Oligoesters of (*R*)-3-Hydroxybutanoic Acid: Transmembrane Transport of Ca^{2+} across Vesicle Bilayers

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ABSTRACT: Oligomers of (*R*)-3-hydroxybutanoate (OHB) have previously been shown to generate nonselective single-channel conductance through planar phospholipid bilayers in patch-clamp experiments (voltage induced). In view of the ubiquitous occurrence of low molecular weight, complexed polyhydroxybutanoate in essentially all living organisms, we have now studied the effect of OHBs on the Ca^{2+} ion permeability through vesicle bilayers (concentration-gradient induced). Thus, 8-, 16-, 32- and 64-mers of HB with and without O-terminal coumarin fluorescence label were incorporated in Quin-2-encapsulating vesicles (ca. 200 nm diameter) by sonication. The physiologically most important Ca^{2+} transport into the vesicles was studied by following the Ca^{2+}-dependent Quin-2 absorption at 264 nm. There was no or erratic Ca^{2+} transport detectable with the 8- and 16-mer, whereas the longer-chain OHBs tested (32- and 64-mers) showed a transport behavior with turnover numbers in the range of 2−3 s^{-1}, very similar to calcimycin. Furthermore, the OHB-mediated Ca^{2+} transport was inhibited by the presence of La^{3+}. A carrier-like mechanism is suggested, involving three OHB molecules in the 32-mer, and Ca^{2+} ions hopping between complexing carbonyl groups. (A channel mechanism with much higher turnover numbers is operative in the corresponding patch-clamp experiments.)

Introduction

Cation transport across lipid bilayer membranes by synthetic carriers or ion channels has been achieved in a variety of imaginative ways during the past 20 years.[2,3] Studies on nonpeptide ion channel mimics have attracted special interest.[4] Most of the nonpeptide channel models were designed to act either as unimolecular 'tunnels' or gramicidin-like, dimerizing 'half-pores'. In contrast, ion channels formed by peptides and synthetic peptide analogues[5] function mainly as well-defined self-assemblies which are similar to the 'barrel-stave-type' model for the one-sided action of amphotericin B.[6]

Previously, we reported the synthesis[7] and preliminary assessment of ion transport activities[8] of oligo[(*R*)-3-hydroxybutanoates] (OHB).[9] Patch-clamp experiments in lipid bilayers resulted in large-conductance, nonselective ion channels.[8b] Poly[(*R*)-3-hydroxybutanoic acid] (PHB), an ubiquitous class of biopolymers,[10] has been the subject of research in one of our groups for many years.[11] Besides their occurrence as high-molecular-weight storage material (sPHB) in microorganisms,[12] PHB has also been found in a low-molecular-weight form (cPHB). cPHB has been detected in eukaryotic and prokaryotic cells, in human aorta tissue, and in blood plasma; it has been characterized further by ^{1}H NMR spectroscopy.[13,14h] Reusch and co-workers have shown that cPHB (ca. 150 units) and $CaPP_i$ (calcium polyphosphate, ca. 75 units) form a complex which may function as an ion channel in genetically competent *Escherichia coli*.[14] The ionophoric ability of this nonproteinaceous calcium-selective channel was unambiguously demonstrated in recent patch-clamp experiments, by comparison of the complex extracted from *E. coli* membrane with a complex that was constituted from synthetic OHB and inorganic polyphosphate.[15] For this purpose, and for our investigations on the structure and synthesis of PHB-derived ion channels, we had prepared linear and cyclic OHBs with chain lengths of up to 128 units as model compounds.[7,11] Furthermore, we have demonstrated that the linear oligomers, like sPHB, have a remarkable tendency to form lamellar crystallites of ca. 50 Å thickness, in which the chains are arranged as 2_1-helix strings consisting of 16 HB units (pitch, 6 Å).[16] Previous assessment of ion transport activity of the 16-mer and longer oligomers has been attributed to the formation of hydrophobic lamellar crystallites in the lipid bilayer (hydrophobic part[17] of ca. 48 Å).

In the present work, we investigated the function of our synthetic OHBs, with different chain lengths and end groups and conjugated to a fluorescent dye (Figure 1), in a lipid vesicle system.

This study avoids the external application of high voltage, which is used in patch-clamp measurements.[18] Our model system consists of lipid vesicles (liposomes) encapsulating the chelating metal dye[19] 2-[(2-bis-[carboxymethyl]amino-5-methylphenoxy)methyl]-6-methoxy-8-bis[carboxymethyl]-aminoquinoline, as K^+ salt (Quin-2) and harboring the HB oligomers in the lipid bilayer (Figure 2). We present evidence that the HB derivatives studied can be incorporated into the bilayer membrane of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles and, depending on their length, can mediate the transport of calcium ions.

Material and Methods

Reagents. Synthetic POPC was obtained from Avanti Polar Lipids. (*R*)-3-Hydroxybutanoic acid derivatives were synthesized by M.G.F.[1,7,20] Quin-2, $CaCl_2$, and calcimycin (A23187) were from Sigma. $CaBr_2$, CaI_2, and $LaCl_3$ were from Fluka (Switzerland). The calcium salt stock solutions were standardized by titration with primary standard ethylenediaminetetraacetate (EDTA).

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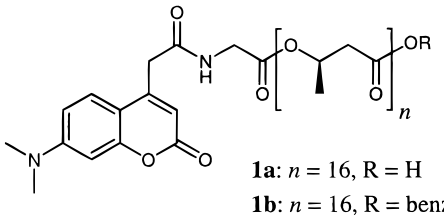
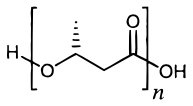


1a: n = 16, R = H
1b: n = 16, R = benzyl
2a: n = 32, R = H
2b: n = 32, R = benzyl



3: n = 8
4: n = 16
5: n = 32
6: n = 64

Figure 1. Chemical structures of the synthetic OHB derivatives **1**−**6** synthesized from (*R*)-3-hydroxybutanoic acid and tested as transporters.

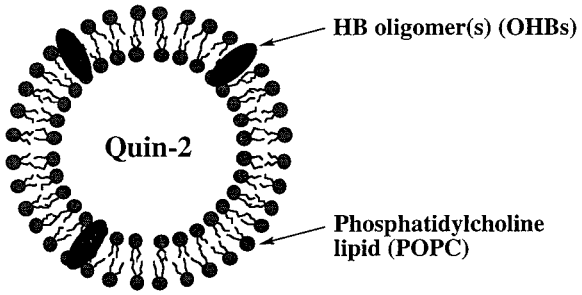


Figure 2. Schematic representation of a vesicle incorporating the synthetic OHB in the lipid bilayer and enclosing the Ca^{2+} indicator Quin-2. The drawing shows a cross-section through a spherical, unilamellar vesicle (not to scale). Typical diameters of the vesicles used are on the order of 200 nm, whereas the thickness of the lipid bilayer is approximately 4 nm. For the ratio POPC/OHB, see Table 1.

Preparation and Characterization of Liposomes. Vesicles were prepared by sonication[21] with use of a microtip of a Branson Sonifier 250 set at 30 W. In a typical experiment, 180 mg (240 mmol) POPC and 0.48 mmol of the OHB derivative in chloroform were carefully dried to produce a thin film inside a 100-mL round-bottom flask. The film was dispersed at room temperature in 6 mL of a buffer solution containing 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), pH 7.4, and 5 mM Quin-2. Sonication was conducted at 10 °C under Ar for 30 min, stopped for 15 min, and continued for 30 min. After warming up to room temperature the dispersion was centrifuged with a microcentrifugette 4214 from ALC (Italy) at 7000 rpm for 6 min to remove titanium particles[22] possibly originating from the tip. The resulting preparation (6 mL) was applied to gel permeation chromatography with Sepharose 4B columns (length, 40 cm; diameter, 1.4 cm) to remove exovesicular Quin-2 and OHB (Figure 3).

The liposome fractions were pooled and the POPC content was quantified by using the Ames assay.[23] The amount of entrapped Quin-2 was determined by spectrophotometric titration with standard $CaCl_2$, after disruption of the vesicles by 1.2 mM deoxycholate (see Figure 5). The content of incorporated OHBs **1** and **2** was determined by UV−vis spectroscopy after lysis of the liposomes with an equal volume of CF_3CH_2OH using a molar extinction coefficient [ϵ] at 380 nm of 15 840 M^{-1} cm^{-1}. The amount of the incorporated OHBs **3**−**6** was derived from the quantity used during the preparation, assuming a similar incorporation yield as for the OHBs **1** and **2**.[24] Preparation of vesicles for control measurements or for calcimycin-mediated transport was performed analogously, but without addition of OHB.

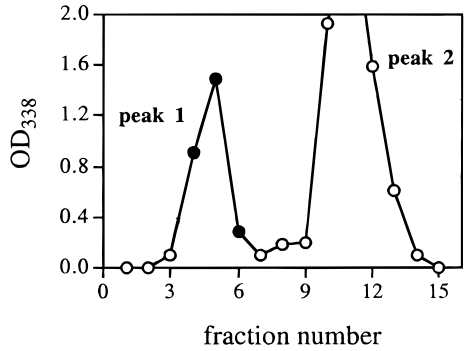


Figure 3. Gel permeation chromatographic separation of Quin-2 trapped in **2a**-containing POPC vesicles from free Quin-2 on Sepharose 4B. Peak 1 corresponds to the vesicles and peak 2 corresponds to the free Quin-2. Three milliliters of the vesicle suspension containing 40 mM POPC, 80 μM **2a**, and 5 mM Quin-2 was first sonicated, centrifugated, and then applied on a Sepharose 4B column (length, 40 cm; diameter, 1.4 cm; flow rate, 0.8 mL/min; 2.5 mL/fraction; elution with 10 mM HEPES, pH 7.4). The pooled fractions are indicated by solid circles.

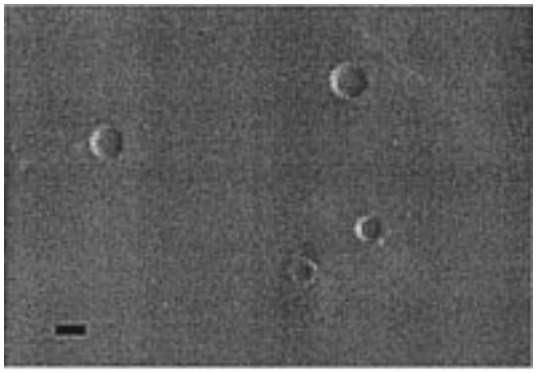


Figure 4. Freeze-fracture electron micrograph of vesicles (3 mM POPC, 12 μM **1a**) dispersed in 10 mM HEPES, pH 7.4. The samples were frozen from room temperature, and the length of the bar is 200 nm.

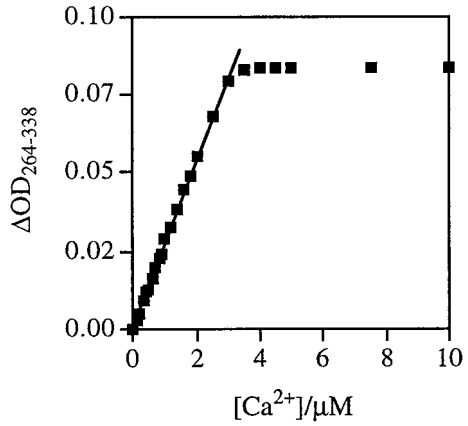


Figure 5. Calibration of the vesicle transport system. Difference absorbance 264 vs 338 nm as a function of Ca^{2+} addition. Vesicles at a POPC concentration of 0.8 mM were lysed with 1.2 mM deoxycholate before addition of Ca^{2+}.

Average vesicle diameters and related parameters were obtained from dynamic light-scattering measurements at scattering angles of 60, 90, and 120°, using a fiber optics−based spectrometer consisting of an argon laser, a digital autocorrelator, and a computer-controlled rotational stage.[25] The stability of the liposomes stored at room temperature with protection from ambient light for several days was ascertained by UV-spectroscopic and light-scattering measurements. Electron micrographs were taken by M. Wessicken using the

Table 1. Physical Properties of the POPC Vesicles

property	value
average diameter[a]	200 ± 20 nm
entrapped volume (at 0.8 mM POPC)[b]	5.5 μL/mL
exposed surface area (at 0.8 mM POPC)[b]	173 379 cm^2/mL
internal Quin-2 concentration	0.55 mM
OHB concentration (at 0.8 mM POPC)	1.8 μM
ratio OHB/POPC[b] molecules	
2a (n = 32)	1:430
1a (n = 16)	1:250
OHB molecules/liposome	
2a (n = 32)	830

[a] As determined by light-scattering measurements. [b] Calculated by taking into account unilamellarity, spherical shape, a bilayer thickness of 37 Å, and a mean head group area of 72 Å^2.

freeze-fracture method (see Figure 4).[26] Some properties of the vesicles used in the present study are specified in Table 1.

Because extensive treatment of phosphatidylcholine vesicles may lead to a degradation of the phospholipid used, e.g., formation of lysophosphatidylcholine and/or phosphatidic acid,[27] in control experiments the effect of the ultrasound treatment was investigated by a thin-layer chromatography (TLC) analysis of the vesicle suspension [Silica Gel 60 F_{254} (Merck), $CHCl_3$/CH_3OH/H_2O (65:25:5 v/v/v); molybdenum blue (Dittmer and Lester)]. No detectable degradation was observed. The stability of OHB **2a** under ultrasound treatment was analyzed as well by TLC [Silica Gel 60 F_{254} (Merck), CH_2Cl_2/Et_2O/CH_3OH (10:3:0.2 v/v/v); molybdenum reagent]. No fragmentation was detectable.

Determination of Ca^{2+} Transport. Ionophore-mediated transport of Ca^{2+} into liposomes was determined by monitoring formation of the Quin-2·Ca^{2+} complex spectroscopically.[28] The absorption measurements were made in a Cary 1E UV−vis spectrophotometer (Varian, Australia). Experiments were conducted at room temperature with a POPC concentration of 0.8 mM and an external Ca^{2+} concentration of 1 mM in a quartz cell (1 cm, 1 mL). Data points were recorded every 6 s. In control studies, POPC liposomes without ionophores were used. For investigation of the calcimycin-mediated transport, the ionophore (0.8 μM) was added to the vesicles in ethanol (0.5%) before addition of Ca^{2+}.

Results and Discussion

Incorporation of OHB into Vesicle Bilayers. The first step in most vesicle preparations involves the dissolution of the selected vesicle-forming lipids (in our case, POPC) in an organic solvent, typically chloroform.[22] After solvent evaporation, a thin lipid film is formed which can be dispersed to closed bilayers (mainly large multilamellar vesicles) by adding buffer solution under mechanical agitation. A reduction in size and lamellarity of the vesicles can then be achieved either by repetitive pressure through polycarbonate membranes with pores of defined sizes[29] or by ultrasound treatment.[21] Because all HB oligomers used in the present study are highly soluble in chloroform and insoluble in water, it may not be surprising that they could be incorporated into the POPC bilayer matrix during vesicle preparation. It is astonishing that polycarbonate membranes could not be used for decreasing the vesicle size and lamellarity. The membranes were immediately blocked, most likely because of strong intermolecular interactions between individual OHB molecules. In contrast, the use of ultrasound turned out to be successful, leading to the formation of OHB-containing vesicles that were useful for the type of transport experiment we wanted to perform.[30] The mean radius of the vesicles prepared was in the range of 90−110 nm, independent of whether OHBs were present.

Figure 3 shows a typical gel permeation chromatogram of ultrasonically prepared POPC vesicles harboring OHBs. Because of the chromophoric group present in **1** and **2**, the coelution of OHBs with vesicles could easily be observed spectroscopically, and the quantification of the OHB present in the vesicles was carried out accordingly. In control experiments, an aqueous dispersion of OHBs was treated by ultrasound and added to preformed vesicles before gel permeation chromatography. These externally added OHBs did not elute. They remained on top of the column, most likely because of strong intermolecular association, leading to the formation of large, insoluble aggregates. This observation indicates that cosolubilization of POPC and OHB in an organic solvent before the preparation of a thin lipid film (see above) is an important step for the incorporation of OHB into the bilayer matrix of the POPC vesicles.

The attempts to incorporate OHBs into POPC liposomes using other standard methods,[22] such as the reverse-phase evaporation method, dialysis of mixed lipid-surfactant micelles, and the solvent injection method (CF_3CH_2OH in the present case), failed. This might be due to the specific solubility properties of OHBs, which are neither soluble in water nor in common polar or apolar organic solvents, but which are very soluble in polyhalogenated solvents. The extraction method, in which preformed vesicles are stirred in the presence of OHBs for several hours, yielded vesicles with a poor incorporation ratio (**1a**: 1:5000 OHB/POPC).[31] In contrast, the sonication method, which was finally applied in the present work, produced liposomes with much higher OHB/POPC ratios (**1a**, 1:250; **2a**, 1:430; see Table 1).

During this study, the question arose whether free carboxylate termini were necessary for incorporation and "anchoring" of OHBs in the phosphatidylcholine membrane, as suggested by previous results, based on patch-clamp data.[8b] In the present study with vesicles, the amount of incorporated OHBs was independent of the chemical nature of the oligomer chain end; the free acid and the benzylester gave similar incorporation yields (data not shown).

Electron micrographs taken by the freeze-fracture technique revealed that the vesicles with OHB were spherical and mainly unilamellar (Figure 4). The same was observed for the vesicles without ionophore.

Calcium Transport. Basically, ion transport across vesicle bilayers can be measured in two different ways: (i) the ions are trapped in the inner aqueous compartments of the vesicles and the ion efflux is measured, for example, by using ion-sensitive electrodes; (ii) the ions are added externally and the ion influx is measured, for example, by monitoring spectral changes of an entrapped ion-sensitive indicator. We chose the second approach, because high-salt concentrations have to be entrapped for the first procedure, which may affect the liposome stability. Furthermore, we were not able to add OHBs to preformed vesicles. The OHBs had to be present with POPC during the vesicle preparation, which would not allow the use of the first method.

Quin-2 was selected as the indicator and Ca^{2+} as the ion to be transported. Quin-2 forms a stable 1:1 complex with Ca^{2+}, which shows a strong absorbance decrease at 264 nm compared with free Quin-2 and an isosbestic point at 338 nm (taken as reference point).[19]

OHB-containing vesicles with entrapped Quin-2 were obtained as described above, but in an aqueous solution

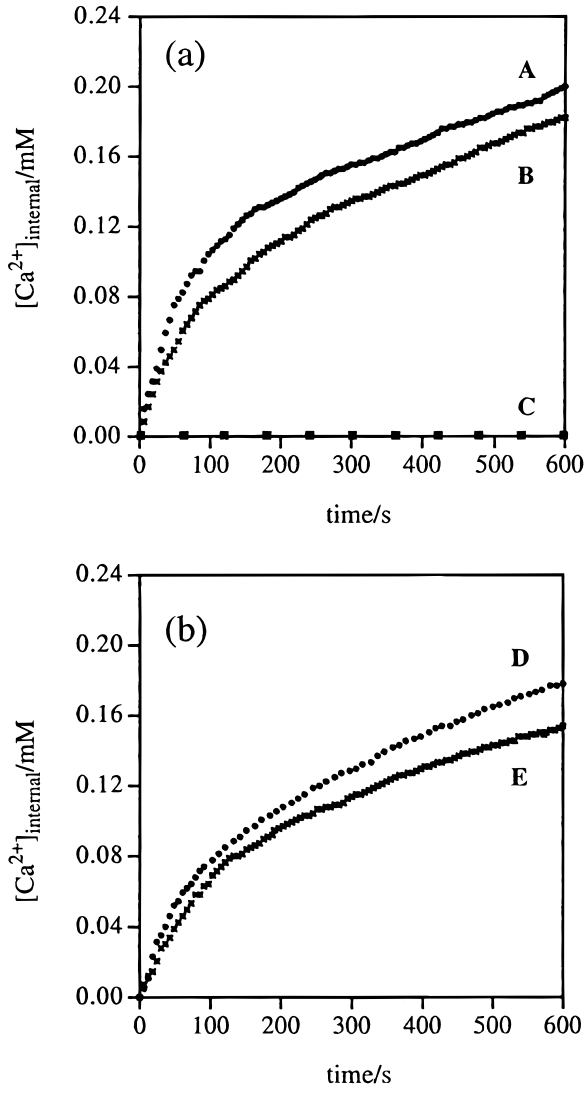


Figure 6. Ca^{2+} influx versus time. (a) Vesicles with calcimycin (●, A), molar ratio calcimycin/POPC 1:1000; vesicles with **2a** (**x**, B), molar ratio **2a**/POPC 1:430; and vesicles without transporter (■, C). (b): Vesicles with **5** (●, D), molar ratio **5**/POPC 1:450; and vesicles with **6** (**x**, E), molar ratio **6**/POPC 1:810. [POPC], 0.8 mM; [$CaCl_2$], 1 mM.

of the indicator. The exovesicular Quin-2 and nonincorporated OHBs were removed successfully by gel permeation chromatography (Figure 3). The quantification of the trapped Quin-2 was performed by titration with Ca^{2+} after lysis of the liposomes by addition of deoxycholate (Figure 5; see also Erdahl et al.[28]).

The passage of Ca^{2+} ions across the POPC bilayers of the vesicles was measured by adding an aqueous solution of $CaCl_2$ (1 mM) to the Quin-2-containing, OHB-loaded vesicle dispersion and followed by recording the time-dependent decrease in absorption of the calcium: Quin-2 complex at 264 nm. Figure 6 shows the Ca^{2+} transport by the OHB **2a** (curve B in Figure 6a), OHBs **5** and **6** (curves D and E in Figure 6b), and the naturally occurring ionophore calcimycin[32] (curve A in Figure 6a). For OHB **2b**, the Ca^{2+} influx curve was similar to curves B and D in Figure 6. In a control study, vesicles with encapsulated Quin-2 but without ionophore in the vesicle wall were exposed to 1 mM Ca^{2+} solution. These vesicles were virtually impermeable to Ca^{2+} (curve C in Figure 6). Therefore, we conclude that the observed calcium transport is strictly ionophore mediated.

Table 2. Ca^{2+} Transport Activity of Synthetic HB Oligomers

compound	activity
3 ($n = 8$)	no
1a−**b**, **4** ($n = 16$)	no[a]
2a−**b**, **5** ($n = 32$)	yes
6 ($n = 64$)	yes

[a] The occurrence of calcium transport was not reproducible.

All HB oligomers tested for their Ca^{2+} transport ability are listed in Table 2. As a result, only those with $n > 16$ showed ionophoric activity.

The transport inability of the 8-mer **3** is in accordance with previous patch-clamp experiments.[8b] The 16-mer (**1**, **4**), which showed single-channel behavior when incorporated into a planar lipid bilayer, was not able to mediate reproducibly calcium transport in the liposomal model system. This discrepancy may arise from the different curvatures and/or experimental conditions in planar bilayers (as used in the patch-clamp experiments) compared with vesicles. Furthermore, compared with previous patch-clamp experiments, anchoring of OHB molecules in the vesicle bilayer has been observed not only for unprotected acid end groups (**2a**, **5**, **6**), but also for the corresponding benzylester (**2b**). In both cases, an anchoring mechanism can be understood on the basis of an association between the OHB and the quarternary nitrogen of POPC (ion−ion in **2a**, **5**, **6**, or ion−dipole attraction[33] in **2b**).

To maintain electroneutrality, transmembrane cation transport should be accompanied by anion transport in the same direction (anion synport) or cation transport in the opposite direction (cation antiport). Different calcium salts ($CaCl_2$, $CaBr_2$, CaI_2) were used to clarify whether OHB is capable of mediating anion transport. Because all initial transport rates were about the same order of magnitude, we concluded that selective anion cotransport did not occur. Furthermore, increasing the Cl^- concentration by addition of NaCl (1 mM) did not change the calcium transport rate. We did not investigate whether cation antiport occurred.

With respect to the known blocking ability of La^{3+} of (i) the PHB/$CaPP_i$ complex of *E. coli*[14i] and (ii) a variety of naturally occurring calcium channels,[34] we investigated the possible inhibitory effect of La^{3+} on our system (using the transporter **2a**). At a $LaCl_3$ concentration of 1 mM, an effective inhibition of Ca^{2+} transport was achieved (Figure 7, curve B).

'Pretreatment' of the OHB-containing vesicles with $LaCl_3$ (1 mM) and subsequent addition of $CaCl_2$ prevented calcium transport activity almost completely (Figure 7, curve C; similar results were obtained with 0.1 mM $LaCl_3$). Calculations estimated that, despite the known high affinity of La^{3+} to phosphate, only between 0.1% (for 0.1 mM $LaCl_3$) and 10% (for 1 mM $LaCl_3$) POPC was complexed with the transition metal.[35] Lanthanum ions are very oxophilic, hard Lewis acids.[36] We therefore presume a high affinity of the ions to the HB-carbonyl groups which results in the observed blocking of the Ca^{2+} ion transport.

Permeabilities and Turnover Numbers. Initial permeabilities were calculated based on eq 1.[37]

$$\frac{[Ca^{2+}]}{[Ca^{2+}]_o} = (1 - e\{-P \cdot A_R \cdot t\}) \qquad (1)$$

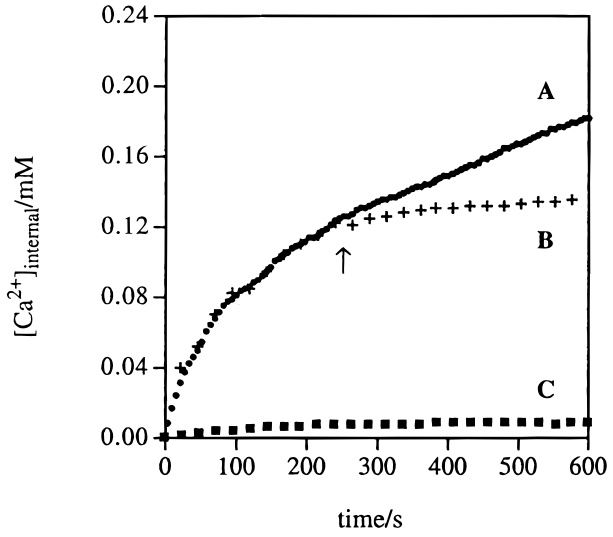


Figure 7. Ca^{2+} influx versus time for vesicles with **2a** (molar ratio **2a**/POPC 1:430). Without addition of lanthanum chloride (●, A); with addition of $LaCl_3$ (1 mM) after 240 s (+, B, arrow) and before $CaCl_2$ (■, C); [POPC], 0.8 mM; [$CaCl_2$], 1 mM.

Table 3. Estimated Permeability Coefficients (*P*) of POPC Vesicles toward Ca^{2+} Ions and Turnover Numbers of OHB Ionophores and Calcimycin

compound	*P* (cm/s)	turnover number[a] (s^{-1})
2a[b]	8.4×10^{-9}	2.4
2b[b]	8.4×10^{-9}	2.4
5[c]	8.3×10^{-9}	2.4
6[d]	5.8×10^{-9}	2.2
calcimycin[e]	9.9×10^{-9}	4.3

[a] Assuming for eq 2, $n = 3$ for **2**, **5** and $n = 2$ for **6** and calcimycin[32] (see text for detail). [b] Molar ratio **2**/POPC 1:430. [c] Molar ratio **5**/POPC 1:450. [d] Molar ratio **6**/POPC 1:810. [e] Molar ratio calcimycin/POPC 1:1000.

This equation was applied to the initial few points of the Ca^{2+} ion uptake data. $[Ca^{2+}]_o$ represents the initial overall concentration of the metal ions on the outside of the reservoir, $[Ca^{2+}]$ stands for the internal Ca^{2+} concentration of the reservoir, *P* denotes the vesicle permeability coefficient, and A_R is the vesicle area divided by the reservoir volume. By using this expression, we are assuming that the calcium concentration in the vesicle interior is essentially zero for $t = 0$. The calculated permeability coefficients are shown in Table 3.

The Ca^{2+} permeability coefficients reported here for the OHB molecules tested (**2**, **5**, **6**) are similar to that determined in the present study for the naturally occurring calcimycin, 9.9×10^{-9} cm/s. This latter value is in good agreement with data from the literature.[28,32] The Ca^{2+} uptake kinetics observed for our experimental system is orders of magnitude higher than the permeability coefficient via uncatalyzed transport, which is in the range of $10^{-13}-10^{-12}$ cm/s.[38] The overall curve shape for the OHB-mediated or the calcimycin-mediated Ca^{2+} transport (see Figure 6) suggests the existence of different rate-limiting factors during early and later periods of the transport process. It was not possible to fit these data with simple first-order kinetics. The best fit to the experimental data was obtained with a biexponential increase, in analogy to what has been reported by Erdahl et al.[28] for the calcimycin system.

Turnover numbers (*T*) for the initial stage of the Ca^{2+} uptake process were also determined and are given in Table 3. They were calculated based on eq 2.[37]

$$T = \frac{P \cdot [Ca^{2+}]_o \cdot n}{[I]} \qquad (2)$$

The concentration of ionophore, [*I*], represents a surface concentration (mol/cm^2) based on the vesicle surface area; *n* denotes the number of monomers that associate and form one active ionophore species (e.g. pore[39]). To transport a calcium ion with a hydrodynamic radius[40] of ca. 2 Å through a pore composed of OHB 32-mer molecules, at least three OHBs are necessary. This was calculated, assuming that an OHB 32-mer is present as a double cylinder of 40 Å length and 9 Å diameter, and that it is oriented perpendicular to the membrane (barrel stave model).[11] From the observation that Quin-2, which has a hydrodynamic radius[41] of ca. 5−6 Å, cannot pass the pore, we concluded that the pore formed has to be smaller than 10−12 Å. Modeling of an aggregation of four OHB 32-mers resulted in a pore with a diameter of ca. 20 Å. Taking these considerations into account, we therefore assume that one single pore is built from three OHB 32-mers. In other words, for the turnover number reported in Table 3, $n = 3$ for the transporter **2** and **5**, and $n = 2$ for carrier 6.[42] The comparison in Table 3 shows that both the synthetic OHBs and calcimycin transport a few Ca^{2+} ions per second in the vesicle system used.

Concluding Remarks

In this article we have shown that OHBs of appropriate chain length display remarkable calcium transport activity in POPC vesicles.

The classification of the unambiguously demonstrated ionophoric activity into the two action modes (i) carrier and (ii) channel could not be easily accomplished.[43] The relatively low turnover number and the known ability of cyclic and linear HB oligomers to act as ionophors in liquid organic membranes[8a] are more compatible with a carrier-like mechanism. On the other hand, the "classical" concept of carrier transport via molecules migrating from one side of the membrane to the other (moving ferry boat) cannot explain the structural dependence of the transport; OHBs able to span a lipid bilayer are required for ion transport (Table 2). This structural dependence of the ionophoric activity, however, is in good agreement with the revised concept of carrier transport, which includes macromolecules (often too large to diffuse) fixed in the membrane while exposing the transport binding sites to the intracellular and extracellular media.[43] These results are in contrast with our patch-clamp experiments,[8b] which resulted in single-channel activity of OHBs in planar bilayers with cations other than Ca^{2+}. Channels typically display turnover numbers in the range of 10^5-10^7 s^{-1}, several orders of magnitude higher than the observed activity in our liposomal system.[43] The occurrence of single-channel activity in planar lipid bilayers on one hand and carrier-like transport properties on the other hand is also known for calcimycin.[44] The influence of a strong electric field may result in a macroscopic membrane phase transition or in a positioning of the ionophore in form of "patches".[45] These patches could be responsible for the appearance of large, water-filled bilayer disruptions that would allow Ca^{2+} transport. This mechanism is unlikely in our system, however, because no leakage of the encapsulated indicator was observed.

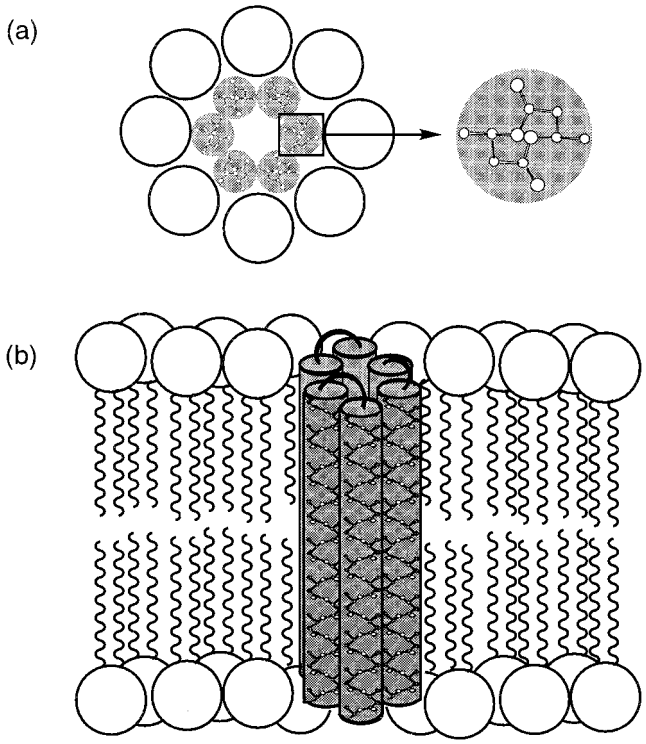


Figure 8. Schematic representation of the postulated pore formation for OHB 32-mers in a phospholipid bilayer. One single pore is composed of three OHB molecules in this model. (a) Top view with enlargement of one helical string; (b) side view.

These results can be rationalized by assuming a schematic model as depicted in Figure 8. In the OHB 32-mer, a relatively small pore is built up by 3 OHB 32-mer molecules, which adapt a possibly helical conformation (see above). The Ca^{2+} transport is most probably achieved by ion-hopping between carbonyl groups inside the pore. These ionic interactions prevent Ca^{2+} passage through the pore in a free diffusion-like manner, which would explain the relatively low turnover number observed and the structural transport dependence (Table 2).

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References and Notes

(1) Fritz, M. G. Ph.D. Thesis No. 12973, ETH-Zürich, 1998.
(2) For example, see: (a) Thomas, C.; Sautery, C.; Castaing, M.; Gray-Bobo, C. M.; Lehn, J. M.; Plumere, P. *Biochem. Biophys. Res. Commun.* **1983**, *116*, 981. (b) Shinar, H.; Navon, G. *J. Am. Chem. Soc.* **1986**, *108*, 5005. (c) Castaing, M.; Lehn, J. M. *J. Membr. Biol.* **1987**, *97*, 79. (d) Nakano, A.; Xie, Q.; Mallen, J. V.; Echegoyen, L.; Gokel, G. W. *J. Am. Chem. Soc.* **1990**, *112*, 1287. (e) Kobuke, Y.; Ueda, K.; Sokabe, M. *J. Am. Chem. Soc.* **1992**, *114*, 7618. (f) Fyles, T. M.; James, T. D.; Kaye, K. C. *J. Am. Chem. Soc.* **1993**, *115*, 12315. (g) Murillo, O.; Watanabe, S.; Nakano, A.; Gokel, G. *J. Am. Chem. Soc.* **1995**, *117*, 7665. (h) Jin, T.; Kinjo, M.; Koyama, T.; Kobayashi, Y.; Hirata, H. *Langmuir* **1996**, *12*, 2684.
(3) Kobuke, Y. In *Advances in Supramolecular Chemistry*; Gokel, G. W., Ed.; JAI Press: Greenwich, 1997; Vol. 4, pp 163−210 and references therein.
(4) Gokel, G. W.; Murillo O. *Acc. Chem. Res.* **1996**, *29*, 425.
(5) For example, see: (a) Clark, T. D.; Buehler, L. K.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1998**, *120*, 651. (b) Lear, J. D.; Schneider, J. P.; Kienker, P. K.; DeGrado, W. F. *J. Am. Chem. Soc.* **1997**, *119*, 3212.
(6) Bolard, J. *Biochim. Biophys. Acta* **1985**, *864*, 257.
(7) Lengweiler, U. D.; Fritz, M. G.; Seebach, D. *Helv. Chim. Acta* **1996**, *79*, 670.
(8) (a) Bürger, H. M.; Seebach, D. *Helv. Chim. Acta* **1993**, *76*, 2570. (b) Seebach, D.; Brunner, A.; Bürger, H. M.; Reusch, R. N.; Bramble, L. L. *Helv. Chim. Acta* **1996**, *79*, 507.
(9) The term oligomer refers to monodisperse compounds. Material with a molecular weight distribution is designated as polymer.
(10) (a) Steinbüchel, A. In *Biomaterials*; Byrom, D., Ed.; Stockton Press: New York, 1991; pp 123−213. (b) Steinbüchel, A. *Nachr. Chem. Tech. Lab.* **1991**, *39*, 1112.
(11) (a) Seebach, D.; Brunner, A.; Bachmann, B. M.; Hoffmann, T.; Kühnle, F. N. M.; Lengweiler, U. D. Biopolymers and Oligomers of (*R*)-3-Hydroxyalkanoic Acids—Contributions of Synthetic Organic Chemists, Ernst Schering Research Foundation, Berlin, 1995; Vol. 28, pp 1−105. (b) Müller, H.-M.; Seebach, D. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 477.
(12) Schlegel, H. G. *Allgemeine Mikrobiologie*, 7th ed.; Thieme Verlag: Stuttgart, 1992.
(13) Seebach, D.; Brunner, A.; Bürger, H. M.; Schneider, J.; Reusch, R. N. *Eur. J. Biochem.* **1994**, *224*, 317.
(14) (a) Reusch, R. N.; Sadoff, H. L. *J. Bacteriol.* **1983**, *156*, 778. (b) Reusch, R. N.; Hiske, T. W.; Sadoff, H. L. *J. Bacteriol.* **1986**, *168*, 553. (c) Reusch, R.; Hiske, T.; Sadoff, H.; Harris, R.; Beveridge, T. *Can. J. Microbiol.* **1987**, *33*, 435. (d) Reusch, R. N.; Sadoff, H. L. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4176. (e) Reusch, R. N. *Proc. Soc. Exp. Biol. Med.* **1989**, *191*, 377. (f) Reusch, R. N. *Chem. Phys. Lipids* **1990**, *54*, 221. (g) Reusch, R. N. *FEMS Microbiol. Rev.* **1992**, *103*, 119. (h) Reusch, R. N.; Sparrow, A. W.; Gardiner, J. *Biochim. Biophys. Acta* **1992**, *1123*, 33. (i) Reusch, R. N.; Huang, R.; Bramble, L. L. *Biophys. J.* **1995**, *69*, 754. (j) Castuma, C. E.; Huang, R.; Kornberg, A.; Reusch, R. N. *J. Biol. Chem.* **1995**, *270*, 12980.
(15) Das, S.; Lengweiler, U. D.; Seebach, D.; Reusch, R. N. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 9075.
(16) (a) Seebach, D.; Bürger, H. M.; Müller, H.-M.; Lengweiler, U. D.; Beck, A. K.; Sykes, K. E.; Parker, P. A.; Barham, P. J. *Helv. Chim. Acta* **1994**, *77*, 1099. (b) Sykes, K. E.; McMaster, T. J.; Miles, M. J.; Parker, P. A.; Barham, P. J.; Seebach, D.; Müller, H.-M.; Lengweiler, U. D. *J. Mater. Sci.* **1995**, *30*, 623.
(17) Fettiplace, R.; Haydon, D. A. *Physiol. Rev.* **1980**, *60*, 510.
(18) Single-channel activity in patch-clamp experiments with OHBs could only be observed when a voltage of ca. 100 mV was applied (see ref 8b).
(19) (a) Tsien, R. Y. *Biochemistry* **1980**, *19*, 2396. (b) Tsien, R. Y.; Pozzan, T.; Rink, T. J. *J. Cell Biol.* **1982**, *94*, 325.
(20) The synthesis of **1** and **2** is described in: Fritz, M. G.; Seebach, D. *Helv. Chim. Acta* **1998**, *81*, 2414.
(21) Huang, C. *Biochemistry* **1969**, *8*, 344.
(22) *Liposomes: A Practical Approach*; New, R. R. C., Ed.; Oxford University Press: New York, 1990.
(23) Ames, B. N. *Methods Enzymol.* **1960**, *8*, 115.
(24) In **1**, the molar ratio of **1** to POPC used during the liposome preparation was 1:250. After gel permeation chromatography, **1**/POPC in the liposome fractions was 1:240 (corresponding to an incorporation yield of ca. 95%). For **2**, liposomes were prepared with **2**/POPC = 1:500, yielding 1:430 in the liposome fractions (corresponding to 85%). Based on these observations with **1** and **2**, a mean incorporation yield of 90% was assumed and taken into account for **3**−**6**.
(25) Gisler, T.; Rüger, H.; Egelhaaf, S. F.; Tschumi, J.; Schurtenberger, P.; Ricka, J. *Appl. Opt.* **1995**, *34*, 3546.
(26) Müller, M.; Meister, N.; Moor, H. *Mikroskopie* (*Wien*) **1980**, *36*, 129.
(27) Hauser, H. O. *Biochem. Biophys. Res. Commun.* **1971**, *45*, 1049.
(28) Erdahl, W. L.; Chapman, C. J.; Taylor, R. W.; Pfeiffer, D. R. *Biophys. J.* **1994**, *66*, 1678.
(29) (a) Olson, F.; Hunt, C. A.; Szoka, F. C.; Vail, W. J.; Papahadjopoulos, D. *Biochim. Biophys. Acta* **1979**, *557*, 9. (b) Mayer, L. D.; Hope, M. J.; Cullis P. R. *Biochim. Biophys. Acta* **1996**, *858*, 161.
(30) Please note that for all vesicle preparations used, gel permeation chromatography followed the ultrasound treatment.
(31) This procedure was performed at room temperature or 50 °C and also in the presence of 10% (v/v) methanol. The incor-

poration rate could not be increased by using these different experimental conditions.
(32) (a) Chapman, C. J.; Puri, A. K.; Taylor, R. W.; Pfeiffer, D. R. *Arch. Biochem. Biophys.* **1990**, *281*, 44. (b) Reed, P. W.; Lardly, H. A. *J. Biol. Chem.* **1972**, *247*, 6970. (c) Westley, J. W. *The Polyether Antibiotics: Naturally Occurring Acid Ionophores*; Dekker: New York, 1982; Vol. 1.
(33) This type of interaction has been discussed before in work on synthetic flux-promoting compounds; see Menger, F. M.; Davis, D. S.; Persichetti, R. A.; Lee, J.-J. *J. Am. Chem. Soc.* **1990**, *112*, 2451.
(34) *Membrane Transport of Calcium*; Carafoli, E., Ed.; Academic Press: London, 1982.
(35) Akutsu, H.; Seelig, J. *Biochemistry* **1981**, *20*, 7366.
(36) Molander, G. A. *Chem. Rev.* **1992**, *92*, 29.
(37) van Zanten, J. H.; Monbouquette, H. G. *Biotechnol. Prog.* **1992**, *8*, 546.
(38) *Phospholipids Handbook*; Cevc, G., Ed.; Dekker: New York, 1993.
(39) In this context, the term pore refers to a geometrical arrangement, without implying any classification of transport mechanisms.
(40) Phillips, C. S. G.; Williams, R. J. P. *Inorganic Chemistry*; Clarendon Press: Oxford, 1965; Vol. 1, p 162.
(41) Zimmerberg, J.; Blumenthal, R.; Sarkar, D. P.; Curran, M.; Morris, S. J. *J. Cell Biol.* **1994**, *127*, 1885.
(42) In analogy to the postulation that at least three 32-mers form a pore by arranging six helices (see Figure 8), we suppose that two 64-mers (corresponding to eight helices) were involved in the active carrier species. It might be that not all "transmembrane helices" are involved in the pore formation.
(43) Gennis, R. B. In *Biomembranes. Molecular Structure and Function*; Cantor, C. R., Ed.; Springer-Verlag: New York, 1989; pp 270−306. Hille, B. *Ionic Channels of Excitable Membranes*; Sinauer Associates: Sunderland, 1984.
(44) Balasubramanian, S. V.; Sidkar, S. K.; Easwaran, K. R. K. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 1038.
(45) For example, see: (a) Osman, P.; Cornell, B. *Biochim. Biophys. Acta* **1994**, *1195*, 197. (b) Ludtke, S. J.; He, K.; Heller, W. T.; Harroun, T. A.; Yang, L.; Huang, H. W. *Biochemistry* **1996**, *35*, 13723.

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