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Detection, synthesis, structure, and function of oligo(3-hydroxyalkanoates): contributions by synthetic organic chemists☆

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Abstract

Two types of the biological macromolecules poly(*R*-3-hydroxyalkanoates) have been identified: the high-molecular-weight microbial storage material (sPHA) and a short-chain variety, consisting of butyrate and valerate residues, complexed with other biomacromolecules such as calcium polyphosphate or proteins (cPHB/PHV). While sPHA has attracted, and still enjoys, a lot of attention from numerous scientists around the world, research on cPHB and the structurally and functionally related polymalate (PMA) is still in its infancy. In this article, we present a review on the chemical synthesis, structure, function and interactions of monodisperse cPHAs, the oligo(3-hydroxyalkanoates), with emphasis on the butyrates (OHB); we report hitherto unpublished results on the enzymatic degradation of cPHB and PMA, on a new analytical method for HB/HV detection in biological samples, and on OHB-mediated Ca^{2+} transport through phospholipid bilayers of artificial vesicles; finally, we discuss possible mechanisms of ion transport through cell membranes, as caused by cPHB. The speculative—and provocative—question is asked whether the structurally simple PHAs may have evolved as storage materials and amphiphilic macromolecules before poly-peptides, -saccharides, and -nucleic acids, in the history of life, or under prebiotic conditions.

Keywords: Oligo- and poly(3-hydroxyalkanoates); Chemical synthesis; Analysis of PHB/PHV; Ion transport through phospholipid bilayer membranes; Vesicles and liposomes; Patch-clamp ion-channel measurements; Complexation of cyclic HB and HV oligomers with hydrophobic and hydrophilic substrates

1. Introduction

Originally, poly((*R*)-3-hydroxy-butanoate and -pentanoate) (PHB, PHV) had caught our attention, more than 15 years ago, as a welcome, cheap source of the constituent hydroxyacids, which we used as chiral building blocks for syntheses [1–3]. In the following years, our interest shifted to structural studies and, most recently, and in close collaboration with Rose N. Reusch, to investigations of the role PHB may play in biochemistry. This development becomes evident by looking at the list of our publications in the PHB area [1–51] (including review articles [13,26], abstracts [7,11,14]), and ETH dissertations [40–51].

When contemplating the history of poly(hydroxyalkanoates) (PHA), including β-polymalate (PMA) (Table 1), it is clear that research was focused on

☆ Dedicated to Professor Heinrich Zollinger on the occasion of his 80th birthday.

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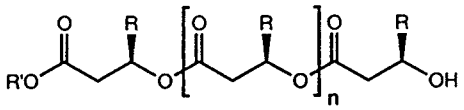
Table 1
Some highlights and historical notes about poly(3-hydroxyalkanoates) (PHA)[a]



Microbial storage material R = Me/Hex, R′ = H, *n* ca. 10^4; biodegradable (Lemoigne, Merrick, Dawes, Doi, Schlegel, Witholt, Steinbüchel, Fuller, Lenz, Sinskey)	*Ca channel* $CaPP_i$ complex; R = Me/Et, R′ = H, *n* ca. 150 (Reusch, Seebach)
BIOPOL® ICI, Zeneca, Monsanto; R = Me/Et, R′ = H, *n* ca 10^4	*Protein-bound* Unknown function; R = Me, R′O = protein, *n* unknown (Reusch)
PHB in genetically modified plants R = Me, R′ = H, *n* ca. 10^4 (Poirier, Sommerville, Monsanto)	*Polymalate* R = CO_2H, PMA, *n* = 30–500, for regulation of DNA-polymerase, in myxomycetes and fungi (Holler)

[a] For references see citations in subsequent figures and accompanying paragraphs. The best studied polymers of this type are poly(hydroxy-butanoate) (PHB), -valerate) (PHV), and -octanoate) (PHO).

genetics, biology, biochemistry, polymer chemistry, material sciences and environmental aspects [52]. One may ask the question what a synthetic organic group like ours may contribute to this field. Well, chemists are in charge of analyses, syntheses, structure determinations. They study properties, reactivities and supramolecular interactions between compounds. Thus, they are able to lay the foundations for models of the biological functions and for applications of natural and unnatural compounds.

2. Syntheses of oligo(3-hydroxybutanoic acids) (OHB) and of oligo(β-malic acids)—mechanism of enzymatic degradation

PHB was first discovered as a high molecular-weight polymer stored in granules (inclusion bodies) by certain microorganisms (Lemoigne, 1923–1927 [53]). Only much later, a short-chain variety forming a complex with calcium polyphosphate was isolated from the cell membrane of genetically competent *Escherichia coli* (Reusch, 1983–1997 [33,54–58]). In order to be able to study the properties of such low-molecular-weight PHB derivatives we have synthesized oligomers (OHB) of specific chain lengths (polymer chemists would call them *monodisperse polymers*), up to the 128mer (mol. weight ca. 11 000 Da, Fig. 1). With these compounds it was possible to calibrate the standards used for molecular-weight determinations of PHB by gel-permeation chromatography (GPC, Fig. 2) and, thus, to determine the chain length of PHB in *E. coli* to correspond to a ca. 140mer [21].

Using the same methodology as for the *isotactic* OHBs we are able to prepare *syndiotactic*, *atactic*, and *block* oligomers of HB, with a given sequence of (*R*) and (*S*)-configuration of the HB moieties along the chain (Fig. 3). Such non-natural OHBs are impor-

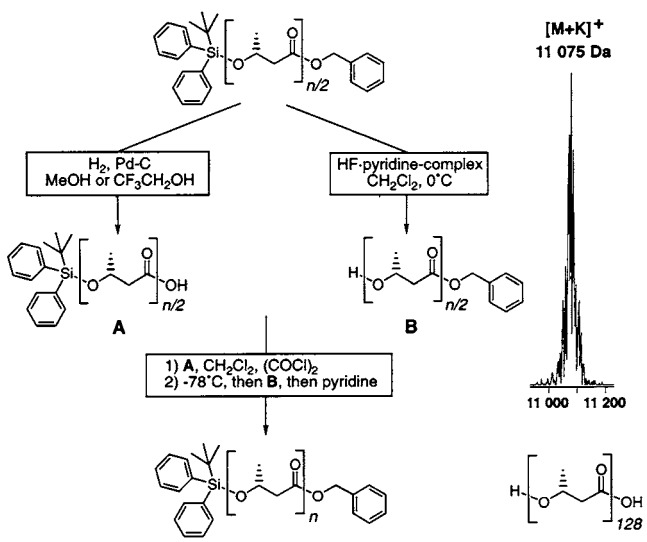


Fig. 1. Synthesis of oligo((*R*)-3-hydroxybutanoates) (OHB) by fragment coupling. The largest 'monodisperse' OHB synthesized so far is the 128mer, a chain of 512 atoms [27]. For shorter chain length, *t*-butyl-ester protection of the C terminus and benzyl-ether protection of O terminus may be used [20,39,42,46,49]. The mass peak shown was obtained by MALDI-TOF mass spectrometry with an α-cyano-4-hydroxycinnamic acid matrix. For early attempts to synthesize OHBs see papers by Masamune et al. [87]. Cyclic OHBs, oligolides, consisting of up to 32 HB and/or HV units (rings of up to 128 ring atoms) were also prepared and identified [5,6,8,9,15,22,25,27,29,32]. For macromolecular bicyclic and dendritic OHB derivatives see [31,32].

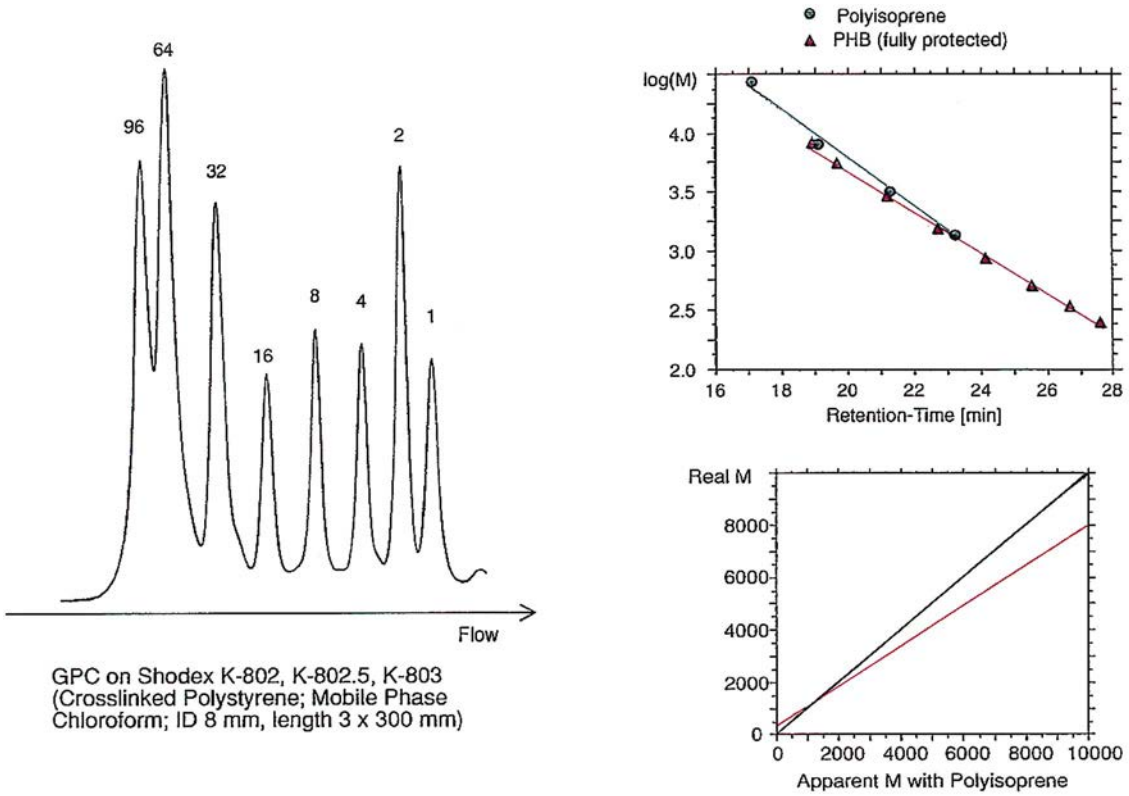


Fig. 2. Gel-permeation chromatography of an OHB mixture containing monomer and some oligomers up to the 96mer, and calibration versus polyisoprene standards [20]; a correlation with polystyrene and polyethyleneglycol standards was also published [20]. For a comparison with mass-spectrometric molecular weights see [16].

tant for two reasons: to study and compare the structures (backbone conformation) [59,60] with that of the *isotactic* (*all*-(*R*)) isomers [15,22,53], and to study the stereoselectivity of enzymatic PHB depolymerization. With a suitable analysis which allows detection of the fragments formed by cleavage of the octamers (Fig. 4), it was thus possible to follow the cleavage by one of the best-known PHB depolymerases (extracellular enzyme from *Alcaligenes faecalis*) and to derive hitherto unknown details about its mechanism of action. The results can be interpreted with a model for the enzyme's active site shown in Fig. 5: at least three, and for maximum rate, four subsites must be occupied by HB units of the chain, cleavage occurs only between two (*R*) HB units, the enzyme is an *endo* esterase. Thus, the *syndiotactic* octamer is stable against cleavage by this depolymerase, while synthetic polymers from *racemic* 3-HB precursors are cut wherever there happen to be two adjacent (*R*)-3-HB residues along the chain. Biodegradability [23] and biocompatibility of various PHA derivatives [53], occurrence of (*R*) and (*S*)-3-hydroxyalkanoates in fatty acid metabolism, and in various biochemical processes (for examples, see Table 2) should, however, not

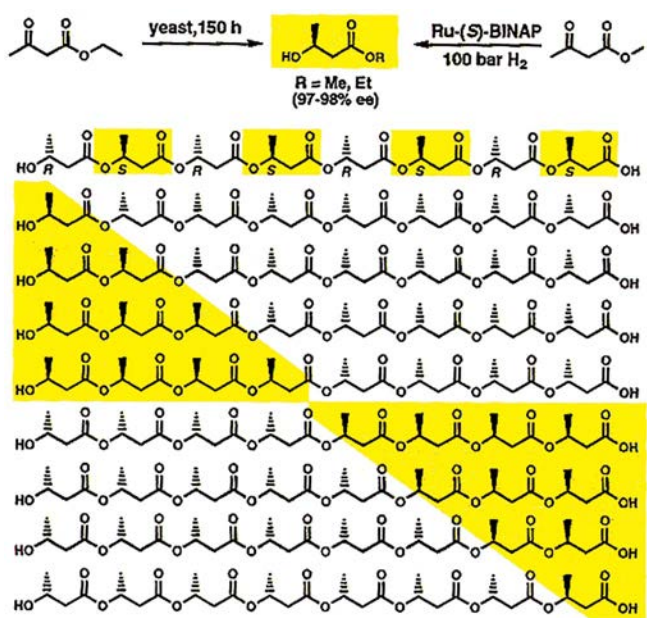


Fig. 3. *Syndiotactic* HB octamer and various octamers with specific sequences of (*R*) and (*S*) HB residues [39]. For the synthesis, the (*R*) HB building block was obtained by esterifying PHB depolymerization, the (*S*) enantiomer either by yeast reduction or by Noyori hydrogenation [88] of 3-keto-butanoate [39,49].

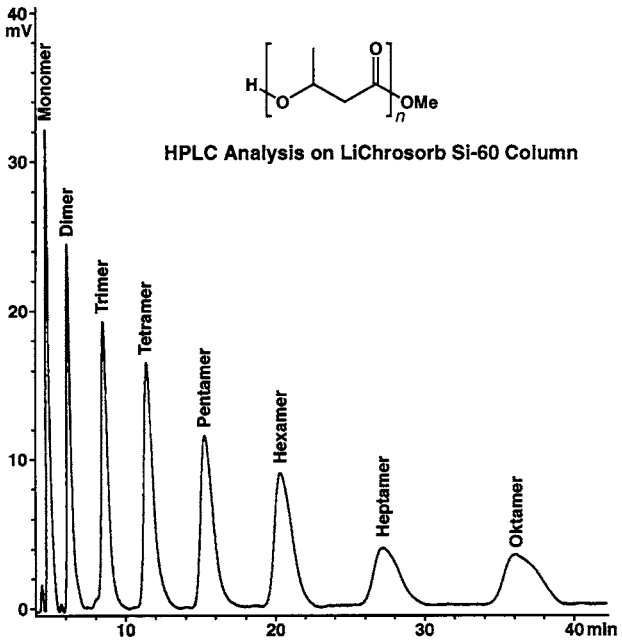


Fig. 4. HPLC-Analysis of an OHB mixture containing monomer to octamer methyl esters; the column used does not separate diastereoisomers, so that, for instance, all octamers shown in Fig. 3 will be detected together [38,49].

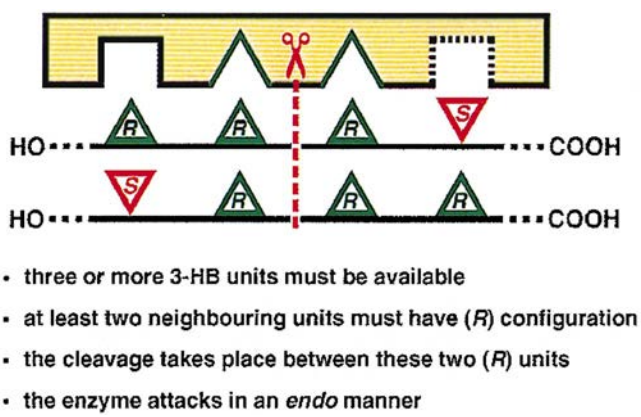
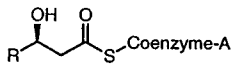
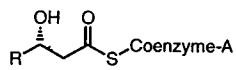
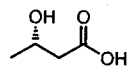
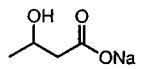
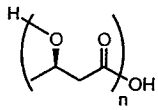


Fig. 5. Model for the active site of PHB depolymerase from *Alcaligenes faecalis*. The maximum rate of cleavage is observed only if all four subsites are occupied by HB units, with the central two subsites having to be occupied by two (*R*)-HB residues [38,39,49]. For most recent biochemical work on PHB depolymerases we refer to a paper by Jendrossek et al., and references cited therein [89].

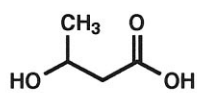
make us consider careless use of these materials, e.g. as food additives (Table 2): it has been shown by Sadler and his colleagues that both enantiomers of 3-HB have teratogenic effects in rodents, albeit at rather high concentrations (Fig. 6).

Yet another polyester-hydrolyzing enzyme was studied with specifically synthesized low-molecular-weight model compounds: certain myxomycetes appear to use β-polymalate (PMA) for regulation of

Table 2
(*R*)-3-Hydroxyalkanoates are intermediates of de novo fatty-acid synthesis, and the (*S*)-enantiomers those of fatty-acid degradation [80–83][a]

Intermediates of fatty acid synthesis	
Synthesis:  Degradation: 	
	only the (*S*)-enantiomer blocks the outward K^+-current in mouse ventricular myocytes in a voltage independent manner [61]
	very low energy diets during therapeutic starvation [62] (*R*)-form utilized by rat heart [63]
	PHA's for use in cream substitutes PHA's as additives to low-fat or no-fat food [64]

[a] Hydroxybutanoic acid is a natural metabolite found in various mammalian body fluids, and its Na salt has been proposed as substitute for glucose as a brain nutrient in humans. The use of PHB as a non-nutritional food additive and taste-improving component in ice cream and yoghurt has been patented.



rac-3-hydroxybutyric acid

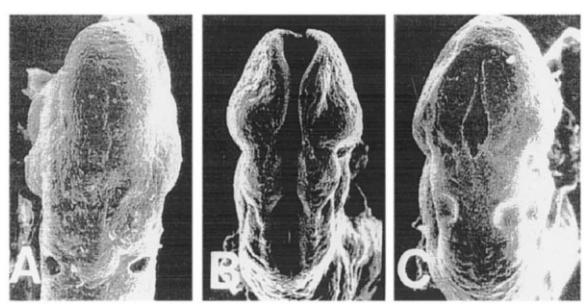


Fig. 6. Teratogenic effect of (*S*) and, to a lesser extent, of (*R*) HB in rodents [90–93]. The HB-induced malfunctions in rodent embryos involves abnormal morphogenesis during neurulation. (A) day 9 conceptures cultured for 24 h in control; (B) effect with 32 mM racemic hydroxybutyrate (neural tube closure defect); C: effect of 32 mM HB plus 7.5 mM ribose media. The picture is taken from Hunter III ES, Sadler TW, Wynn RE. Am J Physiol 1987;253:E72.

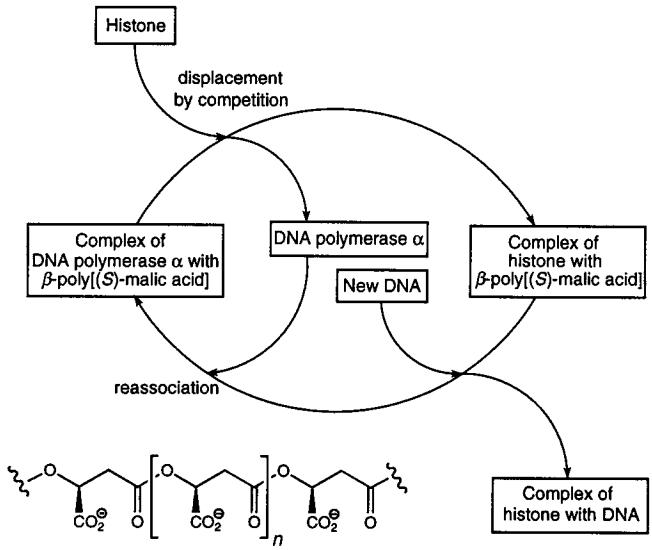


Fig. 7. Proposed function of β-polymalate in *Physarum polycephalum*. Myxomycetes may contain large amounts of β-poly((*S*)-malic acid), PMA, *formally* an oxidation product of PHB. PMA binds to histones and to DNA polymerase, both highly positively charged proteins, and is, thus, thought to be involved in DNA-reproduction regulation [94].

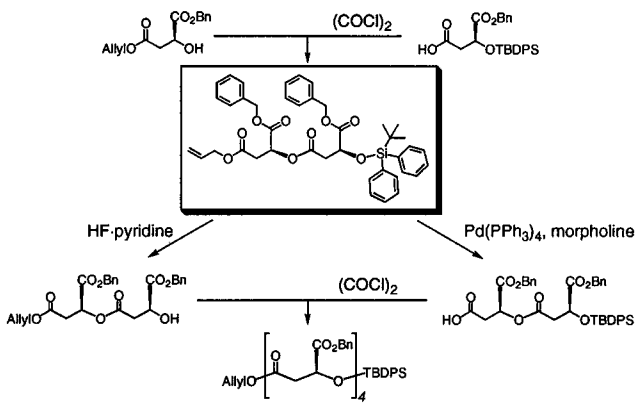


Fig. 8. Synthesis of a malic acid tetramer, using three different protecting groups [95]; allyl for the C-terminal carboxy group, benzyl for the branching carboxy groups and t-butyl-diphenylsilyl for the O-terminal hydroxy group. Activation of the carboxy group for ester-forming fragment coupling by reaction with oxalyl chloride.

DNA reproduction in certain stages of their life cycle (Fig. 7). PMA is an analog of PHB, having CO_2H instead of CH_3 side chains, and like polyphosphate and DNA it is a polyanionic biomacromolecule (up to 450 malate units in the chain). The synthesis of pure linear or cyclic oligomers consisting of β-malate units is *much* more difficult than that of the HB analogs, due to the additional functional group. Three different, so-called orthogonal protecting groups have to be used for the hydroxy and the two different carboxy groups, and after fragment coupling

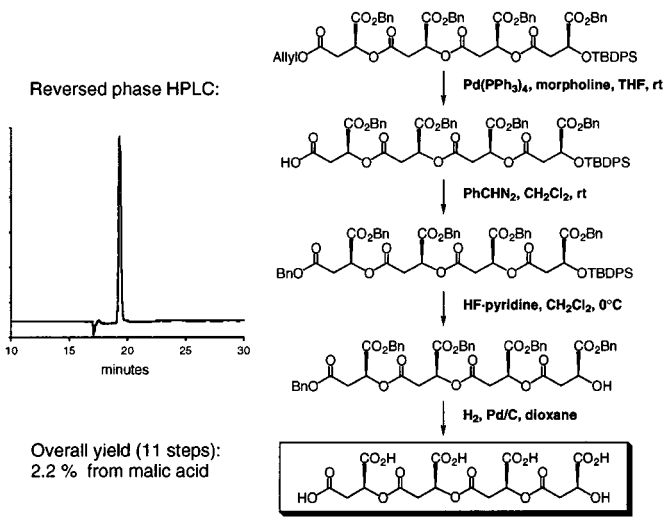


Fig. 9. Deprotection of the *O*-silyl- and ester-protected tetramer to the free hydroxy-pentacarboxylic acid and HPLC characterization thereof [95]. The transesterification of the C-terminal allyl to a benzyl ester group is necessary, in order to make the final hydrogenolytic cleavage (under neutral, heterogeneous conditions) of *all* ester groups possible.

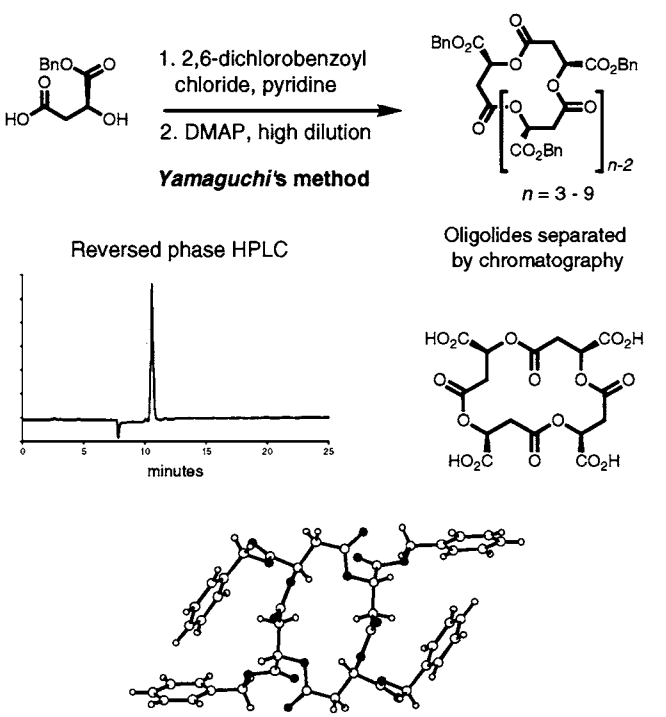


Fig. 10. Formation of mixtures of oligolides from malic acid monoester under Yamaguchi macrolactonization [96,97] conditions [30,95]. Cyclic oligomers containing up to nine malate units have been identified in these mixtures, and some were separated and isolated in pure form. HPLC of the tetrolide tetracarboxylic acid [95] and X-ray crystal structure of its tetrabenzyl ester prove the purity and structure of the tetrolide.

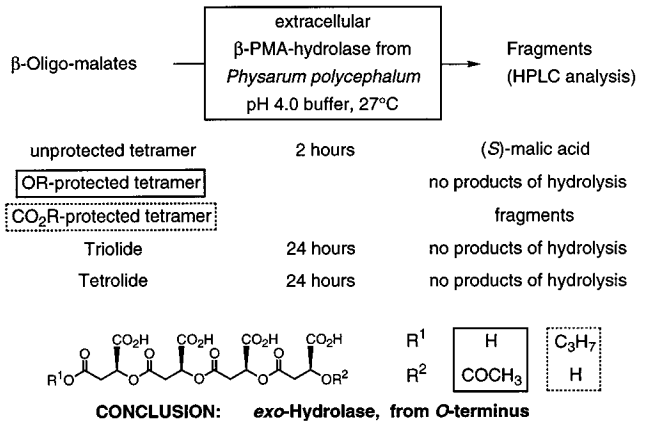


Fig. 11. β-PMA Hydrolase from *P. polycephalum* cleaves the hydroxy-pentacarboxylic acid (Fig. 9) and the hydroxy-propylester-tetracarboxylic acid, but not the *O*-acetyl-pentacarboxylic acid and the oligolide-tetracarboxylic acid (Fig. 10). Thus, the enzyme is an *exo*-hydrolase, cleaving the chain from the O-terminus [95,98].

with side-chain ester groups, the deprotection must be carried out under carefully chosen conditions (preventing transesterifications) and a polycarboxylic acid must be purified. The syntheses, also of cyclic oligomers are outlined in Figs. 8–10, and the RP-HPLC traces shown prove the purity of the compounds. With the linear tetramer (fully deprotected, C-terminal mono-propylester, O-terminal acetate) and the cyclic tetramer we were able to test the extracellular hydrolase from *P. polycephalum*. Although a tetramer may not be an ideal model for a polymer containing dozens or hundreds of malate units, we may draw the following preliminary conclusion from the results obtained (Fig. 11): the hydrolase cleaves the chain from the O-terminus (*exo* esterase).

3. Detection of (*R*) and (*S*)-HB/HV in biological samples by titanate-mediated transesterification—a new analytical tool

There are many methods for the qualitative and quantitative detection of PHAs (Fig. 12, with leading references for PHB and PHV). Extraction of biological samples with boiling chloroform gives solutions of the polyester itself, or of its complex with Ca polyphosphate, which can be directly detected by NMR spectroscopy (Fig. 13). Sometimes, for instance with bovine serum albumin (BSA), it is necessary to add some methanol in the extraction to detect chloroform-soluble material [21]. The most common method is an indirect analysis: the sample is heated with concentrated H_2SO_4 at temperatures up to 120°C, and the crotonic acid formed from HB is detected and quantified by HPLC; PHV is not detected by this method. As shown in Table 3, the crotonate test, applied directly to lyophylized, dry cells, cell fractions, tissues, or protein powders, gives 10–1000 times larger values (0.1–14 mg/g) than those obtained by analyzing chloroform extracts (1–30 μg/g), especially when the samples are heated above 100°C and/or for prolonged periods of time [61–63]. The

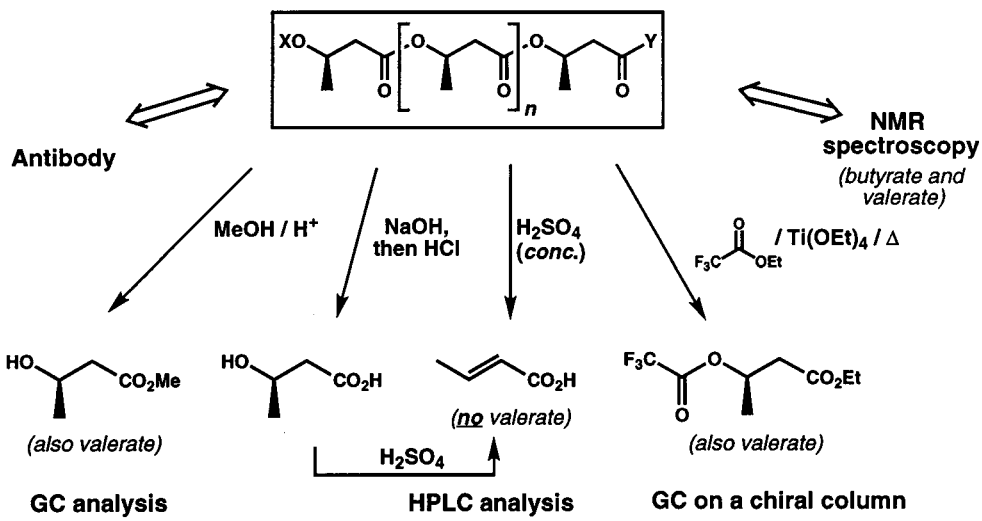


Fig. 12. Analytical methods for the detection of PHB and PHV. The antibody test is the most sensitive one, detecting amounts in the picogram range [99]. By the NMR method only the $CHCl_3$-soluble PHB/PHV is detectable (lower limit in the μg range) [21,100,61]. Esterifying degradation in MeOH/H^+ gives the methyl esters of HB/HV [101,102]. The most commonly used method is degradative elimination to crotonic acid which is detected by HPLC, the Karr method [103]; the conc. H_2SO_4 used destroys HV and does not allow for its detection [104]. For a comparative discussion of the methods see [21]. For the new titanate method see Fig. 14.

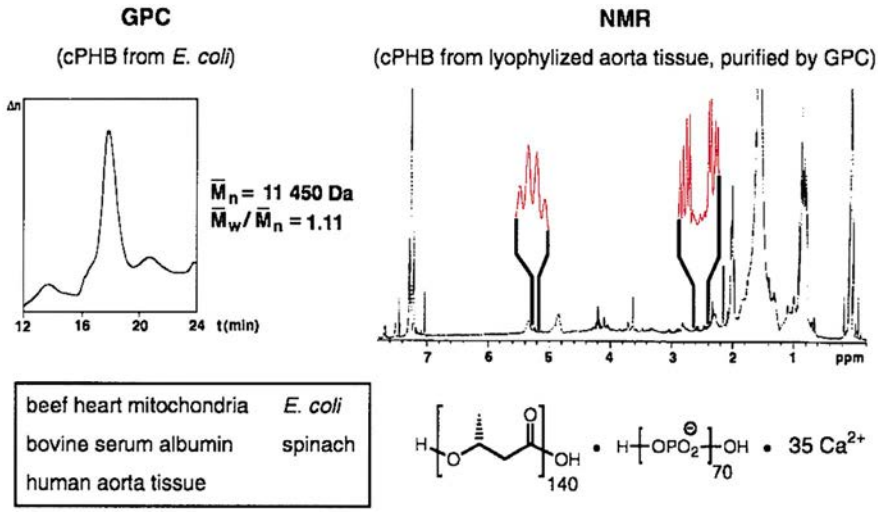


Fig. 13. NMR Detection of PHB ($CDCl_3$ solution) as extracted (with chloroform) from genetically competent *E. coli* and from various eukaryotic sources; GPC trace shows that the PHB from *E. coli* has a rather narrow molecular-weight distribution (for column calibration see Fig. 2); the *formula* of the Ca·PP_i complex from *E. coli* is shown (see also Table 6); the NMR spectrum shows that human aorta tissue contains (besides many other $CHCl_3$-soluble components) oligo- or poly(hydroxybutanoate) [21].

numbers for the same source may vary widely, even when the same analytical method is applied, but there is no doubt that more or less small amounts of PHB are present in all organisms investigated so far.

Although Reusch has checked other possible origins of crotonic acid, such as proteinogenic amino acids [61] (the candidates are threonine, cystein and aspartic acid) or 3-amino-propionic acid (β-alanine), we considered it desirable to have an analytical method available that will (1) cleave ester bonds (→ depolymerization and removal of HB/HV units from the side-chains of serine, threonine, tyrosine, cystein residues), and (2) allow for detection of both enantiomers of 3-hydroxy-butanoic and of 3-hydroxy-valeric acid, without elimination. We chose the titanate-mediated transesterification reaction, which is known not to cause β-elimination [64,65], in such a way that products with characteristic mass spectra are formed and can be detected by a combination of gas chromatography (on a chiral column) and mass spectrometry (GC/MS coupling) (Fig. 14) [66]. With this titanate test we obtained the following results (Table 4): only the $CHCl_3$-extractable fraction of HB can be detected in BSA, which, just like spinach and gorse protein powders, also contains HV; most strikingly, comparable amounts of (*R*) and (*S*) HV are analyzed in BSA and gorse. Since all ester bonds should be cleaved in the course of the Ti-test procedure, there are four possible conclusions: (1) the HB detected by

Table 3
Crotonate test[a] with various biological sources

Organism	μg HB/g dry wt sample	
	$CHCl_3$ insoluble fraction	$CHCl_3$ soluble fraction
E coli (JM 101) whole cells	400[b]	
	4900[c]	1.0[e]
	5500[d]	
Yeast	1200	1.5
Peanuts	1300	4.2
Spinach	4000	12
Beet stem	14 000	30
Gorse	2000	12
Snail hump	680	11
Sheep (intestine	1700	3.2
Cat (muscle)	100	1.6
Bovine lung	280	2.9
Bovine brain	560	15
Porcine heart	360	2.5
Porcine liver	490	2.3

[a] In the crotonate test, the material to be investigated is heated with conc. H_2SO_4 (90–120°C, 0.3–12 h); the resulting crotonic acid is detected by HPLC (0.01 μg/g) [61]. The numbers given for *E. coli* refer to whole cells, those for the other organisms refer to commercial protein powders. The values for the protein samples have been determined by heating to 120°C [61–63].
[b] Experimental conditions: 92°C, 0.3 h.
[c] Experimental conditions: 92°C, 12 h.
[d] Experimental conditions: 120°C, 0.7 h.
[e] Experimental conditions: methanolysis in 3% sulfuric acid [61].

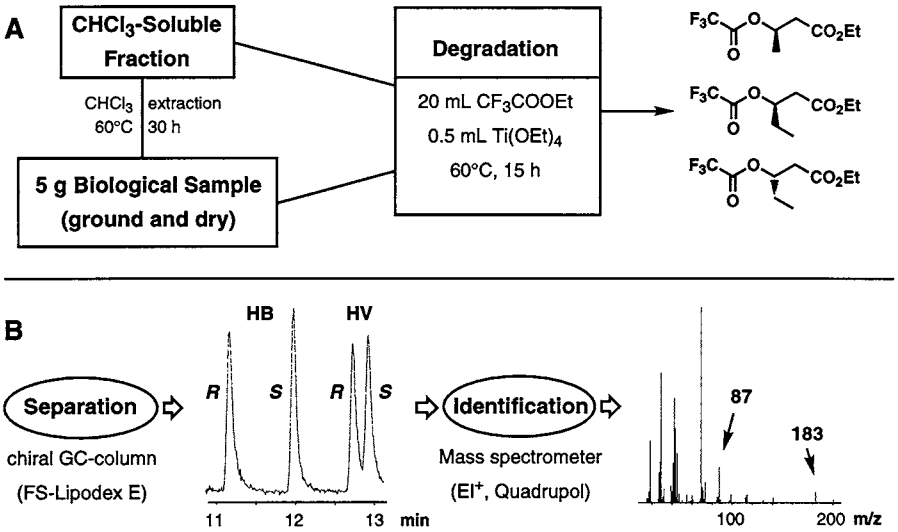


Fig. 14. The titanate test. (A) Procedure for (*R*)/(*S*)-HB and (*R*)/(*S*)-HV detection by titanate-catalyzed degradative transesterification in refluxing ethyl trifluoroacetate (b.p. 61°C). (B) Subsequent detection by separation on a chiral GC column and MS identification of the trifluoroacetoxy ethyl esters [66]. Detection limit ca. 40 ng/g. The scheme shows the procedure for analysis of the $CHCl_3$-extract residue, but it can also be applied directly to the lyophylized biological materials (Table 4).

Table 4
Comparison of crotonate and titanate tests[a] [66]

Sample	μg/g dry wt sample							
	Crotonate test[b]		Transesterification test[c]					
	H_2SO_4	H_2SO_4	$CHCl_3$-soluble			Direct		
	90°C (0.7 h)	120°C (0.7 h)	(*R*)-HB	(*R*)-HV	(*S*)-HV	(*R*)-HB	(*R*)-HV	(*S*)-HV
BSA	20	180	0.3	0.1	0.3	0.3	0.2	0.1
HSA		44				–	–	–
Spinach	80	153	1.4	–	0.4			
Gorse		274	2.3	0.3	0.6			
Porcine liver		83	–	–	–			
Snail		192	0.3	–	–			
Bovine brain	7	13	–	–	–			
Bovine lung		80	0.7	–	–			

[a] The titanate test appears to detect only the $CHCl_3$-soluble fraction, see discussion in accompanying paragraph. Note that HV is found in various samples by this test, and also note that both enantiomers of HV are present, while only the (*R*) form of HB is detected. HSA refers to human serum albumin of the highest purity which is commercially available from Sigma.
[b] Detection limit of crotonate test, 0.01 μg/g.
[c] Detection limit of transesterification test, 0.04 μg/g.

the crotonate test is covalently attached to proteins through amide bonds which are not cleaved by the titanate (e.g. lysine or the N-terminal amino group); (2) HB is part of the peptide backbone (-NH-CHR-CO-O-CH(Me)-CH_2-CO-NH-CHR-CO-, cf. peptolides [34,35,67]); (3) the proteins do not denature in refluxing ethyl trifluoroacetate, and they contain (complexed or covalently bonded) PHB chains entrapped in groves not accessible to $CF_3CO_2Et/Ti(OEt)_4$; (4) the crotonic acid formed with H_2SO_4 does not stem from HB. It is impossible, at this stage, to distinguish between these alternatives. Further investigations are necessary, before we can be sure that there is a post-translational poly(3-hydroxyalkanoylation) of proteins, analogous to other such processes of property modification, some of which are shown in Fig. 15.

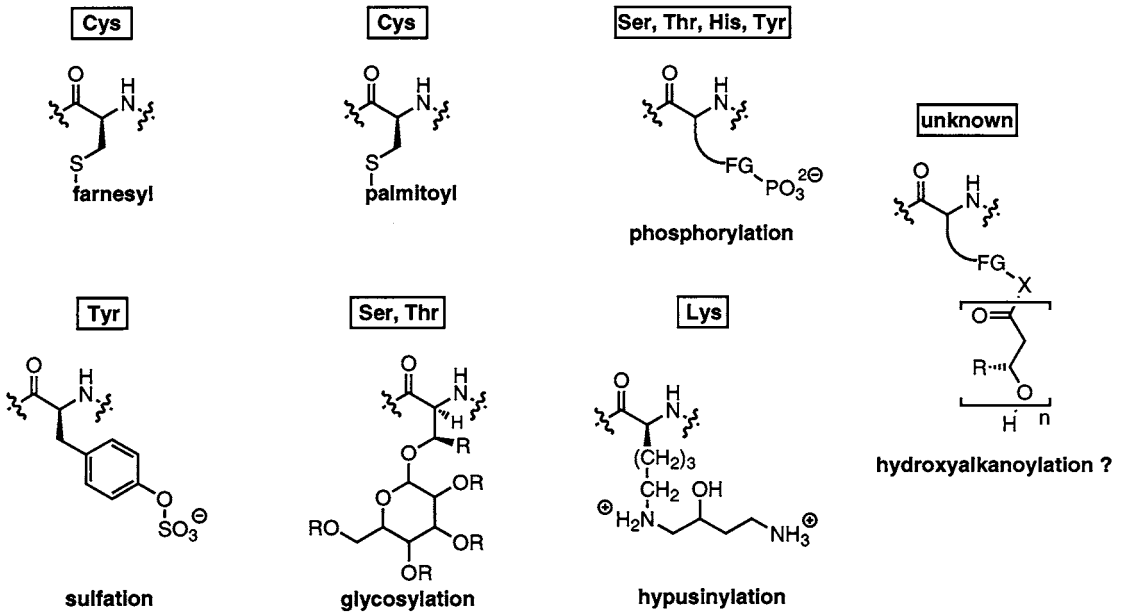


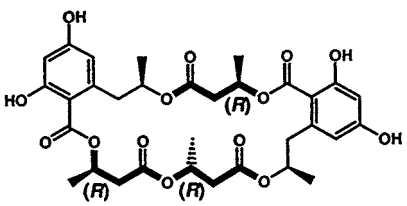
Fig. 15. Proteins undergo post-translational modifications by (enzyme-catalyzed) reactions of side-chain functional groups [86]. Only some of the modified amino acid residues are given here; for a complete list see specialized literature [105]. Is there also a post-translational oligo(3-hydroxyalkanoylation) of proteins?

4. Short-chain or complexing PHB, cell membranes, and ion transport

Two facts emerge upon inspection of a list of biological sources from which the low molecular weight variety of PHB, the so-called complexing or cPHB, has been isolated (Table 5): cell membranes and Ca^{2+} ions are involved. Thus, the shock treatment

Table 5
Occurrence of complexing PHB (cPHB) and of HB derivatives in organisms[a]

E. coli	Inner cell membrane when genetically competent (competence caused by Ca^{2+})
Eukaryotic organisms	Highest concentration in mitochondria; $[Ca^{2+}]$ mitochondrion/cytosol/extracellular, 4:1:10^3
Highest blood serum	5–15 mg/ml, mainly bound to albumin, the transport system for lipids, binding ca. 40% of the serum Ca^{2+} content [84]
Marine fungus	15G156β, cell wall active antifungal and nerve-growth factor activity [85]



[a] Low-molecular-weight PHB and HB derivatives are found in lipophilic environments, such as cell membranes, and in parts of organisms where Ca^{2+} plays an important role. For details see the literature referred to in this table and in review articles [53].

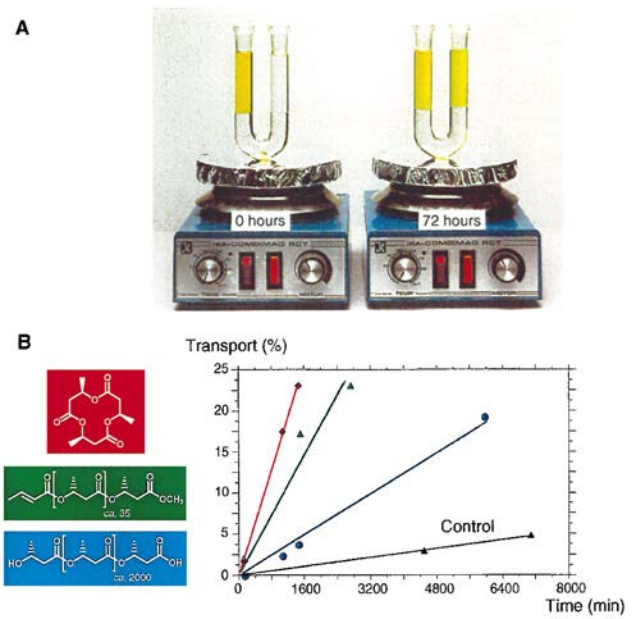


Fig. 16. U-Tube experiments with OHB and PHB show non-ion-selective transport of alkali and alkaline earth ions between aqueous solutions through an organic solvent ('bulk-liquid' membrane), with the yellow picrate counter ions. (A) Experimental set-up. (B) The example shown is the concentration-gradient-driven transport of Cs^+ picrate$^-$ through CH_2Cl_2 by the cyclic trimer of HB (triolide), by a non-monodisperse 35mer and by PHB [17,44]. Simple esters such as methyl butyrate or butyl acetate do not cause ion transport under these conditions.

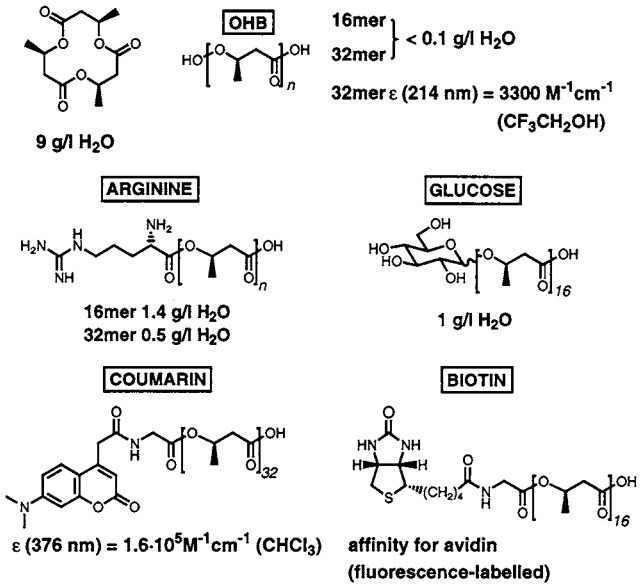


Fig. 17. Various OHB derivatives. Water solubility of various OHBs and of their arginine and glucose derivatives; fluorescence-labelled OHBs [37,51]. The solubilizing and UV-active groups were attached to the O-termini of the OHBs, because there was an indication from patch-clamp experiments that the CO_2H group had to be unprotected for observation of ion transport [28] (cf. Figs. 20 and 21).

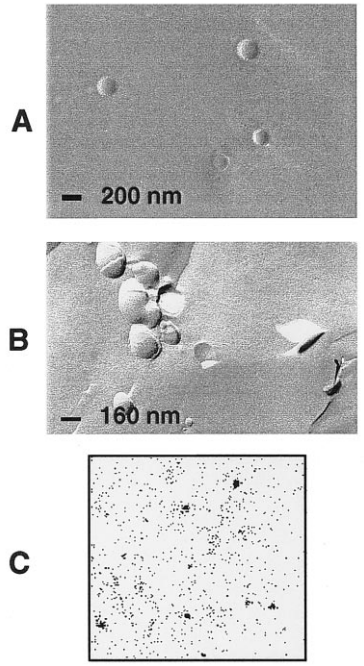


Fig. 19. Structural investigation of unilamellar liposomes with membrane-incorporated OHB derivatives. Electron micrographs and fluorescence-microscopy images of liposomes and liposome aggregations containing OHB derivatives [36,51]. (A) Single, ca. 200 nm diameter vesicles containing OHB. (B) Aggregates of vesicles. (C) Fluorescent liposomes, representation with inverted colors.

transforming *E. coli* to become genetically competent (i.e. able to pass DNA plasmids through the cell membranes) leads to the formation of a complex of the approximate composition $(HB)_{140}{\cdot}(PP_i)_{70}{\cdot}Ca_{35}$ (ca. 10 000 molecules per cell), embedded in the inner cell membrane (vide infra). Also, PHB was extracted from beef-heart mito-chondria in quantities sufficient for NMR detection, and mitochondria, organelles which have been called the 'microorganisms' or 'metabolic factories' within eukaryotic cells, are known to maintain a fourfold Ca^{2+} concentration against the surrounding cytosol. Furthermore, in bovine and human blood cPHB appears to be mainly associated with serum albumin, a protein (ca. 42 g/l of blood) which also contains 40% of the serum's calcium. Finally, macrocyclic, HB-containing compounds with cell-wall-

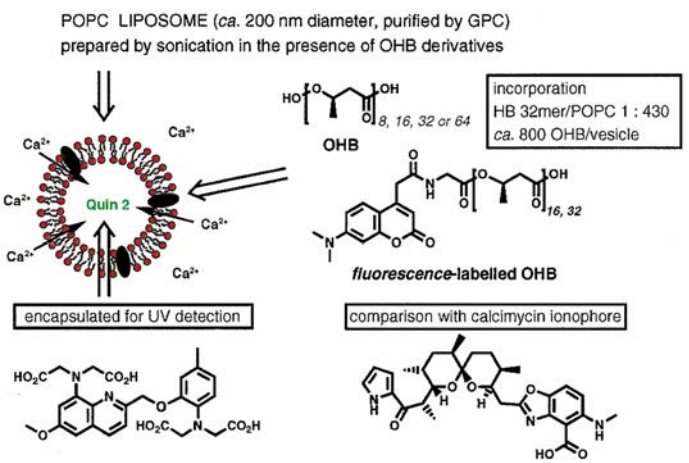


Fig. 18. Incorporation of OHBs and of fluorescence-labelled OHBs into the phospholipid-bilayer membranes of liposomes encapsulating the Ca^{2+}-detecting dye Quin 2 [106,107]; for comparison, liposomes with the ionophore calcimycin [108,109] as membrane component have also been prepared [36,51]. Note that ca. 800 OHB molecules *per* vesicle are incorporated, and thus, fluorescence microscopy allows for detection of ca. 10^{-20} mol of OHB *per* vesicle.

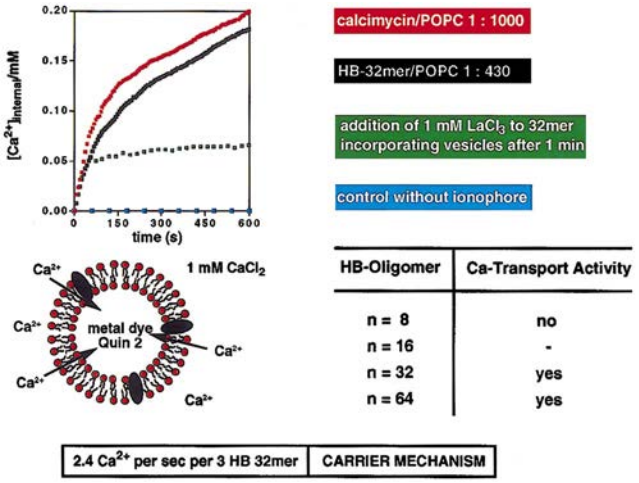


Fig. 20. Concentration-gradient-driven Ca^{2+} transport through liposome membranes mediated by OHB derivatives and by calcimycin (Ca^{2+} influx from a 1 mM $CaCl_2$ solution). Lanthanide ions block the transport almost completely; there is a cut-off of the transport effect with OHB-chain length $\leq$16mer [36,51]. A so-called *carrier mechanism* [110] is derived from kinetic analysis of the transport. POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (lecithine). OHB esters with protected CO_2H group also give rise to Ca^{2+} transport under these conditions (cf. Figs. 17 and 21).

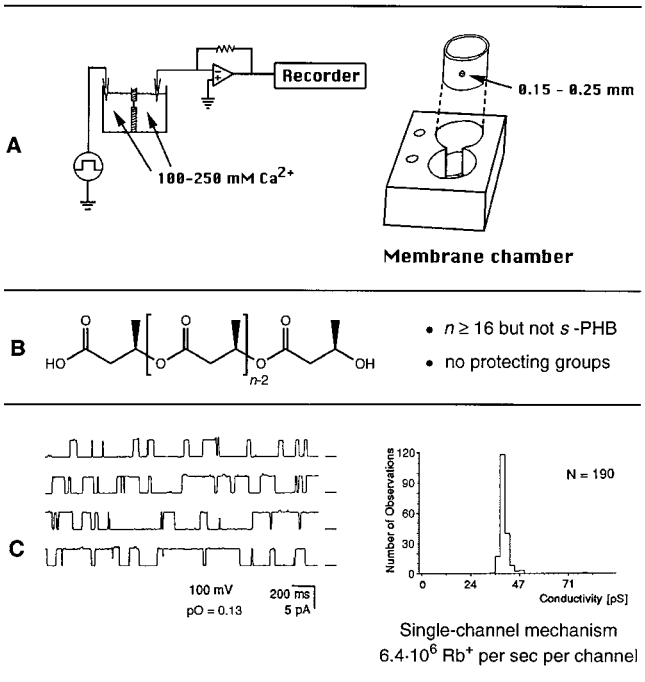


Fig. 21. Patch-clamp technique for studying voltage-driven ion transport through planar phospholipid bilayers and application to OHBs [28]. (A) Experimental set-up. (B) OHBs which give rise to single-channel behaviour of POPC bilayers. The data shown refer to Rb^+ ions; the effect is non-ion selective; a similar concentration of OHB is required to cause single-channel behaviour in the planar bilayer as for carrier transport in liposomes (molar ratio of POPC/OHB 32mer ca. 400:1); again, there is a cut-off with decreasing OHB chain length. A free CO_2H group of the OHB was found to be necessary for observation of single-channel behavior.

related fungicidal activity have been identified. This list of occurrence of cPHB, or of HB/HV/HA derivatives, could be continued. The question arises as to whether cPHB has a dual affinity for both the lipophilic environment of cell membranes, and the positively charged ions, such as Ca^{2+}. Again, the chemist is charged to study the properties of HB derivatives under well-defined conditions, and to provide evidence for such amphiphilic behaviour. We used our linear and cyclic OHBs for investigations of concentration- and voltage-driven ion transport through bulk-liquid membranes [17,44], phospholipid vesicle bilayers [19,36,51] and planar bilayers [28,33,45].

The most simple U-tube experiments (Fig. 16) show that alkali and alkaline-earth picrates are transported by cyclic (particularly the triolide) and linear OHBs and PHB, following the Hofmeister order: ions with the largest radii (Cs^+, Ba^{2+}), i.e. with smallest surface-charge density or highest 'lipophilicity', migrate fastest.

A model system, more closely related to a cell membrane, is the phospholipid vesicle or liposome. In order to improve the bioavailability of the water-insoluble higher OHBs, and in order to detect very small amounts of OHB, we prepared the arginine and glucose derivatives, and the fluorescence- or biotin-labelled compounds shown in Fig. 17. Liposomes were then prepared from the phospholipid POPC in the presence of OHB derivatives and of a Ca^{2+}-complexing UV-active compound (Quin 2) (Fig. 18). Of the methods tested for vesicle generation only the one involving sonication [68] led to OHB incorporation into the vesicle membrane (no spontaneous incorporation was observed). The vesicles, encapsulating Quin-2 and containing OHBs in their bilayer (Fig. 19) were purified by GPC, transferred into a Ca^{2+}-containing solution, and the calcium influx was followed spectroscopically (Fig. 20). The results are: (1) the larger OHBs, such as the 32mer, mediate ion transport at a rate comparable to that caused by the ionophore calcimycin (A23187), the 8- and 16mers do not; (2) the trivalent La^{3+} blocks the transport almost completely; (3) kinetic analysis reveals that a carrier mechanism is operative (ca. 2.5 Ca^{2+} ions/s per three 32mers).

Along a third line of work, we did so-called patch-clamp experiments with OHBs, which were incorporated into planar phospholipid bilayers, to study voltage-induced ion migration between two aqueous solutions (Fig. 21). The characteristic features of this system are: (1) alkali and alkaline-earth ions can pass; (2) only OHBs $\geq$16mer, but not the high-molecular-weight PHB give rise to ion migration, with the 32mer causing a 'textbook' single-channel behaviour;

Table 6
Preparation of POPC planar bilayer containing a calcium–polyphosphate–OHB 128mer complex[a] [33]

1. Mix 128mer of (*R*)-hydroxybutyrate (1 μg/ml) in $CHCl_3$ and $Ca(PP_i)$ (ca. 60mer, finely pulverised)
2. Evaporate solvent
3. Heat residue in a microwave oven (3 × 1 min)
4. Add 10 μg POPC in $CHCl_3$
5. Sonicate in an ultrasound bath (20 min, 0–22°C)
6. Filter through a 0.2-μm Teflon syringe filter
7. Add portion of the filtrate to POPC/cholesterol (5:1) in decane (40 μg/ml)
8. Allow $CHCl_3$ to evaporate
9. Paint remaining mixture in aperture of cuvette for patch-clamp experiment

[a] Commercial inorganic polyphosphate was used to prepare Ca^{2+} polyphosphate; for preparation of OHB see Fig. 1; the OHB concentration in the resulting bilayer is ca. 1/100 of that required for the experiments with OHBs alone, cf. Figs. 20 and 21. Ratio phospholipid/HB_{128}, 10 000:1.

(3) there is a very high flux, for instance 6.4×10^6 Rb^+ ions/s per channel.

The switch from a carrier mechanism for the slow ion transport in the concentration-driven vesicle experiments to a channel mechanism with high throughput in the planar-bilayer experiment under the influence of a rather high voltage (cf. Fig. 20 with Fig. 21) is not unique to OHBs: it has also been observed with calcimycin [69] under the two types of conditions.

Finally, we have used the 128mer OHB (Fig. 1) to prepare a complex with inorganic calcium polyphosphate (ca. 60mer), incorporated it into a POPC/cholesterol (17%) planar bilayer (Table 6) and performed patch-clamp experiments (Fig. 22). The voltage/current plot of the resulting Ca^{2+}-specific ion transport from -120 to $+120$ mV potential across the membrane is indistinguishable from that measured with a PHB·Ca·PP_i complex extracted from the cell membranes of genetically competent *E. coli*. Thus, we have constructed the natural channel complex from totally synthetic materials. Both, the presence of polyphosphate [70] and the existence of a non-proteinaceous PHB ion channel in *E. coli* had been a matter of controversy in the medicinal/biological/biochemical community, prior to publication of our experiments [33].

There is no doubt that at least *E. coli*, possibly also all the other organisms in which PHB has been found, can synthesize cPHB, albeit the enzymes making it are unknown (no homology in the genes of *E. coli* and of the storage-PHB-producing microorganisms, such as *A. eutrophus*, has been found in several independent searches by different people over the years).

5. Possible structures of the ion channels involving oligo(3-hydroxybutanoates) and cPHB

We have demonstrated that OHBs mediate ion transport through hydrophobic environments, such as planar phospholipid bilayers and liposome membranes, in two different ways: by a concentration-gradient-driven *carrier mechanism* or by a voltage-driven *single-channel mechanism*. In addition, there must be

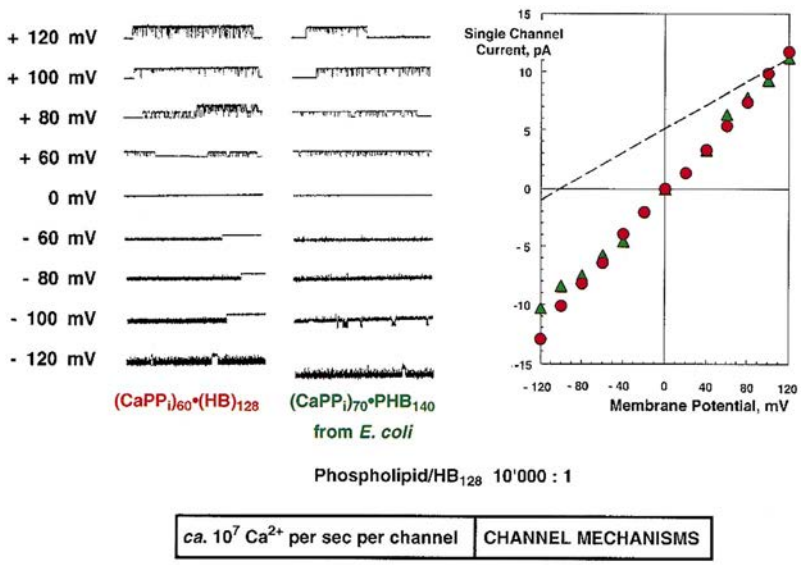


Fig. 22. Patch-clamp electrograms obtained with planar phospholipid bilayers containing the fully synthetic Ca·PP_i·OHB complex (Fig. 21) or the complex from *E. coli*. The superimposable voltage–current correlation is proof for the identity of the two channels [33]. The channel formed by this Ca·PP_i·OHB complex is Ca^{2+}-ion-specific, while the transport of ions by OHBs alone is not (cf. Figs. 16, 20 and 21). The Ca^{2+} transport is inhibited by La^{3+}.

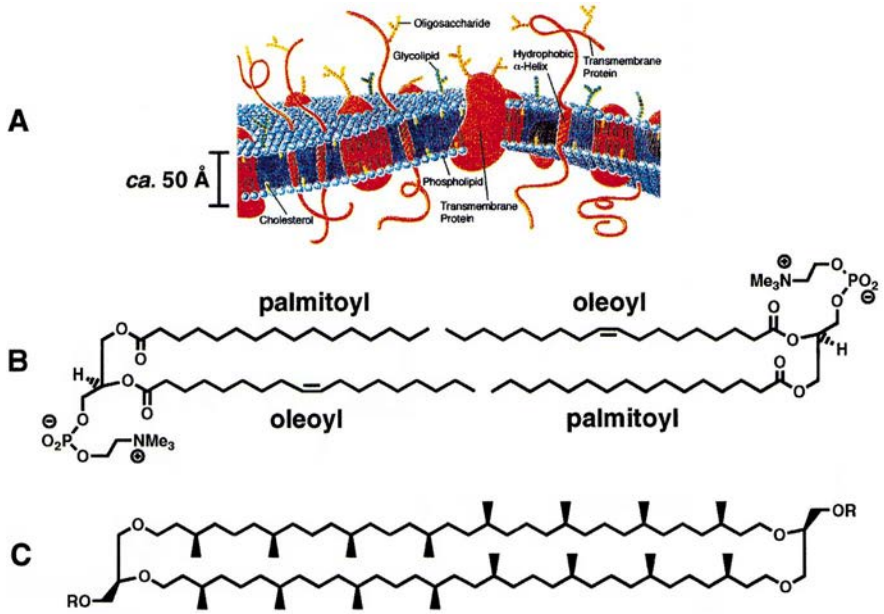


Fig. 23. (A) Schematic representation of the cell membrane. (B) POPC, component of a phospholipid bilayer. (C) Macrocyclic, terpenoid glycerol ether used by *archaebacteria* as cell-wall component. The thickness of a cell membrane, as given by the extension of its components, is ca. 50 Å or 5 nm. The cartoon-type representation of the membrane is taken from Ref. [111].

at least two different supramolecular structures, one for the *non-ion-selective* transport system which is effected by the OHBs alone, and another one for the *ion-selective*, PHB·Ca·PP_i-complex channel. Thus, the *function* of PHB has been clearly established—how about the *structure* of the pore and channel? The challenge was then, to collect information (chemical facts) about possible structures.

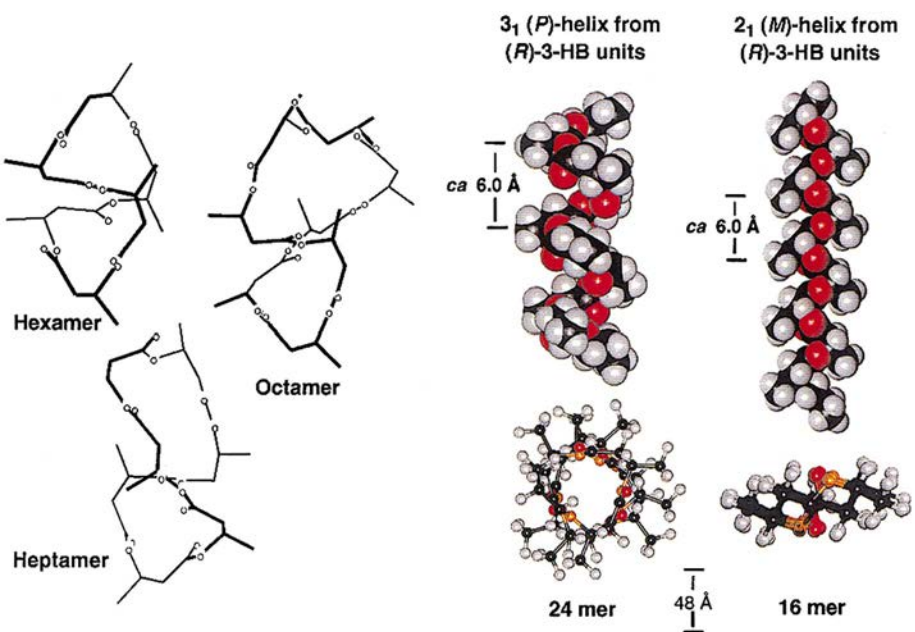


Fig. 24. Two different helices of PHB which have been identified experimentally: one is a left-handed 2_1 helix as present in stretched fibers and *lamellae* (cf. Fig. 25) of the polymer [53], the other is a right-handed 3_1 helix, single turns of which are found in X-ray crystal structures of oligolides [4,5,8,15,22,25,29,39,43,48]. A 24mer in the 3_1- and a 16mer in the 2_1-helical conformation could span a bilayer (ca. 50 Å); in a fully extended form, a 10mer would suffice. For further structures see recent discussions by Marchessault et al. [59,60]. The space-filling models were produced with the program Insight II [112], the presentations of the hexa-, hepta- and octamer with MacMoMo [113].

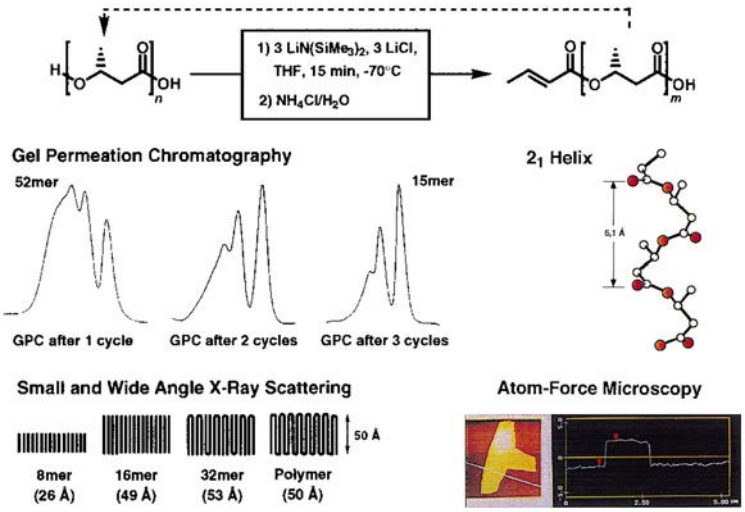


Fig. 25. Determinations of the thickness of lamellar crystallites formed by PHB and OHB. Repetitive chemical 'shaving' of PHB with a very strong base [6,20], X-ray scattering [20] and AFM [24] give a remarkably uniform picture: a preference for ca. 50 Å-thick *lamellae*, consisting of ca. 16 HB units (corresponding to eight 6 Å pitches, assuming a 2_1 helix!); the 32mer must be in a hairpin arrangement. 'Shaving' with ester-cleaving enzymes [114,115] and with tertiary amines [116] has also been reported.

The basic material of which inner and outer cell membranes and—in eukaryots—membranes of organelles (such as mitochondria) and membranes around the nucleus are built consists of compounds with hydrophobic aliphatic chains and hydrophilic charged head groups; the thickness of these membranes is ca. 50 Å (Fig. 23). How does the polyester PHB fit into this environment, to mediate ion transport, and what is known about its shape? The polymer has been shown by stretch-*fiber* X-ray diffraction to form a left-handed 2_1 helix (two HB units for each pitch of 6 Å; right-hand side of Fig. 24). Various independent measurements have revealed that the thickness of lamellar *crystallites* of OHBs and PHB is in the 40–70 Å range, with a preference near 50 Å (Fig. 25), a striking similarity with the cell-membrane thickness. Another folding pattern has been recognized in the single-crystal X-ray structures of cyclic

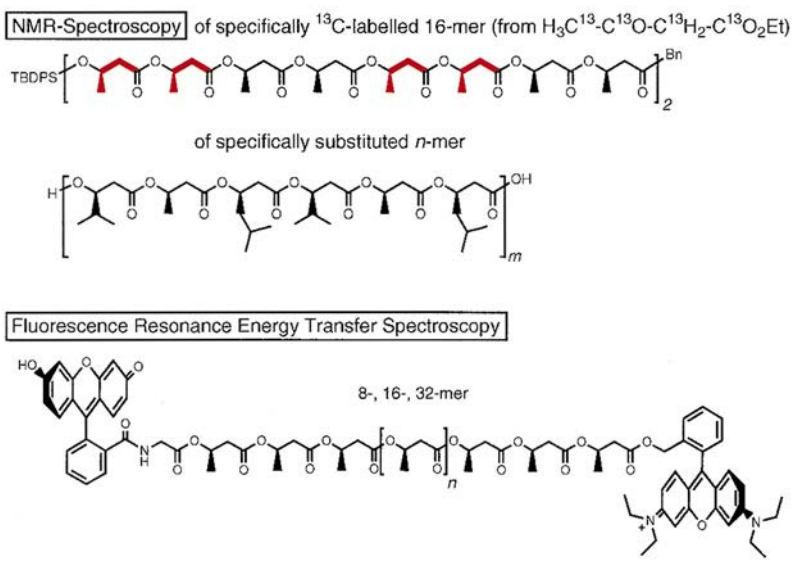


Fig. 26. OHB derivatives and analogs which could be used for determination of the solution structure by Nuclear Magnetic Resonance (NMR) or Fluorescence Resonance Energy Transfer (FRET) spectroscopy. The syntheses of such compounds, specifically labelled by isotopes, by substituents, or by chromophores, is underway in our laboratory [117].

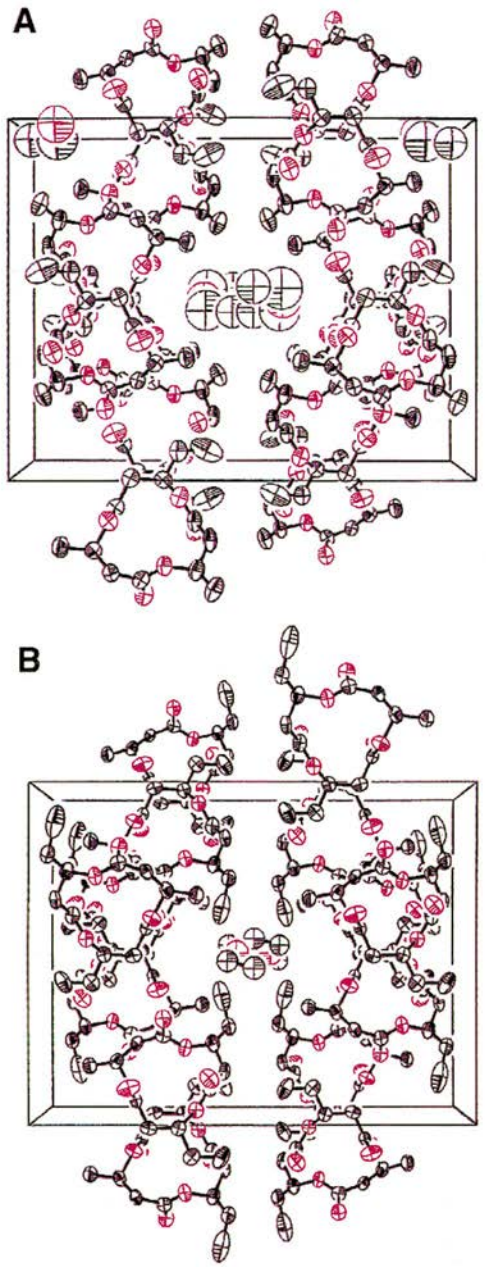


Fig. 27. In the crystalline state, triolides of HB usually stack in tubes, with the C=O dipoles parallel to the stacking direction [18,20,22,29,39]. When triolides of HB/HV ('mixolides') crystallize from ether, space between such stacks is filled with solvent molecules [22]. Interestingly, these crystals, when put under vacuum, turn pale, with loss of the entrapped ether molecules, but without disintegrating (the process is reversible). (A) 'mixolides' with HB/HV content 73:27; 0.5 equiv. Et_2O; (B) 'mixolides' with HB/HV content 33:67; 0.25 equiv. Et_2O in channel.

OHBs: a right-handed 3_1 helix (three HB units for each pitch of 6 Å; left and center of Fig. 24). This 3_1 helix is covered or spiked with methyl groups and thus has a lipophilic surface. The C=O bonds of the 2_1 helix are perpendicular, those of the 3_1 helix parallel to the corresponding helix axis, thus, the carbonyl oxygen atoms of the former are accessible for complexing ions, those of the latter are not (Fig. 24). Unlike the 2_1 helix, the 3_1 helix has a resulting dipole moment along the axis ($\oplus$ pole at the C, $\ominus$ pole at the O-terminus). The chain length necessary to span a phospholipid bilayer with either of these helical conformations (16 or 24 HB residues) shows an intriguing resemblance (or should we say relationship or even correspondence?) with the cut-off chain length in the ion-transport experiments (Figs. 20 and 21).

More relevant to the bilayer environment (a two-dimensional hydrocarbon solvent) is, of course, the *solution structure* of OHBs and PHB/PHV which must not be the same as in the solid state, and which may be solvent-dependent. The larger OHBs and PHB are extremely soluble in chlorinated solvents, such as methylene chloride, chloroform, dichloroethane, and in trifluoroethanol, but not in the more common organic media, such as methanol, ethanol, ethyl acetate, acetone, tetrahydrofuran or the hydrocarbons hexane, octane, benzene or toluene [71], and not at all in water (vide supra, Fig. 17). Investigations by solid-state [12] and liquid-state NMR spectroscopy [22,72,73] and optical measurements (specific rotation $[\alpha]_D$ [22], ORD [74], CD [51] have not provided conclusive evidence for the solution structure. We are therefore preparing specifically labelled OHB derivatives (^{13}C, side chains, chromophors, see Fig. 26), suitable for studying the structure in solution.

Interactions of OHBs with non-polar molecules (cf. central sheet of phospholipid bilayer) as well as with salts (cf. ion transport) have been studied by X-ray crystallography. In the crystal, molecules of HB and HV trimers (triolides, dipole moment 4.6 Debye [15]) stack in antiparallel columns, with the Me/Et groups sticking out laterally (cf. the 3_1 helix in Fig. 24). Co-crystallization of HB/HV triolides with diethylether gives rise to crystals in which the non-polar host molecules are incorporated between such columns (see the 'channels' in Fig. 27A,B). Co-crystallization of HB triolide with alkali and alkaline-earth thiocyanates, on the other hand, produces crystals (Fig. 28) in which the triolide molecules are arranged in kind of channel walls, with their C=O dipoles pointing towards the polar interior which, in turn, contains the cations with part of their water solvation shell; the SCN^- counter ions stick in the 'walls', with their nitrogen atom near the cation; note that the water molecules (red circles in Fig. 28B) in the complex with Ba^{2+} (large blue circles) are positioned in such a way that we could describe the section shown as a water channel with two concentric linings, an inner one of Ba^{2+} $(SCN^-)_2$ and an outer one of HB triolide and THF molecules. In summary, we can state that the complexes shown in Figs. 27 and 28 may be

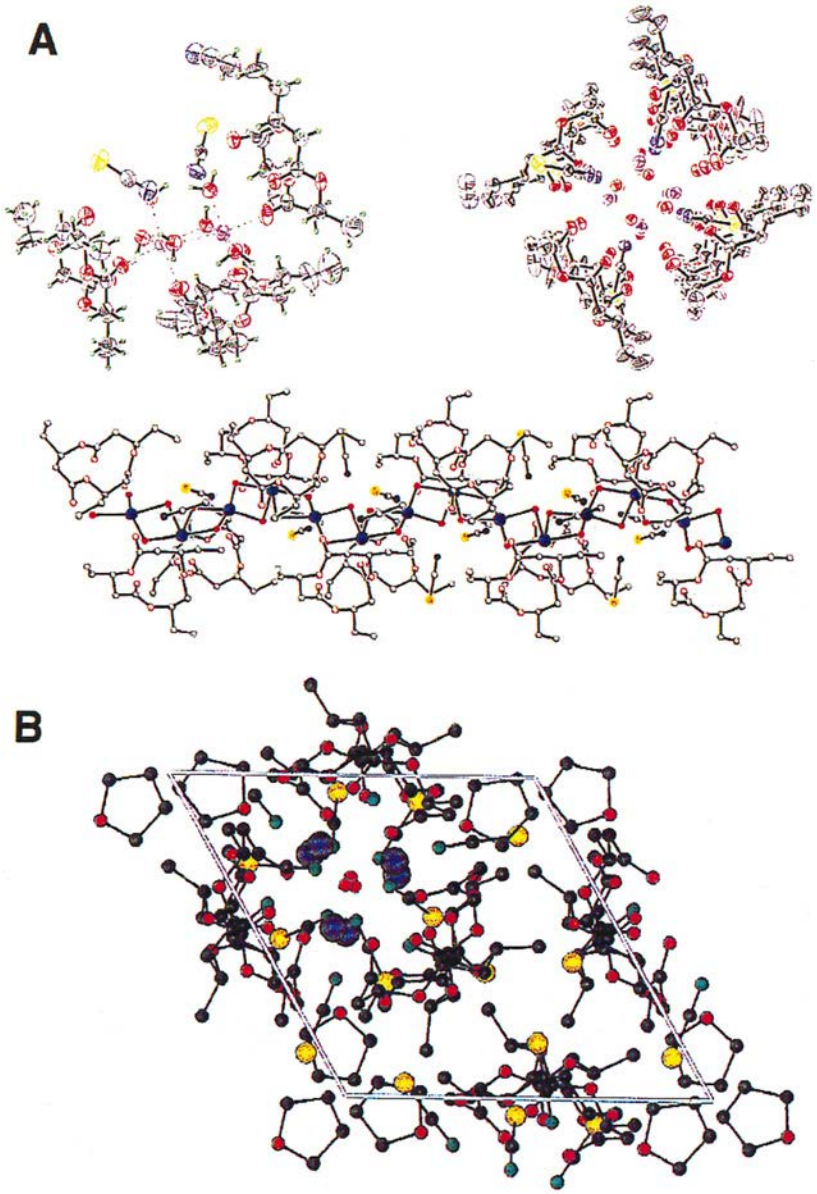


Fig. 28. Co-crystallization of HB triolide with thiocyanate salts (Na^+, K^+, Ba^{2+}) gives complexes which are ideal models for ion channels [9,18,22]: the cations 'enter' with part of their water shell and force the triolide molecules to adapt to their needs, 'flocking' around the channel wall, with the donor carbonyl oxygens providing ligands to the ions within—quite different from the stacked, 'self-satisfying' arrangement found in the absence of ions (cf. Fig. 27). (A) Complex [HV-triolide·NaSCN·$2H_2O$·$0.5CH_3CN$], ligand spheres of Na ions, top and side view of the 'Na channel' [22]. (B) Complex [2HB-triolide·$2Ba(SCN)_2$·$2H_2O$·THF], view along the 'H_2O channel' [18].

considered as structural manifestations of the amphiphilic properties of PHB; the complexes of HB triolide with salts are perfect models for ion channels.

With the structural information gathered so far, we can take a very simplistic—still highly speculative—approach in proposing models for the actual structure of PHB carrier and channel. (1) In the absence of $Ca{\cdot}PP_i$ the 2_1-helical OHBs, which must be at least 16mers (single full passage), better 32mers (hairpin), move around in the hydrophobic bilayer ('two-dimensional hydrocarbon medium', ca. 0.2 mol OHB/mol POPC). (2) There is a tendency for the OHB molecules to aggregate ('crystallize, poor solubility'); three 32mer hairpins form a small pore for carrier-like Ca^{2+} transport (Fig. 29A), an unknown number causes a perturbation of the bilayer structure which (in a strong electric field) leads to single-channel behaviour (Fig. 29B). (3) In the presence of $Ca{\cdot}PP_i$, OHB or cPHB forms a strong complex with the macromolecular salt; considering that a few HB

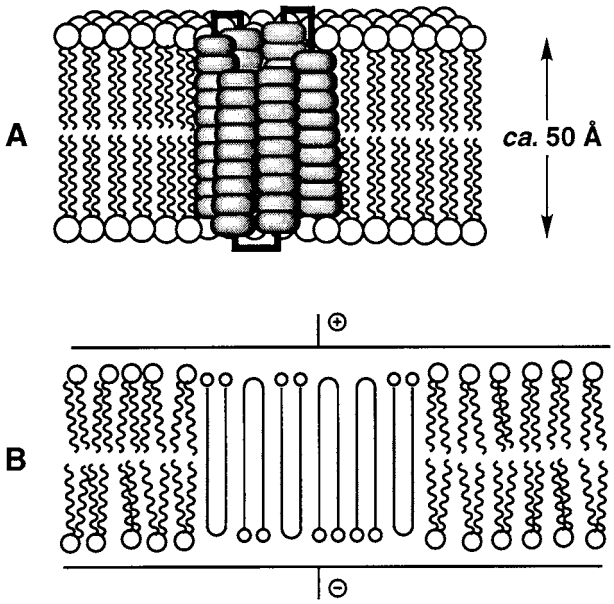


Fig. 29. Proposals for OHB-induced ion-transport mechanisms. (A) A small pore may be formed from three 32mers in a 2_1-helical hairpin conformation, cf. the liposome experiments (Fig. 20) [36,51]. (B) Several 32mers may assemble in the two-dimensionally liquid membrane (under the influence of the strong electric field?), to form an island (a plaque, a perturbation zone) through which ions can rush until disassemblage occurs ('single-channel' opening and closing), cf. the patch-clamp experiments (Fig. 21) [28].

residues will be necessary for each turn, a 150mer could pass the bilayer at least eight times ($8 \times 16 = 128$!), and thus provide a tube or lining around a 70mer, $Ca_{35}(P_i)_{70}$ (Figs. 30 and 31B).

An alternative proposal for the structure of the PHB·Ca·PP_i channel was derived by modelling a complex in which a cPHB forms a helix (17 units per turn,

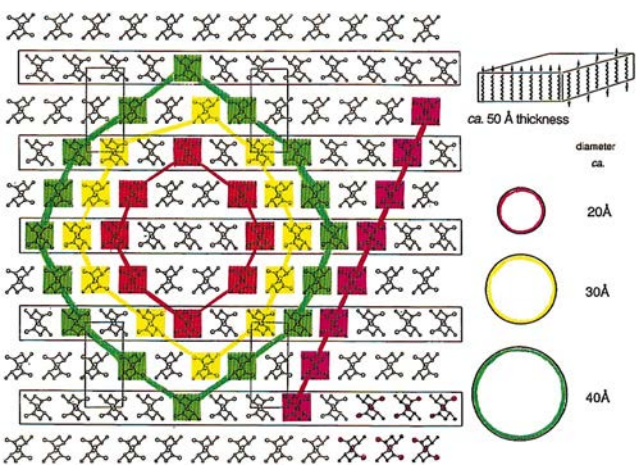


Fig. 30. Tubes could be formed from 8, 12, and 16 2_1-helical lamellar 16mer stretches. These could house a calcium polyphosphate (or even a DNA single- or double-stranded helix?) [20]. Embedded in a phospholipid bilayer, such a complex would provide for Ca^{2+} migration, cf. the Ca-specific single-channel behaviour (Table 6, Fig. 22) [33].

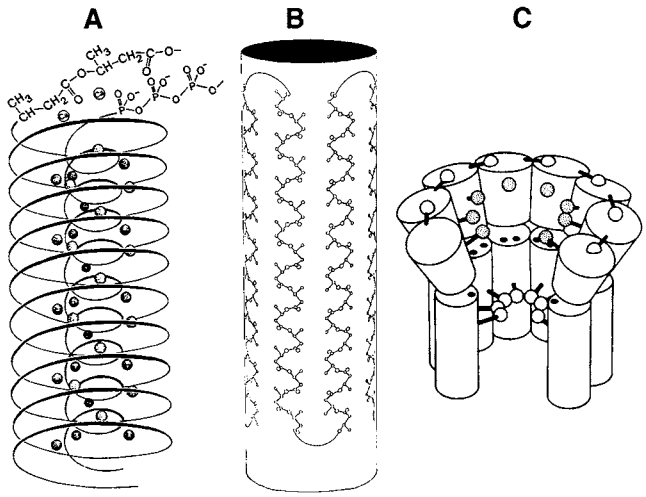


Fig. 31. The spiral model A [33,118] and the column model B [20,22,33] for a Ca^{2+} polyphosphate complex with PHB. Comparison with the alamethicin protein channel C for alkali ion transport [119,120], built by the same design as the column tube (instead of the 2_1 PHB helices, there are protein α-helices).

4 Å pitch) with all C=O groups turned inside and all methyl groups outside, housing a Ca·PP_i helix (Fig. 31A). There is no way, with the data available, to distinguish between the two models. The spiral model shown in Fig. 31A has an ideal inside-polar/outside-lipophilic structure to fit into the lipophilic environment of the bilayer, but it has an entropic disadvantage. The column model is derived from experimental structural parameters obtained with OHBs and PHB; it follows from the structure proposed for the simpler Ca·PP_i-free PHB-ion-transport system, and it is based on the same structural design as well-known protein ion channels (Figs. 31C and 32).

In view of the various hypotheses about the origin of life [75–77] and in view of the work on prebiotic chemistry [78,79] it is intriguing to pose the question

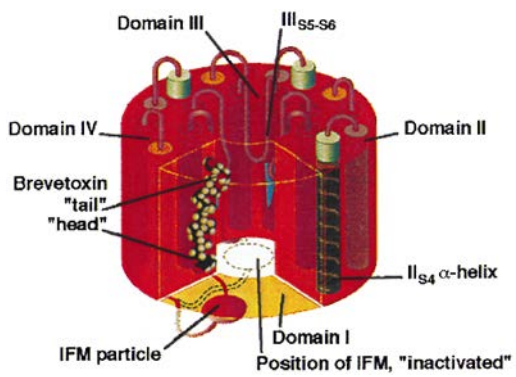


Fig. 32. Model of the structure of a voltage-gated sodium channel consisting of 24 transmembrane α-helices (eight inner, 16 outer ones), taken from Ref. [121]. The design is analogous to that of the alamethicin channel (cf. Fig. 31C) and to one of the proposed structures for the channels involving PHB (cf. Figs. 29A, 30 and 31B).

Table 7
A comparison of biomacromolecules [13,26,34] with their oligomeric analogs, and the building blocks arising from the primary metabolism of organisms [86]. Are oligomers and polymers of HB the first biomacromolecules in the history of life or are they just a fifth, less important class of biomacromolecules, besides proteins, saccharides, isoprenoids, and nucleic acids (not to mention lignins, polyphosphate or sulfur)?

Monomers	Oligomers	Polymers
Metabolism	Regulation, recognition, signalling	Catalysis, storage, transport, structure, information
Hydroxy-alkanoic acids	cP(3-HB) (cf. channel component)	sP(3-HB) (cf. storage)
Amino acids	Oligopeptides (cf. endorphins)	Polypeptides (cf. enzymes, silk)
Monosaccharides	Oligosaccharides (cf. blood group determinants)	Polysaccharides (cf. cellulose, starch)
Acetic acid, isoprene derivatives	Isoprenoids (cf. steroids)	Polyisoprenoids (cf. rubber)
Nucleotides	Oligonucleotides (cf. tRNA)	Polynucleotides (cf. DNA, RNA)

whether PHB-containing channels preceded protein channels or vice versa (Table 7). It is at least imaginable that phospholipids and an activated polymerizable PHB precursor were formed prior to proteins, under prebiotic conditions!

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