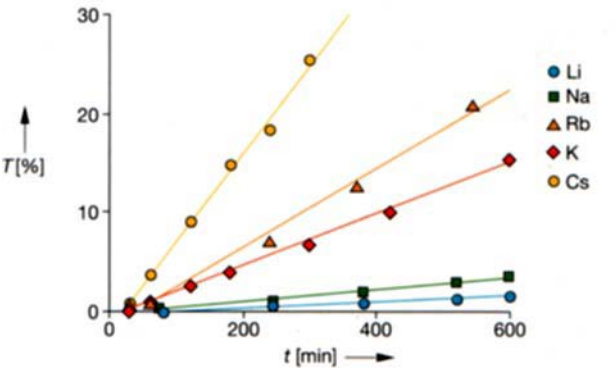


Fig. 13. Top: Apparatus for performing transport experiments through a "fluid membrane". The ions are transported out of a Tris-buffered (0.1 M) solution initially containing 1 mM alkali metal picrate/0.1 M alkali metal chloride, in the left side of the U-tube, through a 0.1 M triolide/CH_2Cl_2 solution into the other arm of the U-tube, whose water phase becomes progressively more yellow as the experiment proceeds. The picrate concentration is determined spectroscopically at λ_{max} = 356 nm. Bottom: Relative transport velocity T of the various alkali metal ions. These represent a so-called lipophilic series (without selectivity for a certain ionic radius!) [104b].

polypeptides, and polysaccharides. All four are distinguished by the facts that a) their monomeric building blocks are fundamental metabolic intermediates of the organisms, b) the respective oligomers display important effects, for example, as cofactors, hormones, or vitamins, in signal transduction or information transfer, or in recognition mechanisms, and c) the high molecular weight forms possess important functions, for example, as catalysts and receptors, as information carriers, as transport vehicles, storage materials, or structural elements. According to these criteria, poly(hydroxyalkanoates) must now also be regarded as a fifth important class of biopolymers. a) Their building blocks, the hydroxycarboxylic acids, in the simplest case hydroxybutyric acid, are a part of fatty acid metabolism; like the isoprenoids and polyketides[260] they are eventually derived from acetic acid. b) The oligomers, called c-P(3-HB) above, occur as components of complexes with other molecules, with effects as yet unknown; c-P(3-HB) is involved in the modification of cell membrane properties (phase transition, fluidity, permeability). c) The high molecular weight s-P(3-HB) is a microbial storage material (carbon reservoir, NADH-equivalents). In Table 8, examples are given of the five classes of biopolymers, and the polymers are contrasted with their low molecular weight equivalents.

Last of all, we come to the question of why low molecular weight c-PHA was discovered so late, and why its importance is only now slowly being recognized. Clearly, it was overlooked in the lipid fractions of cell extracts because of its chemical (heat, acid, base) and biochemical (ester-cleaving enzymes) instability. c-P(3-HB) occurs in *E. coli*, for example, only in very small amounts; it is synthesized to achieve a specific effect (membrane modification) and is then degraded again. It is probably subject to rapid anabolism/catabolism in the organism. The situation is therefore similar to that for hormones, whose stable representatives have been known and identified for a long time, while such important molecules as those of the arachidonic acid cascade (also lipids!), namely the leukotrienes, prostaglandins, thromboxanes etc. have only been isolated, characterized, synthesized, and their full importance recognized in the last few years (several have half-lives in serum or in tissues of less than a minute!).

Table 8. Comparison of monomers, oligomers, and polymers in chemistry and biochemistry of natural products.

Monomer *metabolic intermediates* biosynthesis and degradation from and to other low molecular weight building blocks	**Oligomers** *action* e.g. as hormone, cofactor, vitamin, information carrier, signal, recognition	**Polymers** *function* catalyst, receptor energy, storage material, transport vehicle, structural elements
amino acids	oligopeptides e.g. endorphines,bradykinin	polypeptides, e.g. fatty acid synthase hemoglobin, immunoglobulins skin, nails, hair, silk
monosaccharides, sugars	oligosaccharides e.g. blood group determinants heparin, sugar part of the glycoprotein dextrin	polysaccharides e.g. cellulose, starch chitin, glucan, glycogen murein
acetic acid, mevalonic acid isopentenyl pyrophosphate	isoprenoids carotinoids, rhodopsin, vitamin A, E, squalene, steroids, steroid hormones	polyisoprenoids, rubber, gutta-percha
purine and pyrimidine bases nucleosides, nucleotides, phosphate	oligonucleotides NADH, NADPH, tRNA	polynucleotides *m* and *r*RNA, DNA
hydroxycarboxylic acids	low molecular weight P(3-HB) (c-PHB, complexation)	high molecular weight P(3-HB) (s-PHB, storage)

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[1] a) The expression poly(hydroxy acid) (PHA) was coined in 1983 by Findlay and White (R. H. Findlay, D. C. White, *Appl. Environ. Microbiol.* **1983**, *45*, 71) when it was realized that homopolymeric poly-(*R*)-3-hydroxybutyric acid occurred as the exception rather than the rule. Polymers with various hydroxy acid units are far more common. b) Recently the German term Polyhydroxyfettsäuren (PHF) has been proposed by Steinbüchel (A. Steinbüchel, *Nachr. Chem. Tech. Lab.* **1991**, *39*, 1112-1124)
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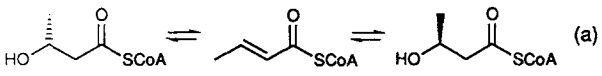

(a)

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[61] As well as sugars, carboxylic acids (up to and including caproic acid [5]) can be utilized, as can (*S*)-3-hydroxybutyric acid itself. (H. W. Ulmer, R. Gross, P. Weissbach, R. C. Fuller, R. W. Lenz, *Polym. Prepr. (Am. Chem. Soc. Div. Polym. Chem.)* **1989**, *30*, (*2*), 402). As an anaerobe, *A. eutrophus* can also use a mixture of H_2, CO_2 and air as C source, which was originally even considered as a raw material for the industrial production of P(3-HB) (D. Byrom, *Trends Biotechnol.* 1987, 5, 246).
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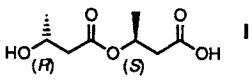
[72] The authors could only isolate an enriched depolymerase from *A. eutrophus*. They maintain that the lack of evidence for a dimer can possibly be ascribed to the action of an esterase, which copurifies with the depolymerase [33].

[73] (*R,S*) dimer, or (3*R*)-3-[(3′*S*)-3′-hydroxybutyryloxy]butyric acid **I**:



I

The (*R,S*) dimer is identical with the compound described as 3-L-[3-D-hydroxybutyryloxy]butyric acid (= LD dimer) in earlier publications [47, 52].

[74] Exoenzymes attack the polymer chains from the ends, in contrast to endoenzymes, which can also cleave within the polymer chain (*Lexikon der Biochemie*, (Eds. : H. D. Jakubke, H. Jeschkeit), VCH, Weinheim, **1974**).

[75] Merrick et al. have shown that the depolymerase is normally deactivated by an inhibitor whose action can be neutralized by an activator protein. The inhibitor can also be destroyed or removed by treating the P(3-HB) granules with trypsin, or extracting with alkali [55, 56]. The activities of the other enzymes are also affected by certain metabolic products. Thus, for example, NADH, pyruvate, and in some organisms oxaloacetate [35] or 2-oxo-glutarate inhibit 3-hydroxybutyrate dehydrogenase [43].

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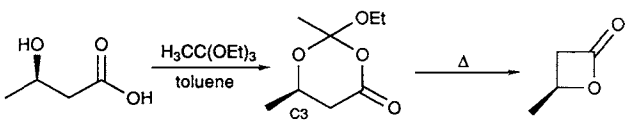

II

acid used as starting material. The original synthetic method, and an improved version: a) A. Griesbeck, D. Seebach, *Helv. Chim. Acta* **1987**, *70* 1320; b) R. Breitschuh, D. Seebach, *Chimia* **1990**, *44*, 216; other synthetic methods: c) A.R. Olson, R.J. Miller, *J. Am. Chem. Soc.* **1938**, *60*, 2687; d) J.R. Shelton, D.E. Agostini, J.B. Lando, *J. Polym. Sci. Part A1* 1971, 9, 2789; e) R.A. Gross, Y. Zang, R.M. Thomas, R.W. Lenz, *Polym. Prepr.(Am. Chem. Soc. Div. Polym. Chem.)* **1988**, *29(1)*, 596.

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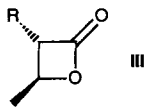

III

1226; H. Schlemper, dissertation (Extrem starke Oligophosphazen-Basen, Synthesen und Anwendungen), Universität Freiburg, **1990**. Similar experiments have been carried out earlier with α,β-substituted β-propiolactones, see J. Mulzer, T. Kerkmann, *J. Am. Chem. Soc.* **1980**, *102*, 3620.

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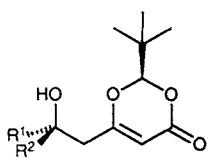
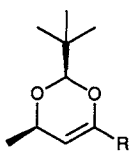
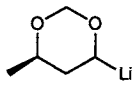


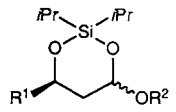
IV



V



VI



VII

Ed. Engl. **1989**, *28*, 472; D. Seebach, U. Misslitz, P. Uhlmann, *Chem. Ber.* **1991**, *124*, 1845. Compound **V**: U. Gysel, dissertation (Chromatographische Enantiomerentrennung von 1,3-Dioxinonen und Synthese von enantiomerenreinen 1,3-Dioxinen aus 3-Hydroxybuttersäure, dissertation no. 9473), ETH Zürich, **1991**. Configurationally stable 4-lithium-1,3-dioxanones with structure **VI** have been synthesized in several steps from P(3-HB). In THF, the (4*R*, 6*R*) derivative is stable at −78 °C, and the(4*R*, 6*S*) epimer is stable at 0 °C (R. J. Linderman, B. D. Griedel, *J. Org. Chem.* **1991**, *56*, 5491). Lactols of type **VII** have recently been synthesized by hydrosilylation of the corresponding 3-hydroxy acids. They can be diastereoselectively alkylated with allylsilane and suitable Lewis acids (A. P. Davis, S. C. Hegarty, *J. Am. Chem. Soc.* **1992**, *114*, 2745). Numerous pheromones have been synthesized by Mori et al. from 3-hydroxybutyrate and 3-hydroxyvalerate (K. Mori, *Tetrahedron* **1989**, *45*, 3233-3298).

[140] *Agricultural and Synthetic Polymers, Biodegradability and Utilization* (*ACS Symp. Ser.* **1990**, *433*).

[141] J. D. Evans, S. K. Sikdar, *CHEMTECH* **1990**, 38.

[142] In contrast to this, it is emphasized repeatedly at conferences and seminars on the subject that greater application of biodegradable polymers is not to be desired. On the one hand, they can be disposed of without commercial use, and on the other, their degradation products may pollute waterways (e.g. by eutrophication).

[143] Conference on Degradability of Polymers and Plastics, London, **1973**.

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shown that in *A. eutrophus* polyphosphate synthesis and P(3-HB) synthesis are connected [76].

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[251] International Symposium on Bacterial Polyhydroxyalkanoates ISBP'92, Göttingen, (FRG), June 1–5, **1992**; Programme and Abstracts, including list and addresses of the participants.

[252] The "s" stands for storage.

[253] The manuscripts of the lectures and posters are published in a special volume of *FEMS-Microbiol. Rev.* **1992**, *103*, 91-376.

[254] The "c" stands for complexed, following a proposal of R. Reusch [251,253].

[255] Pages 151–158 of the book by Dawes [4] and the detailed literature list contained.

[256] Microorganisms are known which can store polyphosphates of molecular weights up to over 10^6 [255].

[257] Literature list to chapter 11 in the book cited in [4].

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