46. Channel-Forming Activity of 3-Hydroxybutanoic-Acid Oligomers in Planar Lipid Bilayers

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Dedicated to Prof. Dr. *Edgar Heilbronner* on the occasion of his 75th birthday

(30.X.95)

Monodisperse and polydisperse oligomers and polymers of 3-hydroxybutanoic acid (3-HB) containing 8, 16, *ca.* 28, 32, *ca.* 60, 64, 96, and *ca.* 3000 monomer units were incorporated into palmitoyl-oleoyl-phosphatidyl choline (POPC) planar bilayers. At concentrations of 0.1–5% of oligo(3-HB), the resulting phospholipid bilayers showed typical single-channel behavior for Rb^+ and Ba^{2+} ions, using the patch clamp technique. Thus, channel-forming activity of a pure polyester has been demonstrated for the first time (*Figs. 1, 3,* and *6*). Single-channel activity depends upon the following structural parameters of the 3-HB derivatives: unprotected OH and COOH groups on the chain ends; chain length ≥ 16 monomer units; no *high*-molecular-weight as in P(3-HB). The results are discussed in view of the Ca^{2+}-specific channel formed with the P(3-HP)/Ca·PP_i complex from genetically competent *Escherichia coli* and in view of the ubiquitous occurrence of low-molecular-weight P(3-HB) in prokaryotic and eukaryotic organisms. A simple model for the channel-causing structure is proposed, based on the proven tendency of oligo- and poly(3-HB) to form *ca.* 50-Å thick lamellar crystallites.

1. Introduction. – Poly[(*R*)-3-hydroxybutanoate] (P(3-HB)) is a stereoregular polyester produced as a storage material by prokaryotic microorganisms under growth-limiting conditions. It can accumulate in quantities of up to 90% of the cell dry mass and usually has a molecular weight of *ca.* 10^5 to 10^6 g/mol [1]. Since its discovery by *Lemoigne ca.* 70 years ago [2], P(3-HB) and related poly(β-hydroxyalkanoates) have attracted growing interest both in academia and industry [3]. The main reason for the interest in these polymers is their biocompatibility and biodegradability which allows them to be used as biodegradable substitutes for conventional plastics[3]).

The biosynthesis of high-molecular-weight P(3-HB) in *Alcaligenes eutrophus* has been the subject of the most intensive studies. The enzymes involved have been identified and expressed in other microorganisms and even in plants using genetic technology [5]. The P(3-HB) accumulation in the microorganism begins when the limitation of certain nutrients occurs, and carbon sources are available in excess. Degradation is initiated if

[1]) Part of the Ph. D. Thesis of *A. B.*, Dissertation No. 11239, ETH Zürich, 1995.

[2]) Part of the Ph. D. Thesis of *H. M. B.*, Dissertation No. 10436, ETH Zürich, 1993.

[3]) The copolymer poly[(*R*)-3-hydroxybutanoate/(*R*)-3-hydroxyvalerate] (P(3-HB/3-HV)) has acquired some economic importance, because it has properties similar to those of polypropylene, P(3-HB/3-HV) is produced by large-scale fermentation processes with the bacterium *Alcaligenes eutrophus* on a 1000-tons-per-annum scale by *ZENECA Bio Products* and is marketed under the tradename *BIOPOL*™ [4]. See also [5].

too little carbon source is present, and, hence, acetyl-CoA can be provided by the enzymatic breakdown of P(3-HB).

A. eutrophus is only one of such bacteria, various others are known to produce high-molecular-weight poly(hydroxyalkanoates) [1]. One notable exception is the family of enterobacteria, which are not known to accumulate P(3-HB) as a high-molecular-weight storage material[4]).

Besides storage properties, no physiological significance has been assigned to P(3-HB) in microorganisms until 12 years ago. It was, therefore, rather surprising, when P(3-HB) was detected in the plasma membranes of different types of *Gram*-negative and *Gram*-positive bacteria (among them also in the enterobacterium *Escherichia coli*), albeit in minute amounts, when these bacteria were genetically transformable[5]) [8]. The P(3-HB) detected in these bacteria showed a molecular weight of *ca.* 13,000 g/mol, corresponding to *ca.* 150 monomeric units and was predominantly accumulated in the inner cell membrane. *E. coli* and *Azotobacter vinelandii* also showed a different structure of the inner cell membrane during the stage of genetic competence as detected by freeze-fracture electron microscopy [9]. The P(3-HB) isolated from competent bacteria by $CHCl_3$ extraction has been found to be always accompanied by calcium polyphosphate [10] ($Ca \cdot PP_i$, which is insoluble in $CHCl_3$) of an estimated chain length of 60–70 monomeric units [11]. These findings led to the postulation of a P(3-HB)/$Ca \cdot PP_i$ complex which may be located in the inner cell membrane. Because the complex components could only be detected in genetically competent bacteria, it was speculated that this complex could act as a nonproteinogenic transmembrane ion channel, which is responsible for Ca^{2+}, PP_i, or even DNA uptake of the cell [10]. Further experiments with materials from eukaryotic organisms showed that minute amounts of the low-molecular-weight P(3-HB) appear to be rather ubiquitous, that it is located preferentially in the membranes of mitochondria and microsomes, and that it is extractable, again together with $Ca \cdot PP_i$ [12] [13]. It was also shown that P(3-HB) is present in human blood plasma [14]. The ubiquitous occurrence of low-molecular-weight P(3-HB) in prokaryotic and eukaryotic cells was further confirmed by ^{1}H-NMR methods, and the absolute configuration of the monomeric unit 3-hydroxybutanoic acid could be assigned as (*R*) for the polymers extracted from *E. coli* and from spinach [13] [15].

Recent work of *Reusch et al.* has shown that the P(3-HB)/$Ca \cdot PP_i$ complex found in competent *E. coli* can act as a voltage-activated, Ca^{2+}-selective ion channel. After purification by gel-permeation chromatography, the complex extracted from the microorganism was incorporated in planar bilayers of synthetic phospholipids where it showed many of the characteristic properties of proteinaceous Ca^{2+} channels [16].

By a segment-coupling method, almost monodisperse 3-HB oligomers of type **A** and **B** have been obtained by *Seebach et al.* [17] [18]. It was our initial aim to look for complexation properties of these 3-HB oligomers with synthetic $Ca \cdot PP_i$ in order to obtain a completely synthetic analog of the naturally occurring P(3-HB)/$Ca \cdot PP_i$ complex. Incorporated into a planar lipid bilayer, such an analog would possibly lead to

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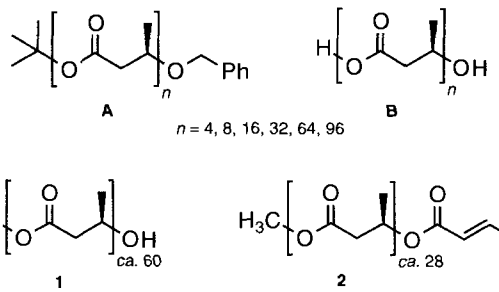
[4]) Under certain nutritional conditions, the *Escherichia coli* strain K12 can produce up to 1.2% of the dry mass as P(3-HB) and even up to 5.8%, if the bacteria contain the R-plasmid RP1; but neither the location of the polymer in the bacteria nor the chain length have been determined [6]. With genetically modified *E. coli*, P(3-HB) may also be produced [7].

[5]) 'Genetic transformation' indicates the process of a cell to take up DNA from the external medium.



similar experimental characteristics as the P(3-HB)/Ca · PP_i complex extracted from competent *E. coli*.

Although we did not detect any significant ion-channel characteristics with synthetic P(3-HB), treated with Ca · PP_i, 3-HB oligomers of a certain chain length were able to induce channel-forming activity in planar lipid bilayers.

Herein, we report for the first time channel-forming activity of a pure polyester, P(3-HB), which, in addition, occurs to be ubiquitous in prokaryotic and eukaryotic cells and seems to play an important physiological role.

2. Single-Channel Measurements. – Our initial goal was to obtain single-channel-forming complexes by mixing the synthetic 3-HB oligomer **B** ($n = 96$) together with synthetic Ca · PP_i *in vitro* in order to obtain an analog of the naturally occurring P(3-HB)/Ca · PP_i complex. We, therefore, followed the procedure of *Reusch et al.* for the reconstitution of the complex from P(3-HB) recovered from competent *E. coli* and synthetic Ca · PP_i [16]. A mixture of the two polymers was sonicated in $CHCl_3$, and a bilayer was formed from the filtered solution and palmitoyl-oleoyl-phosphatidyl choline (POPC) added as described in the *Exper. Part.* However, no channel activity was detected when the final P(3-HB) concentration was in the sub-‰ region (this concentration is sufficient for single-channel conductance, if the naturally occurring P(3-HB)/Ca · PP_i complex is used). However, when the concentration of the 3-HB oligomer **B** ($n = 96$) was raised above 0.1% (*w/w* POPC), and the treatment with Ca · PP_i was omitted, single-channel-like conductances were observed by applying a voltage of 60–120 mV. This stepwise current fluctuation could be obtained repeatedly with a $(3\text{-HB})_{96}$ concentration of 0.1–5% with symmetrical 60 mM RbCl or 250 mM $BaCl_2$ buffer solutions. When the $(3\text{-HB})_{96}$ solution in $CHCl_3$ was treated with Ca · PP_i by ultrasonication, no significantly different behavior was detected. All the oligomers of type **A** and **B** were subsequently tested for single-channel activity. It turned out that, of the tested oligomers, only the ones of type **B** with $n \geqslant 16$ have channel-forming activity in planar lipid bilayers. Typical records and the corresponding conductivity histograms[6]) of the single-channel currents are shown in *Fig. 1*. High-molecular-weight P(3-HB) ($M_w = 4.4 \cdot 10^5$, $M_n = 2.6 \cdot 10^5$) as well as oligomers **B** with $n \leqslant 8$ were not able to induce channel openings, only erratic current fluctuations (which can be also observed in control experiments on POPC membranes without 3-HB oligomers) were observed (*Fig. 2*). It was also not possible to obtain

[6]) For better comparison of the measurements done at different voltages, the values for the conductivity instead of the measured current are given in the histograms.

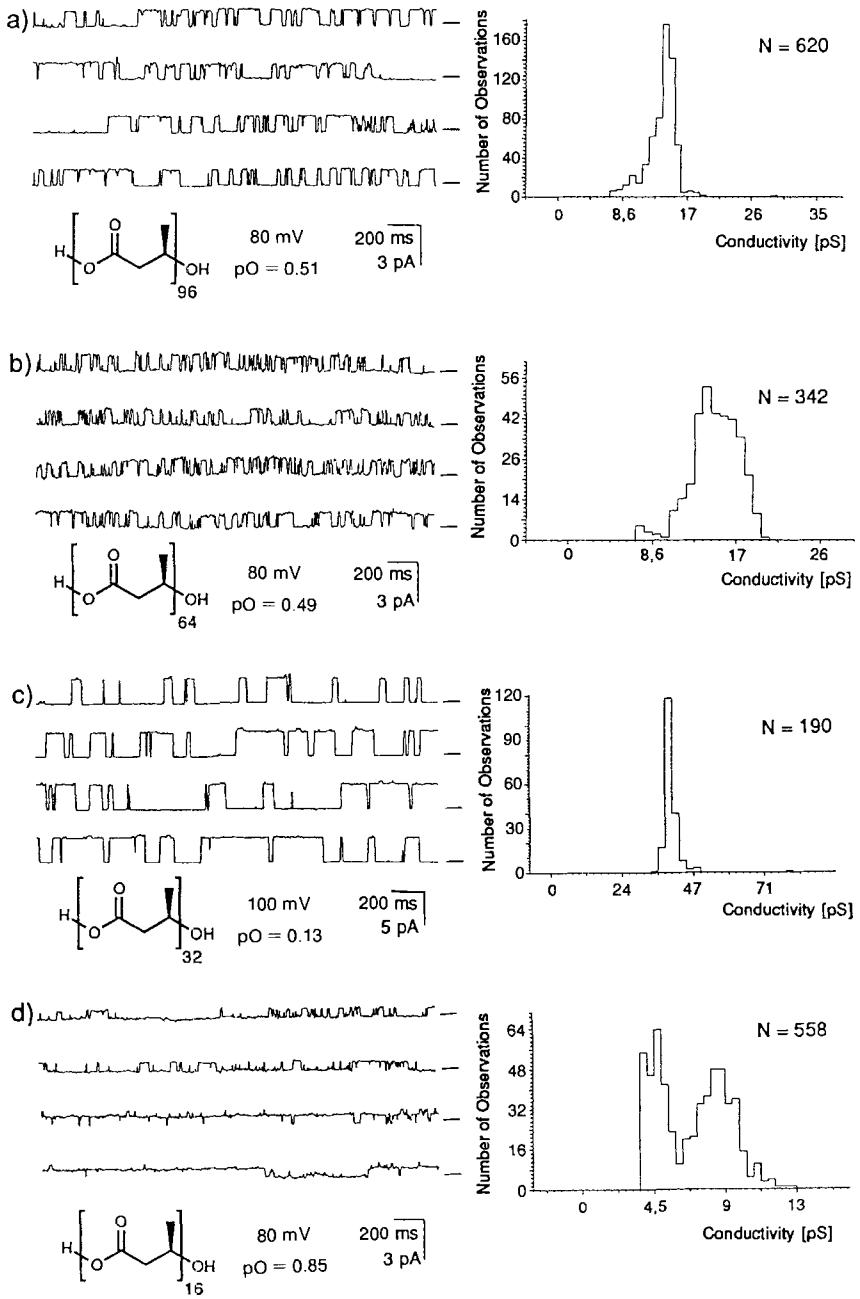


Fig. 1. *Single-channel currents of 3-HB oligomers. Left:* representative current fluctuations obtained, when the given voltage was applied at a planar bilayer made from POPC containing 0.1 to 1% *w/w* of the indicated synthetic oligomer **B**. The solutions were symmetrical 60 mM RbCl, 5 mM $MgCl_2$, 10 mM Hepes/CsOH, pH 7.2. The solid horizontal bar in each record indicates the current level with the channel closed. pO is the probability of the channel in the open state. *Right:* corresponding conductivity histograms. N indicates the total number of observations that have been analyzed.

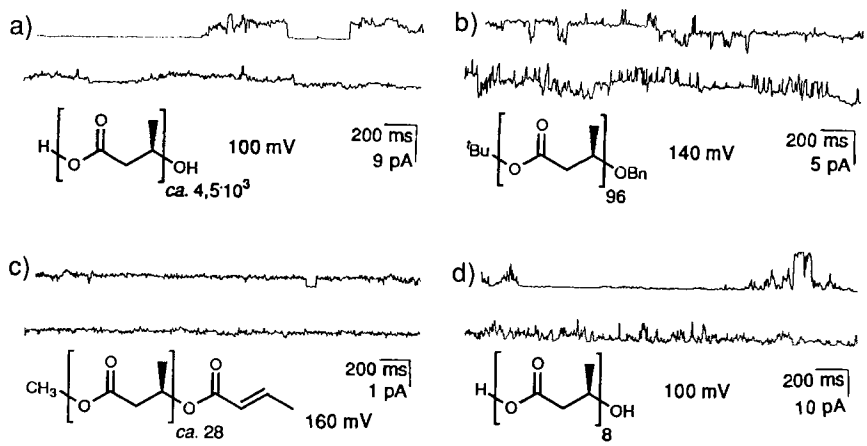


Fig. 2. *Representative electrograms obtained, when the given voltage was applied at planar bilayers made from decane solutions of POPC mixed with the indicated 3-HB derivatives* (conditions as in *Fig. 1*). The lipids were mixed with 5% *w/w* (*a–c*) or 1% *w/w* (*d*) of the corresponding 3-HB derivative.

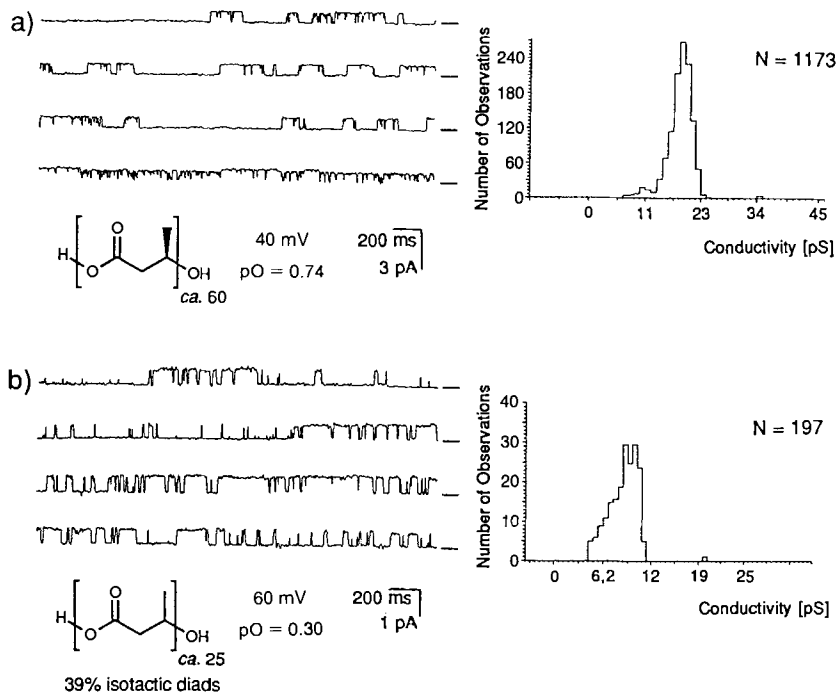


Fig. 3. *Representative current fluctuations and corresponding conductivity histograms obtained, when the given voltage was applied at a planar bilayer (POPC) which contained 5% w/w of broadly distributed 3-HB oligomers* (conditions as in *Fig. 1*)

stepwise current fluctuations by applying a voltage to membranes containing up to 5% of the 3-HB oligomers of type **A** as well as of the broadly distributed oligomer **2**. The 3-HB oligomers do not have to be monodisperse in order to induce single-channel activity. A sample of low-molecular-weight P(3-HB) **1** (X_n *ca.* 60), which was obtained by acid-catalyzed degradation of high-molecular-weight P(3-HB), showed the behavior typical for oligomers **B** ($n \geqslant 16$) when incorporated into a planar bilayer (although only at a concentration of 5% (*w*/*w* POPC)) as shown in *Fig. 3,a*. Low-molecular-weight P(3-HB) does not even have to be strictly stereoregular in order to have channel-forming activity: a planar lipid bilayer with 5% (*w*/*w* POPC) of broadly distributed (M_w *ca.* $4 \cdot 10^3$, M_n *ca.* $6 \cdot 10^2$) P(3-HB) with 39% isotactic diads[7]) showed conductivity at the single-channel level (*Fig. 3,b*).

The general requirements for 3-HB oligomers, in order to show single-channel behavior when incorporated into a planar bilayer, are shown in *Fig. 4*. Single-channel recordings, as shown in *Fig. 1*, have been obtained with the appropriate oligomers at different

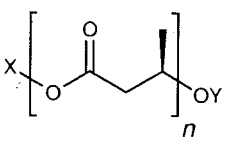


Fig. 4. *General formula for 3-HB oligomers of type* **A** *and* **B** *that have been examined in planar lipid bilayers.* Single channel recordings were obtained with $n \geqslant 16$, X = H, Y = H. No single-channel behavior is obtained for $n \leqslant 8$ (X and Y variable), or for X = *t*-Bu and Y = $PhCH_2$ (*n* variable) as well as for high molecular weight P(3-HB) with $n > 3000$.

voltages and were reproduced several times. Nevertheless, the channel activity thus obtained was too unstable for a reliable, more precise characterization such as selectivity or blocking experiments. With the synthetic oligomers **B** ($n = 16$–96) only a restricted number of membranes have been obtained, that were stable enough for determination of the voltage-current relationship over a broad range. The current shows a linear trend from the applied voltage, when the measurements at the different voltages are conducted on one single membrane (*Fig. 5*). Measurements on more than one membrane for one and the same 3-HB oligomer led to quite different mean values of the conductivity as can be seen by comparison of *Figs. 1* and *6*: membranes containing 0.5% of the oligomer $(3\text{-HB})_{96}$ showed a mean conductance of *ca.* 14 pS at 80 mV (*Fig. 1,a*), whereas an experiment with a membrane containing 5% $(3\text{-HB})_{96}$ gave a value of *ca.* 22 pS at 90 mV

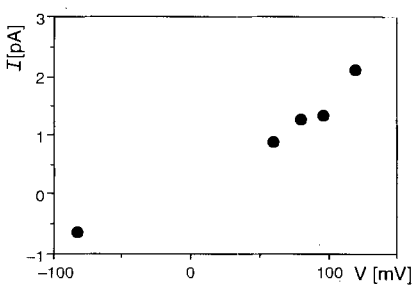


Fig. 5. *Current-voltage relationship for single channels obtained with the deprotected oligomer (3-HB)*$_{96}$ (0.5% *w*/*w* in POPC; experimental conditions as in *Fig. 1*). The currents at the different voltages were obtained from one active membrane. The current displays a linear trend depending upon the applied voltage; however, a statistical correct analysis cannot be done because of the small number of measurements.

[7]) The sample was obtained by methylaluminoxane-catalyzed polymerization of *rac*-β-butyrolactone and fractionation of the obtained polymer and was kindly provided by *R. H. Marchessault*, McGill University, Montreal, Canada [19].

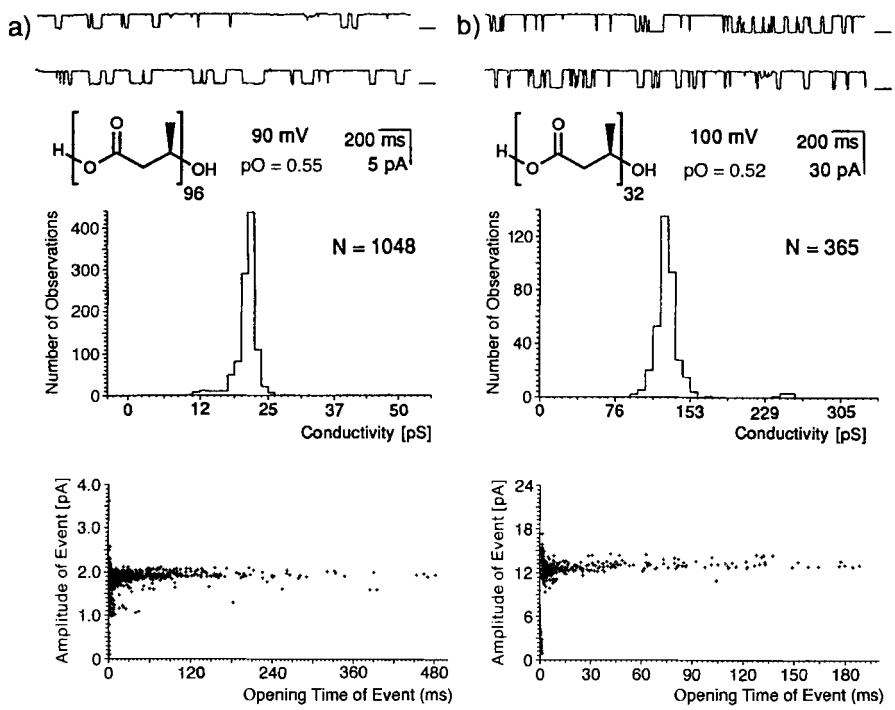


Fig. 6. *Single-channel recordings obtained, when the indicated voltage was applied at planar bilayers from POPC solutions treated with the given 3-HB oligomers* (conditions as in *Fig. 1*). The corresponding conductivity histograms and a plot of the amplitude *vs.* opening time of the recorded events is given below. *a*) 5% *w*/*w* $(3\text{-HB})_{96}$. *b*) 1% *w*/*w* $(3\text{-HB})_{32}$.

(*Fig. 6,a*). This effect seems not to be based on the different P(3-HB) concentrations used. In two independent experiments at 100 mV with membranes containing each 1% of the oligomer $(3\text{-HB})_{32}$, single channels with a conductance of *ca.* 40 pS (*Fig. 1,c*) as well as *ca.* 130 pS (*Fig. 6,b*) could be obtained. In each of the two experiments, only one conductance level was observed thus ruling out that the higher conductance in the second experiment (*Fig. 6,b*) is due to a multiple opening of the channel observed in the former one (*Fig 1,c*).

3. Discussion. – Almost all of the membranes showing single-channel-like behavior exhibited only one discrete conductance level. Mutiple openings, characteristic for natural channel proteins or the P(3-HB)/Ca·PP$_i$ complex extracted from competent *E. coli* [16], were detected only very rarely.

Currents across a bilayer at the single-channel level caused by synthetic ion channels are well known, particularly for synthetic peptides (see *e.g.* [20]). Artificial non-peptide ion channels have been reported as well [21] [22].

Altogether the single-channel records obtained with the selected 3-HB oligomers are similar to channels obtained from ionic and non-ionic detergents like *Triton X-100* or *SDS* [23]. The appearance of single-ion channels was also reported in unmodified lipid-bilayer membranes made from 1,2-distearyl-glycero-3-phosphocholine at the phase-transition temperature [24]. It has to be mentioned that lipid bilayers made from phosphatidyl

choline show an increase in ion-permeability at the chain melting phase transition, while this phenomenon is usually not observed with membranes made from other lipids like, *e.g.*, 'phosphatidyl ethanol amine' [25]. As phosphatidyl choline bilayers are practically impermeable for ions above and below the transition temperature, *Marsh et al.* attributed the enhanced permeability at the phase-transition temperature to areas of mismatch in molecular packing which occur at the interfacial regions between fluid and ordered domains [26]. This model is supported by computer simulation of bilayers at the phase-transition temperature [27].

As outlined above, the occurrence of single-channel behavior for amphiphilic non-peptide compounds is well established. To our knowledge, P(3-HB) is the first polyester that exhibits the ability to make bilayers permeable to ions[8]); almost all of the reported synthetic 'pore-builders' are polyether derivatives[9]). It is also striking that only oligomers showing a similar molecular-weight range to the low-molecular-weight P(3-HB) isolated from competent *E. coli* are able to induce single-channel-like activities in synthetic planar bilayers, and that the effect is only observed when the end groups are unprotected.

Considering the high concentration of P(3-HB) required (0.1–1%) for the detection of stepwise current fluctuations, it is unlikely that single P(3-HB) molecules can cause pores in the planar bilayer responsible for the ion flux; the P(3-HB) content in the membrane has to pass a certain critical value in order to make it permeable for ions. This is reminiscent of the observation in *E. coli*, where freeze-fracture electron micrographs revealed an alteration in the membrane structure with increasing P(3-HB) content [9].

It is known that P(3-HB) forms lamellar crystallites when crystallizing from dilute solutions[10]). The lamellar thickness of such crystallites is in the range of 40 to 60 Å depending on the crystallization conditions [32]. By transmission electron microscopy, fibre X-ray scattering, and atomic force microscopy measurements, it has been shown that the synthetic 3-HB oligomers **A** and **B** also crystallize in the form of lamellar crystals with a lamellar thickness of *ca.* 50 Å for $n \geqslant 16$, and *ca.* 26 Å for $n = 8$ [18] [33]. Furthermore, the favorable dihedral angles along the oligo(3-HB) backbone were determined from a set of crystalline, cyclic 3-HB oligomers. The library of angles thus available has been used to model a 2_1 helix of (*M*)-helicity as well as a 3_1 helix of (*P*)-helicity [17] [34]. The 2_1 helix is in good accordance with the crystalline structure of P(3-HB) derived from fibre X-ray-scattering measurements [35] and has a pitch of *ca.* 6 Å. Thus, the lamellar thickness of *ca.* 50 Å would correspond to a 2_1 helix consisting of at least 16 monomeric units.

The thickness of the hydrophobic part of a planar lipid bilayer obtained from a decane solution can be determined from electrical capacitance measurements and has been reported for phosphatidyl choline membranes as *ca.* 48 Å [36].

With these data in hand, the occurrence of stepwise current fluctuations on bilayers treated with 3-HB oligomers could be explained in the following way: P(3-HB) has the

[8]) Interestingly, P(3-HB) has been shown to form ion-conducting Li-salt complexes (the preferred electrolyte for certain Li batteries is a composite of the polyether polyethyleneglycol (PEG) with $LiClO_4$) [28]. Additional evidence of the salt-complexing abilities of P(3-HB) was provided by crystal structures obtained from complexes formed by the cyclic trimer of 3-HB and (*R*)-3-hydroxypentanoic acid with alkali and alkaline-earth salts [29]. The ability of linear and cyclic oligomers of 3-HB to transport alkali and alkaline-earth metal ions across liquid organic membranes has also been reported [30].

[9]) An interesting exception is the *C*-heptadecyl-substituted calix[4]resorcine which has been described as a pore builder by *Kobuke* and coworkers [22].

[10]) For review articles on the structure of crystalline polymers, see *e.g.* [31].

material property to form lamellar crystallites with a thickness of *ca.* 50 Å. Such crystallites should also be formed during evaporation of the $CHCl_3$ from a P(3-HB)/phospholipid/decane mixture. These hydrophobic crystallites appear to fit properly into a planar bilayer, where the incorporation of a P(3-HB) particle would be stabilized by formation of H-bonds between the free end groups of the oligomers of type **B** and the polar head groups of the phospholipids. Such an incorporation should take place preferentially, if the 3-HB oligomer consists of at least *ca.* 16 monomeric units, if the end groups are not protected, and if a reasonable number of carboxylate and alcohol termini are available for the 'anchoring' of the P(3-HB) particle in the phospholipid bilayer (which is not the case for high-molecular-weight P(3-HB)). A schematic representation showing the oligomer **B** ($n = 32$) incorporated into a phospholipid bilayer is given in *Fig. 7.*

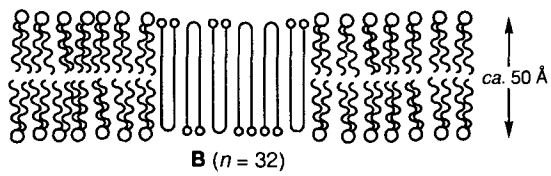


Fig. 7. *Schematic representation of the oligomer* **B** ($n = 32$) *incorporated into a planar phospholipid bilayer*

This would mean that a membrane is formed that contains 'islands' of crystalline P(3-HB) within the liquid crystalline phospholipid phase. This is reminiscent of the morphology of a membrane at the phase-transition temperature. At the interfacial regions between the phospholipids and the P(3-HB) crystallites, discrete current fluctuations could occur, when a certain voltage is applied, similar to the phenomena observed for phospholipid bilayers at their phase-transition temperature [24] [26] [27]. At this point of our investigation, this is a speculative model which is being tested by further experiments.

It is worth mentioning here that *Reusch et al.* were able to reconstitute active Ca^{2+}-selective ion channels from competent *E. coli* into planar membrane, where the final concentration of P(3-HB) with regard to the phospholipids was far below 0.1% [16]. Therefore, the channel activity observed with the $CHCl_3$ extract from genetically competent *E. coli* cannot be induced only by the P(3-HB) component of the complex extracted from the bacteria. Models for this naturally occurring ion channel have been proposed [10] [12] [13] [16] [18] [37], but the exact structure has not yet been resolved.

The authors thank Prof. *G. Stark* and *U. Zeidler* (University of Konstanz) for the possibility to conduct some experiments in their laboratory. For financial support, we thank the ETH-Zürich (ETH project No. 0-20-007-90), the *Sandoz Pharma AG* (CH–4002 Basel), *NIH* grant GM 33375 (*R. N. Reusch*), and *NSF* for a *CAA* award to *R. N. Reusch.*

Experimental Part

1. *Synthesis of 3-HB Oligomers.* The P(3-HB) derivatives **A** and **B** were synthesized as described in [17] [18].

2. *Acid-Catalyzed Partial Depolymerization of P(3-HB).* A mixture of 20 g of P(3-HB) (233 mmol of HB units) and 737 mg (3.88 mmol) of $TsOH \cdot H_2O$ in 200 ml of 1,2-dichloroethane was heated to reflux for 4 d. After cooling to r.t., the mixture was washed successively with sat. aq. $NaHCO_3$, 1 M HCl, and brine (twice), then dried ($MgSO_4$), and filtered. After evaporation, Et_2O was added to the solid residue, and the suspension was stirred vigorously; the solid was filtered off and washed with Et_2O. Drying under high vacuum gave 16.0 g (80%) of a white powder **1** with a m.p. of 149–150°. For the determination of the degree of polymerization, a sample was dissolved in $CHCl_3$ and treated with an excess of CH_2N_2 in Et_2O. The solvent was removed after fading of the yellow color. The number of average degree of polymerization (X_n) was determined as *ca.* 60 from the ratio of the area under the signals for the methyl ester and the 3-Me groups (C–Me/O–Me) obtained by ^{1}H-NMR (*Varian Gemini 200*, 200 MHz, solvent $CDCl_3$).

Partial Depolymerization of P(3-HB) by Pyrolysis. High-molecular-weight P(3-HB) (50 g) was heated to 210° for 100 min under Ar, cooled to r.t. and dissolved in $CHCl_3$. After treatment with activated charcoal, the solvent was removed, and the residue suspended in Et_2O and stirred vigorously. The solid was filtered off and washed with Et_2O. Drying under high vacuum yielded 38.2 g (76%) of a white powder. Treatment with an excess of CH_2N_2 as described above gave the corresponding methyl ester **2** with $X_n \approx 28$ as determined by ^{1}H-NMR.

3. *Planar Bilayer System*[11]). Synthetic 1-palmitoyl-2-oleoylphosphatidyl choline (POPC) was purchased from *Avanti Polar Lipids Inc.* All experiments were conducted at r.t. The experimental setup consisted of a PVC 'bilayer chamber' (model *BCH-13*) and a 'bilayer cuvett' (model *CUV-13*) made of *Delrin* from *Warner Inst.* The side to which the voltage was supplied was designated as the '*cis*'-side; the '*trans*'-side was held at virtual ground. Positive currents in the *Figs.* reflect positive charges moving from the '*cis*'- to the '*trans*'-side. Currents were measured with an *Axopatch 200A* integrating patch clamp and *CV-201A* headstage from *Axon Inst.* Channel currents were filtered at 1500 Hz and recorded on video tapes after pulse co-modulation (*PCM-200, A. R. Vetter Co*). The analysis was done with a personal computer and the *pCLAMP* version 5.5 software (*Axon Inst.*). The currents were filtered at 100 Hz (*Frequency Devices*) and digitalized at 1 kHz with *FETCHEX* (*Axon Inst.*). Channel currents were measured and analyzed with *FECHAN* and *PSTAT* (*Axon Inst.*) Symmetrical buffer solns. were used.

In a typical experiment, 1 mg of POPC (dissolved in *ca.* 100 µl of $CHCl_3$) was mixed with a soln. of 2 to 10 µg of the corresponding 3-HB oligomer in *ca.* 1 ml of $CHCl_3$. After the addition of 25 µl of decane, the $CHCl_3$ was evaporated in a stream of N_2. The resulting lipid soln. was used to form a bilayer across a 0.25-mm aperture in the *Delrin* cup separating two aqueous bathing solns. After the voltage (as indicated in the *Figs.*) was applied for periods of several seconds to minutes, discrete current fluctuations could be observed. Often, a membrane breakdown occurs without prior indication of single-channel behavior.

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