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Influence of Membrane Structure on Ion Transport Through Lipid Bilayer Membranes

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Summary. Charge-pulse relaxation studies with the positively charged PV-K^+ complex (cyclo-(D-Val-L-Pro-L-Val-D-Pro)$_3$) and the negatively charged lipophilic ion dipicrylamine (DPA^-) have been performed in order to study the influence of structural properties on ion transport through lipid bilayer membranes. First, the thickness of monoolein membranes was varied over a wide range using different *n*-alkanes and solvent-free membranes. The thickness (*d*) of the hydrocarbon core of these membranes varied between 4.9 and 2.5 nm. For both transport systems the partition coefficient β was found to be rather insensitive to variations in *d*. The same was valid for the translocation rate constant k_{MS} of PV-K^+, whereas a strong increase of the translocation rate constant k_i of DPA^- with decreasing *d* was observed. In a further set of experimental conditions the structure of the lipids, such as number and position of the double bonds in the hydrocarbon chain and its chain length as well as the nature of the polar head group, was varied. The translocation constant k_{MS} of PV-K^+ transport was found to be much more sensitive to these variations than k_i of DPA^-.

Much larger variations in k_i and k_{MS} were observed in membranes made from lipids with ether instead of ester linkages between glycerol backbone and hydrocarbon chain. The results are in qualitative agreement with the surface potentials of monolayers made from corresponding lipids. Increasing amounts of cholesterol in membranes of dioleoylphosphatidylcholine caused a strong decrease of k_{MS} (PV-K^+), whereas k_i was found to be rather insensitive to this variation.

In monoolein membranes cholesterol causes a decrease of k_{MS} up to sixfold and a increase of k_i up to eightfold. The partition coefficient β of DPA^- was insensitive to cholesterol, whereas β of PV-K^+ was found to decrease about eightfold in these membranes. The influence of cholesterol on k_{MS} is discussed on the basis of viscosity changes in the membrane and the change in k_i of DPA^- and β of PV-K^+ on the basis of a possible change of the dipole potential of the membranes. The other sterols, epicholesterol and ergosterol cause no change in the kinetics of the two probes.

The different influence of membrane properties like thickness, viscosity, and dipole potential on the two transport systems is discussed under the assumption that the adsorption planes of the two probes have different positions in a membrane. Possibly because of a larger hydrophobic interaction, the adsorption plane of PV-K^+ is located more towards the hydrocarbon side and that of DPA^- more towards the aqueous side of the dipole layer.

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Many transport phenomena of biological membranes can be investigated using artificial lipid bilayer membranes as model systems. Ion transport processes are particularly well suited because they are conveniently monitored electrically with high sensitivity. The sensitivity of this experimental approach is such that apparently minor changes in the chemical make-up or in other parameters of a lipid bilayer manifest themselves in large, measurable effects. For instance, the rate constants of ion transport as determined by electrical relaxation techniques can give information on the fluidity of the membrane interior. Other techniques allow the determination of the thickness of a membrane with high reliability.

The influence of membrane thickness on ion transport processes such as those involving pores [24], carrier molecules [3], or lipophilic ions [8] have been investigated. It has been found that the lifetime of the gramicidin A channel in membranes formed from the same lipid was strongly influenced by the chain length of the solvent [24]. On the other hand, the influence of solvent on carrier-mediated ion transport is much smaller. In particular, the rate constants of translocation of the complexed and the uncomplexed carrier molecule were found to be rather insensitive to the membrane thickness [3]. For negatively charged lipophilic ions such as tetraphenylborate or dipicrylamine (DPA^-), a strong decrease of the translocation rate constant with increasing membrane thickness has been found, while the partition coefficient seems to be almost insensitive to the change in thickness [8]. It emerges that different ion transport systems respond differently to changes in membrane thickness, a fact that is not readily explained.

Nevertheless, studies of the transport kinetics of lipophilic ions and of ion carrier molecules have contributed significantly to the understanding of the structure and dynamics of lipid bilayer membranes [1, 2, 9, 10, 12, 23, 27, 29, 31, 37–39]. (For a review *see* [21].)

Ion transport mediated by such carrier molecules as valinomycin, macrotetrolides, or enniatins [23, 37] is much more complicated than the transport of lipophilic ions. The latter occurs in just three steps, namely, adsorption of the ion from the aqueous phase to the membrane-solution interface, translocation through the hydrocarbon interior to the opposite interface, and desorption into the aqueous phase [27]. A similar mechanism obtains for the transport of the cation complexes of the cyclodecapeptide PV (Fig. 1). This similarity is due to the fact that the PV-ion complexes have, compared to valinomycin, a high stability constant in the aqueous phase

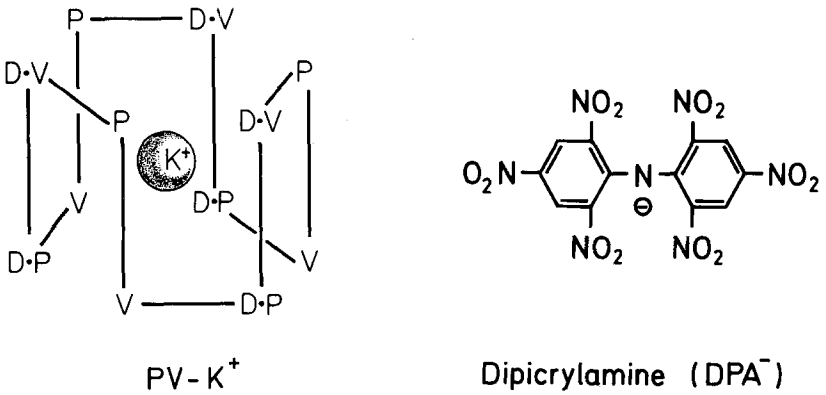

Fig. 1. (*a*): Schematic representation of the potassium complex of the peptide cyclo-(D-Val-L-Pro-L-Val-D-Pro)$_3$ or PV [18]. *P, V* are L-prolyl and L-valyl, *D.P, D.V* are D-prolyl and L-valyl residues. (*b*): Structure of the dipicrylamine anion (DPA^-). This compound ($pK_a = 2.66$ [17]) is ionized under the conditions used in this work

and a low dissociation rate constant [5]. These complexes can therefore be used as positively charged probes of the membrane interior much in the same way as the negative ions tetraphenylborate [1] or dipicrylamine (DPA^-) [8]. The way bilayers of different thicknesses or made of different lipids influence the kinetic parameters of negatively or positively charged lipophilic ions can give valuable insight into structure and dynamics of lipid membranes.

This paper describes experiments designed to give information on lipid bilayer membranes using PV-K^+ complexes and DPA^-as probes. In addition to studying the effects of the solvent used for membrane formation, the dependence of the transport kinetics on the nature of the lipid used was investigated. To that end, the polar head groups and the fatty acid residues of the lipids were varied extensively. In a further set of experiments the influence of the addition of sterols to the lipids on the transport parameters was studied. Kinetic studies with lipophilic ions and with carriers can be performed using the well established voltage-jump relaxation method [27, 37] or by the more recent charge pulse relaxation technique [7, 9]. The latter method was used throughout this study. Its main advantages are a minimal pertubation of the membrane ($V_m = 6$–8 mV) and an increased time resolution, which is given by the measuring circuit and is not limited by the membrane conductance.

Materials and Methods

Black lipid bilayer membranes were obtained from various lipids by two different methods. (i) Solvent-containing membranes were formed from a 1–3 % (w/v) lipid solution in *n*-alkane (Merck, Darmstadt, G.F.R., standard for gas chromatography) across a circular hole, either 1 or 2 mm in diameter, as described earlier [11]. The size of the hole had no influence on the probe concentration in the membrane or on the relaxation time constants. (ii) Bilayer membranes from monolayers ("solvent-free" membranes) were formed in the usual way [4] across a small hole (0.2–0.3 mm in diameter) in a thin Teflon foil. The temperature was kept at 25 °C throughout.

Membranes were formed from various lipids. The monoglycerides (Nu Check Prep, Elysian, Minn.) had the following fatty acid residues: palmitoleoyl (Δ^9-$C_{16:1}$), petroselinoyl (Δ^6-$C_{18:1}$), oleoyl (Δ^9-$C_{18:1}$), vaccenoyl (Δ^{11}-$C_{18:1}$), eicosenoyl (Δ^{11}-$C_{20:1}$) and erucoyl (Δ^{13}-$C_{22:1}$). The lipids contained about 98 % of the l-isomer and gave a single spot on a thin layer chromatogram. Most of the phospholipids used in this study were synthesized by K. Janko [6, 26]. The 1,2-diacyl-*sn*-glycerol-3-phosphorylcholines had the same fatty acid residues as above-mentioned for the monoglycerides. In addition, phosphatididylcholines with the following fatty acid residues were used: phytanoyl (3,7,11,15-$C_{16:4CH_3}$) and one with mixed chains, L-1-oleoyl-2 stearoyl-3-phosphatidylcholine. An ether phosphatidylcholine (DL-1-0-oleyl-2-0-palmityl-3-phosphatidylcholine) was obtained from Calbiochem, San Diego, Cal. Egg phosphatidylcholine as well as phosphatidylserine and phosphatidylinositol from ox brain were isolated and purified by standard methods [35, 36]. DL-dioleoyl phosphatidylethanolamine and its ether analog were synthesized as described previously [6]. All lipids were checked by thin layer chromatography and found to be pure.

Some experiments were performed using dioleoyl phosphatidylcholine/sterol or monoolein/sterol mixtures with the following sterols: cholesterol (cholest-5-*en*-3β-ol Eastman reagent grade), epicholesterol (cholest-5-*en*-3α-ol, Merck, analytical grade) and ergosterol (cholest-5,7,22-trien-24-methyl-3β-ol, Sigma). PV [18] and dipicrylamine (Fluka, Buchs, Switzerland, puriss.) were used as concentrated stock solutions in chloroform or in ethanol, respectively. Small amounts of the stock solutions were added to the aqueous solutions to get a final concentration of 5×10^{-7} M (PV) and of 3×10^{-8} M (DPA^-). These concentrations were chosen in order to obtain a linear relationship between the concentrations of the probes in the aqueous phase and in the membrane and to avoid boundary potentials [30]. The unbuffered aqueous solutions contained either 1 M KCl (experiments with PV) or 0.1 M NaCl (experiments with dipicrylamine). The membrane experiments were performed 20–30 min after blackening. Since the time constant of the fast relaxation process in the presence of PV was up to four times larger when the phosphatidylcholine membranes were "prepainted", this procedure was not used. In addition the cell used for membrane formation was cleaned with ethanol before each experiment. With this procedure, the experimental data for PV-K^+ were fairly constant.

The charge pulse experiments were carried out as described in previous publications [7, 9]. The membrane capacitance was charged up to a voltage of about 10 mV by a brief current pulse (20 to 50 nsec duration) through silver/silver-chloride or platinized platinum electrodes. The ensuing voltage transient across the membrane was recorded with an oscilloscope (Tektronix 7633/7A 13/7A22 storage oscilloscope). The evaluation of the data from the oscillographic records was performed as described earlier [7, 9]. It is seen from Eq. (5) that for the calculation of the total concentration N_t of PV-K^+ complexes and DPA^- in the membrane the specific capacitance has to be known. For most systems used here the specific capacitance has been determined previously [2–4, 26]. For the others, C_m was measured by applying rectangular voltage pulses of 10 mV to the membrane. The capacitive current was measured as a voltage drop across an external resistance with a storage

oscilloscope (Tektronix 5115/5A22). The specific capacitance of the membrane was then calculated as described earlier [6].

Description of the Transport Model

The transport models for the PV-K^+ complex and for lipophilic anions like DPA^- and tetraphenylborate and the application of the charge pulse method to both models have been presented in full detail in previous publications [5, 9]. Therefore, we present here only the main equations which relate the experimental data to kinetic and equilibrium parameters of the models. PV forms complexes with cations like K^+, Rb^+, and Cs^+ in the aqueous phase [5]. The stability constants of these complexes is rather high, and their dissociation rate constants in organic solvents [14] (and most likely also in aqueous solutions) appear to be very slow. Therefore, the PV-K^+ complex may be regarded as a positively charged lipophilic ion. Despite the similarity of the lipophilic anion and the PV-K^+ models the nomenclature to describe them differs somewhat. These differences are maintained here in order to stress the point that PV-K^+ may, in principle dissociate while the lipophilic anions do not. The transport of lipophilic ions such as PV-K^+ or DPA^- across lipid bilayer membranes occurs in two distinct reactions. These are the adsorption-desorption reaction between the aqueous phase and the membrane interface (rate constants k_{MS}^{am} and k_{MS}^{ma} for PV-K^+, k_{am} and k_{ma} for DPA^-) and the jump of the charged molecules across the potential barriers in the membrane (rate constants k_{MS} and k_i respectively) [5, 27]. For charge pulse experiments at small voltages ($V_m \ll 25$ mV), the decay of the voltage across the membrane is governed by two exponentials with $\tau_1 \ll \tau_2$ [9]:

$$V_m(t) = V_m^0 a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) \tag{1}$$

with

$$a_1 + a_2 = 1. \tag{2}$$

The relaxation times τ_1 and τ_2 and the relaxation amplitudes a_1 and a_2 are known functions of the rate constants k_{MS}^{am}, k_{MS}^{ma} and k_{MS} (k_{am}, k_{ma} and k_i, respectively). In the systems with dipicrylamine studied here, the behavior of V_m at long times is governed by slow diffusion of the lipophilic ion in the aqueous phase near the membrane surfaces [9]. This means that only the short relaxation time τ_1 may be obtained from the experiments. τ_2 was on the order of seconds for DPA^- and could not be used to determine k_{am} and k_{ma}.

For the PV-K^+ complex it is not known whether the slow relaxation process is due to the exchange of complexes across the membrane/water interface, due to a slow dissociation of molecules in the membrane, and/or due to a similar process in the aqueous phase. Because of these different possibilities, we consider here only the fast process which is related to the movement of PV-K^+ complexes across the central barrier of the membrane. τ_2 for PV-K^+ was on the order of a few hundred msec corresponding in principle to a k_{ma} of $<10\ \text{sec}^{-1}$. Under conditions where one decay process is much faster than the other, the theoretical expressions for τ_1 and a_1 [9] reduce to:

PV-K^+ complex	dipicrylamine (DPA^-)	
$\tau_1 = \dfrac{1}{2k_{MS}(1+bN_t)}$	$\tau_1 = \dfrac{1}{2k_i(1+bN_t)}$	(3)

$$a_1 = \frac{bN_t}{1+bN_t} \tag{4}$$

$$b = \frac{F^2}{4RTC_m}. \tag{5}$$

N_t is the total concentration ($\text{mol} \cdot \text{cm}^{-2}$) of PV-$K^+$ or of lipophilic anion adsorbed to both membrane surfaces at equilibrium; C_m is the specific membrane capacitance, F the Faraday constant, R the gas constant, and T the absolute temperature. From the experimental value of N_t determined at a given aqueous concentration of PV-K^+ complexes c_{MS} or of lipophilic ions c, the partition coefficient β can be calculated:

PV-K^+	DPA^-	
$\beta = \dfrac{N_{MS}}{c_{MS}} = \dfrac{N_t}{2c_{MS}} = \dfrac{k_{MS}^{am}}{k_{MS}^{ma}}$;	$\beta = \dfrac{N_t}{2c} = \dfrac{k_{am}}{k_{ma}}$.	(6)

N_{MS} is the concentration of PV-K^+ complexes in one membrane interface at equilibrium ($N_t = 2N_{MS}$). β is equal to the thickness of an aqueous layer containing the same amount of PV-K^+ complexes or lipophilic ions as one membrane interface.

The first relaxation process was found to be purely exponential in all systems considered here. This is in principle not obvious because in the case of high surface concentration of lipophilic ions and carrier-ion complexes ($N_t \approx 1\ \text{pmol cm}^{-2}$) boundary potentials [30] may occur, which tend to create more than one relaxation even for low voltages (P. Läuger, *personal communication*).

Results

a) Influence of the Membrane Thickness

In order to study the influence of the membrane thickness on the kinetics of the transport of PV-K^+ across lipid bilayer membranes glycerolmonoleate was dissolved in different *n*-alkanes to form the membrane. In addition "solvent-free" membranes from the same lipid were used for the kinetic studies. The results are summarized in Table 1. The data for DPA^- [8] are also included. In the series *n*-octane to *n*-hexadecane the time constant of the fast relaxation process as well as its amplitude a_1 does not change appreciably. Deviations occur only in the case of "solvent-free" membranes obtained from monolayers. This is caused by a decrease of the concentration of PV-K^+ complexes in the membrane rather than by a change of the translocation rate constant k_{MS}. The decrease of N_t may be caused by the large excess of lipids which is needed for bilayer formation [4], thus lowering the aqueous concentration of PV-K^+ and DPA^-, respectively.

The negligible influence of the membrane thickness on the kinetics of PV-K^+ transport matches the findings with valinomycinmediated Rb^+ transport [3] where also almost no change was found. But it is in sharp contrast to the results obtained with the negatively charged DPA^- (*see* Table 1) or tetraphenylborate [2] systems, where the translocation rate constants strongly increase with increasing specific capacitance of the membranes. This increase of k_i in the case of DPA^- is about 13-fold while the membrane thickness changes a factor of approximately two (from 4.8 to 2.5 nm).

The partition coefficient for PV-K^+ complexes between membrane and aqueous phase was calculated according to Eq. (6). For the determination of the concentration c_{MS} of complexes in the aqueous phase the following relation holds:

$$c_{MS} = \frac{K c_M \cdot c_0}{K c_M + 1}. \tag{7}$$

K is the stability constant of the complexes in the aqueous phase, c_M the ion concentration and c_0 the total PV concentration. For $K \simeq 10\,\text{M}^{-1}$ [5], $c_M = 1\,\text{M}$, and $c_0 = 5 \times 10^{-7}\,\text{M}$, one obtains a value of c_{MS} of about $4.5 \times 10^{-7}\,\text{M}$. This concentration has been used for the calculation of β throughout this study.

Table 1. Kinetic parameters of PV-K^+ and DPA^- transport through membranes formed from glycerol monooleate dissolved in *n*-alkanes of increasing chain length and through solvent-free membranes at 25 °C [a]

Solvent	C_m nF cm^{-2}	PV-K^+					DPA^-	
		τ_1 μs	a_1	k_{MS} 10^3 s^{-1}	N_t pmol cm^{-2}	β 10^{-3} cm	k_i 10^3 s^{-1}	β 10^{-3} cm
n-Octane	394	14	0.83	6.2	2.0	2.2	0.68	2.3
n-Decane	390	14	0.84	5.8	2.2	2.4	0.71	2.5
n-Dodecane	416	13	0.84	6.1	2.3	2.6	0.91	2.2
n-Tetradecane	469	14	0.82	6.8	2.2	2.4	1.2	1.8
n-Hexadecane	585	18	0.77	6.3	2.1	2.3	4.7	2.3
Solvent-free	745	27	0.64	6.8	1.4	1.6	8.5	1.3

[a] The values for DPA^- were taken from ref. 8 and those for specific capacitances C_m from ref. 4. The aqueous phase contained 5×10^{-7} M PV and 1 M KCl. The partition coefficients β for the solvent-free membranes represent a lower limit because of the presence of a large excess of lipid which probably reduces aqueous PV or DPA^- concentration.

b) Variation of the Lipid Structure

Tables 2–5 summarize the experimental results obtained with a variety of lipids. Tables 2 and 3 contain the data of PV-K^+ and DPA^- transport across membranes formed from phosphatidylcholines, monoglycerides, a phosphatidylethanolamine as well as from two negatively charged lipids, phosphatidylserine and phosphatidylinositol. The fatty acid residues of the phosphatidylcholines and the monoglycerides were varied over a wide range. The chain length of monounsaturated fatty acid residues was increased from C_{16}- to C_{22}-chains in the case of the phosphatidylcholines and a C_{16}- to a C_{22}-chain in the case of the monoglycerides. While the partition coefficient β is almost insensitive to these variations, there is a strong influence on the rate constants k_{MS}(PV-K^+) and k_i(DPA^-). For phosphatidylcholines, k_{MS} and k_i increase about fivefold and ninefold, respectively, when the chain length of the fatty acid residue decreases from C_{22} to C_{16}. Similar results were obtained with membranes from monoglycerides differing in the chain length of their hydrocarbon backbone. For PV-K^+ the rate constant of translocation decreases between C_{16} and C_{22} about 200-fold, but the influence on k_i(DPA^-) is much smaller (12-fold).

The results of experiments with the cis and trans isomers of the Δ^9-$C_{18:1}$ fatty acid are also included in Tables 2 and 3. This change has an almost

Table 2. Kinetic parameters of PV-K^+ and DPA^- transport through membranes formed from different lipids dissolved in *n*-decane[a]

Fatty acid residues		PV-K^+					DPA^-	
	C_m nF cm^{-2}	τ_1 μs	a_1	k_{MS} 10^2 s^{-1}	N_t pmol cm^{-2}	β 10^{-3} cm	k_i 10^2 s^{-1}	β 10^{-3} cm
Phosphatidylcholines								
Diphytanoyl (3, 7, 11, 15-$C_{16:4CH_3}$)	366*	2500	0.51	0.97	0.40	0.44	5.9	3.7
Dipalmitoleoyl (Δ^9-$C_{16:1}$)	387*	65	0.54	35	0.49	0.54	8.5	18
Dipetroselinoyl (Δ^6-$C_{18:1}$)	399*	410	0.52	5.8	0.46	0.51	8.8	23
Dioleoyl (Δ^9-$C_{18:1}$)	374*	140	0.59	15	0.58	0.64	4.3	37
Divaccenoyl (Δ^{11}-$C_{18:1}$)	368*	290	0.48	9.0	0.36	0.40	3.7	18
1-Oleoyl-2-stearoyl	370*	390	0.50	6.3	0.40	0.44	5.5	16
Dieicosenoyl (Δ^{11}-$C_{20:1}$)	358*	200	0.62	9.3	0.63	0.70	1.7	47
Dierucoyl (Δ^{13}-$C_{22:1}$)	327*	120	0.83	6.9	1.7	1.9	0.90	50
Egg	339*	150	0.53	16	0.40	0.44	4.2	29
Monoglycerides								
Palmitoleoyl (Δ^9-$C_{16:1}$)	445**	5.3	0.75	240	1.4	1.6	13	0.12
Petroselinoyl (Δ^6-$C_{18:1}$)	378**	19	0.90	26	3.6	4.0	10	2.7
Oleoyl (Δ^9-$C_{18:1}$)	390**	14	0.84	58	2.2	2.4	7.1	2.5
Vaccenoyl (Δ^{11}-$C_{18:1}$)	396**	17	0.87	38	2.9	3.2	6.2	2.1
Elaidinoyl (trans Δ^9-$C_{18:1}$)	375	43	0.85	18	2.2	2.4	11	2.0
Eicosenoyl (Δ^{11}-$C_{20:1}$)	345	51	0.90	10	3.2	3.6	5.5	2.1
Erucoyl (Δ^{13}-$C_{22:1}$)	318**	290	0.93	1.3	4.2	4.7	1.1	3.3
Phosphatidylethanolamine								
Oleoyl (Δ^9-$C_{18:1}$)	372*	2000	0.78	0.55	1.4	1.6	25	11
Phosphatidylserine								
Brain	350	440	0.48	5.8	0.35	0.39	6.6	0.65
Phosphatidylinositol								
Brain	355	160	0.39	19	0.24	0.27	4.2	0.96

[a] The results for DPA^- were taken from ref. [8] and from Table 3. The aqueous phase contained 5×10^{-7} M PV and 1 M KCl, $T = 25$ °C. The values of the specific capacitance C_m denoted with one or two asterisks were taken from ref. [6] or ref. [3], respectively.

Table 3. Kinetic parameters of DPA^- transport through membranes formed from different lipids dissolved in *n*-decane[a]

Fatty acid residues	C_m nF cm^{-2}	τ_1 ms	a_1	k_i 10^2 s^{-1}	N_t pmol cm^{-2}	β 10^{-3} cm
Monoglycerides						
Palmitoleoyl (Δ^9-$C_{16:1}$)	445	0.34	0.13	13	0.069	1.2
Petroselinoyl (Δ^6-$C_{18:1}$)	378	0.36	0.29	10	0.16	2.7
Oleoyl (Δ^9-$C_{18:1}$)	390	0.52	0.27	7.1	0.15	2.5
Vaccenoyl (Δ^{11}-$C_{18:1}$)	396	0.62	0.24	6.2	0.13	2.1
Elaidinoyl (trans Δ^9-$C_{18:1}$)	375	0.35	0.23	11	0.12	2.0
Eicosenoyl (Δ^{11}-$C_{20:1}$)	345	0.67	0.26	5.5	0.13	2.1
Erucoyl (Δ^{13}-$C_{22:1}$)	318	2.90	0.37	1.1	0.20	3.3
Phosphatidylinositol						
Brain	355	1.0	0.13	4.2	0.058	0.96

[a] The aqueous phase contained 3×10^{-8} M DPA^- and 0.1 M NaCl; $T = 25$ °C. The results for monoolein membranes were taken from ref. [7] and the values of the specific capacitance from ref. [3].

Table 4. Kinetic parameters of PV-K^+ and DPA^- transport through membranes formed from phosphatidylcholines differing in the number of double bonds in the fatty acid residues[a]

Fatty acid residue	PV-K^+						DPA^-	
	C_m nF cm^{-2}	τ_1 µs	a_1	k_{MS} s^{-1}	N_t pmol cm^{-2}	β 10^{-3} cm	k_i s^{-1}	β 10^{-3} cm
Dioleoyl (Δ^9-$C_{18:1}$)	624	190	0.48	1.4×10^3	0.60	0.66	6.0×10^3	33
Dilinoleoyl ($\Delta^{9,12}$-$C_{18:2}$)	645	24	0.20	1.7×10^4	0.17	0.19	7.8×10^3	15
Dilinolenoyl ($\Delta^{9,12,15}$-$C_{18:3}$)	665	1.8	0.12	2.4×10^5	0.10	0.12	8.6×10^3	5.0

[a] The lipids were dissolved in *n*-hexadecane. The aqueous phase contained 5×10^{-7} M PV and 1 M KCl; $T = 25$ °C. The data for DPA^- were taken from Table 5 and the specific capacitance for dioleoylphosphatidylcholine from ref. [4].

Table 5. Kinetic parameters of DPA^- transport through membranes formed from phosphatidylcholines with fatty acid residues of different degrees of unsaturation[a]

Fatty acid residue	C_m nF cm^{-2}	τ_1 μs	a_1	k_i s^{-1}	N_t pmol cm^{-2}	β 10^{-3} cm
Dioleoyl (Δ^9-$C_{18:1}$)	624	42	0.49	6.0×10^3	0.65	33
Dilinoleoyl ($\Delta^{9,12}$-$C_{18:2}$)	645	45	0.30	7.8×10^3	0.29	15
Dilinolenoyl ($\Delta^{9,12,15}$-$C_{18:3}$)	665	51	0.12	8.6×10^3	0.10	5.0

[a] The lipids were dissolved in *n*-hexadecane. The aqueous phase contained 10^{-8} M DPA^- and 0.1 M NaCl; $T = 25$ °C. The experimental data for dioleoyl phosphatidylcholine were taken from ref. [8] and the value of the specific capacitance for the same system from ref. [6].

negligible influence on $k_i(DPA^-)$. A much stronger dependence on this structural variation was found for $k_{MS}(PV\text{-}K^+)$. For the lipid with branched C_{16}-chains diphytanoylphosphatidylcholine a 36-times smaller k_{MS} was observed than with the straight C_{16} chain. In this case, too, the influence on k_i is comparatively low.

Tables 2 and 3 also contain the results obtained for lipids with other polar head groups, like phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Whereas the rate constant k_{MS} for the translocation of the $PV\text{-}K^+$ complex is much smaller (30 to 100-fold) in phosphatidylethanolamine membranes than in the other neutral membranes, $k_i(DPA^-)$ changes to a smaller extent. Melnik *et al.* [31] found a similar low value for k_{MS} at solvent-free membranes formed from bacterial phosphatidylethanolamine, but their partition coefficient β is about 20 times smaller than ours. This is possibly caused by the large excess of lipid needed to form stable membranes, thus lowering the aqueous PV-concentration. The values for $k_{MS}(PV\text{-}K^+)$ and $k_i(DPA^-)$ for the negatively charged lipids phosphatidylserine and phosphatidylinositol do not differ very much from those obtained with egg phosphatidylcholine. However, it is interesting to note that the partition coefficients β for $PV\text{-}K^+$ are very small for the two charged lipids phosphatidylserine and phosphatidylinositol at large ionic strength (1.6×10^{-5} cm and 1.3×10^{-5} cm), while the opposite was found for β of DPA^-. At large ionic strength, β for this lipophilic ion and phosphatidylserine membranes is at least 20 times higher ($\beta = 0.25$ cm) than it is for the neutral lipids [8].

In order to determine the number of elementary charges per unit area for phosphatidylinositol membranes, experiments with DPA^- and different concentrations of NaCl were performed. The partition coefficient β changed from 1.3×10^{-2} cm at 1 M NaCl to 0.96×10^{-3} cm at 0.1 M NaCl and to 1.1×10^{-4} cm at 10^{-2} M NaCl, while k_i remains virtually constant within the limits of experimental error. The data for β correspond to a charge density of one elementary charge per 0.57 nm^2 and a β_0 for large ionic strength of 0.35 cm. The fit was performed using the Gouy-Chapman equation [8] and assuming that no Na^+ ions are adsorbed to the phosphatidylinositol membranes.

It was reported earlier [8] that a number of experiments with DPA^- were performed using phosphatidylcholines with C_{18}-chains and a different degree of unsaturation. It was found that there is a strong increase of k_i with increasing number of double bonds. But in the same series there was also a large increase of the specific capacitance of the membranes indicating a lowering of the thickness and/or an increase of the dielectric constant of the hydrocarbon region. So far it has not been clear whether the influence on k_i was due to the increasing membrane fluidity or to the decreasing thickness. Therefore, a series of experiments were performed with PV-K^+ and DPA^- (Tables 4 and 5) using the same lipids dissolved in *n*-hexadecane where the specific capacitance increase should be small. It is seen from Table 4 that the influence of the number of double bonds on k_{MS} of PV-K^+ is extremely high. From one to three double bonds in a C_{18}-chain k_{MS} increases about 200-fold, whereas β decreases sixfold. The influence of the degree of unsaturation in a C_{18} chain on the transport kinetics of DPA^- is much smaller; k_i increases less than 50% in the series one to three double bonds. The findings with PV-K^+ are quite similar to the earlier observation with the valinomycin-Rb^+ system [3], where also a strong influence of the degree of unsaturation on k_{MS} and k_S was found.

Table 6 summarizes experiments with lipids differing in the nature of the linkage between hydrocarbon chain and glycerol backbone. The results obtained earlier with DPA^- [8] are also included. A comparison of experiments with ester and ether analogs of phosphatidylcholine and phosphatidylethanolamine is of some interest because it is known that the nature of this linkage influences the surface potential of monolayers to a large extent [33]. Changes of the dipolar potential drop in a membrane-water interface may influence the ion permeability of membranes [20, 22, 25, 31]. Table 6 shows that there is a strong influence of the kind of linkage of the hydrocarbon tail on the rate constants of translocation k_{MS} and k_i. For PV-K^+, k_{MS} is generally larger for ether linkage, while the opposite is

Table 6. Kinetic parameters of PV-K^+ and DPA^- transport through membranes formed from lipids with ester or ether linkage of the hydrocarbon tail[a]

Lipid		PV-K^+					DPA^-	
	C_m nF cm^{-2}	τ_1 ms	a_1	k_{MS} s^{-1}	N_t pmol cm^{-2}	β 10^{-3} cm	k_i s^{-1}	β 10^{-3} cm
1-Oleoyl-2-stearoyl 3-phosphatidylcholine	370	0.36	0.55	630	0.48	0.53	550	16
1-O-Oleyl-2-O-palmityl 3-phosphatidylcholine	352	0.043	0.57	5000	0.50	0.56	24	31
Dioleoyl phosphatidyl-ethanolamine	372	2.0	0.78	55	1.4	1.6	2500	11
Di-O-oleyl phospha-tidylethanolamine	357	0.043	0.69	3600	0.85	0.95	350	16

[a] The lipids were dissolved in *n*-decane. The aqueous phase contained 5×10^{-7} M PV and 1 M KCl, $T = 25$ °C. The data for DPA^- were taken from ref. [8] and the values of the specific capacitances from ref. [6].

true for $k_i(DPA^-)$. The partition coefficients for both "probes" are influenced to a much smaller extent.

c) Membranes Containing Sterols

Table 7 contains the results obtained for the two "probes" with membranes containing the sterols cholesterol, epicholesterol and ergosterol. The molar lipid/sterol rations in the membrane forming solution ranged from 5 : 1 to 1 : 4. In order to avoid a thinning of the membranes, as has been observed with decane [3, 8, 19], *n*-hexadecane was used as solvent. Table 7 shows that epicholesterol and ergosterol do not influence appreciably the transport kinetics of PV-K^+ and DPA^-. Similar observations have also been made with stigmasterol and cholesterololeat [2].

In contrast, cholesterol influences monoolein and phosphatidylcholine membranes to a much larger extent. While for PV-K^+ in the monoolein/cholesterol membranes k_{MS} and β change, for DPA^- only k_i increases with increasing cholesterol content. Cholesterol added to the membrane forming solution of dioleoyl phosphatidylcholine membranes changes only k_{MS} of PV-K^+ and leaves the kinetics of DPA^- nearly unaffected.

Table 7. Kinetic parameters of PV-K^+ and DPA^- transport through membranes formed from lipid/sterol mixtures dissolved in *n*-hexadecane is the sterol/lipid mole fraction in the membrane-forming solution[a]

	PV-K^+					DPA^-	
X	τ_1 ms	a_1	k_{MS} s^{-1}	N_t pmol cm^{-2}	β 10^{-3} cm	k_i s^{-1}	β 10^{-3} cm
Dioleoyl phosphatidylcholine/cholesterol							
0	0.19	0.47	1400	0.60	0.66	6000	33
0.17	0.22	0.44	1300	0.52	0.57	5800	37
0.33	0.32	0.48	820	0.61	0.67	5600	46
0.50	0.76	0.46	360	0.56	0.62	6200	34
0.67	2.06	0.42	140	0.49	0.55	6500	36
0.80	8.16	0.43	35	0.50	0.55	6300	35
Dioleoyl phosphatidylcholine/epicholesterol							
0.50	0.15	0.46	1800	0.58	0.64	–	–
0.80	0.11	0.48	2400	0.62	0.68	6700	34
Dioleoyl phosphatidylcholine/ergosterol							
0.50	0.20	0.48	1300	0.61	0.67	–	–
0.80	0.17	0.46	1600	0.56	0.62	6200	30
Monoolein/cholesterol							
0	0.019	0.77	6300	2.1	2.3	4700	2.3
0.17	0.021	0.76	5900	2.0	2.2	5400	2.3
0.33	0.039	0.67	4200	1.3	1.5	7700	2.2
0.50	0.088	0.52	2700	0.70	0.77	15000	2.8
0.67	0.150	0.40	2000	0.43	0.47	26000	2.5
0.80	0.320	0.30	1100	0.27	0.31	38000	2.5
Monoolein/epicholesterol							
0.50	0.018	0.74	7100	1.8	2.0	3700	2.5
0.80	0.017	0.72	8200	1.6	1.8	4100	3.2

[a] The aqueous phase contained 5×10^{-7} M PV and 1 M KCl; $T = 25$ °C. The results for DPA^- and the values for the specific capacitances were taken from ref. [2].

Discussion

In this publication we have varied the composition of lipid bilayer membranes over a wide range. For all the systems reported we were able to measure the kinetics of two charged probes, the negatively charged DPA^- and the positively charged PV-K^+ complex. The latter is considered as a lipophilic cation because of its high stability constant in the aqueous phase [5] and its low dissociation rate constant [14]. It was therefore possible to

evaluate for the two probes the rate constants of translocation (k_i and k_{MS}) as well as the partition coefficient β. The rate constant of exchange between the energy minima in the membrane-water interface and the aqueous phase could not be determined because of slow aqueous diffusion (DPA^-) [9] or of the possible slow dissociation of PV-K^+ in the membrane and/or in the aqueous phase.

In one set of experiments the influence of the solvent used for bilayer formation on the transport kinetics of the two probes were studied. Going from *n*-octane to *n*-hexadecane and to solvent-free membranes, the membrane thickness d changes from 4.8 nm (390 nF cm^{-2}) to 2.5 nm (745 nF cm^{-2}). For DPA^- this change has a large influence on the rate constant of translocation, increasing it by a factor of 13 going from the thickest (4.8 nm) to the thinnest (2.5 nm) membrane. This strong increase in k_i may be explained by a lowering of the electrostatic energy w in the middle of the membrane with a decreasing membrane thickness from d^* to d [34]:

$$\Delta w = w(d) - w(d^*) = h\left(\frac{1}{d^*} - \frac{1}{d}\right) \tag{8}$$

$$h = \frac{e_0^2}{4\pi\varepsilon_0\varepsilon_m kT} \ln\left(\frac{2\varepsilon_w}{\varepsilon_w + \varepsilon_m}\right) \simeq 17.8 \text{ nm} \qquad (T = 298 \text{ K}). \tag{9}$$

e_0 is the elementary charge, ε_0 the permittivity of free space, $\varepsilon_m \simeq 2.1$ and $\varepsilon_w \simeq 78.5$ are the dielectric constants of the membrane and of water, respectively, k is the Boltzmann constant, and T the absolute temperature; w is expressed in units of kT.

The theoretically predicted values of k_i/k_i^* (k_i^*, d^* for *n*-decane as solvent) fit the experimentally observed data very well for DPA given in Table 1 for membranes formed from monoolein dissolved in the different *n*-alkanes. Only for solvent-free membranes from this lipid k_i/k_i^* is about a factor of two to three lower than predicted by $\exp(\Delta w)$. This finding indicates that there is no particular structural difference between membranes formed from a lipid dissolved in different *n*-alkanes other than the membrane thickness. Similar considerations may also be applied to solvent-free membranes. This result also fits very well the considerations of Haydon [20], who has presented arguments that the packing of the polar head groups is nearly the same in solvent containing and solvent-free membranes. Thus, from the thickness dependence of k_i one may conclude that the only effect of the solvent is to increase the thickness of the hydrophobic layer leaving the packing of the membranes essentially unchanged. The observed thickness dependence of k_i for DPA^- is in sharp

contrast to the findings with PV-K^+ (Table 1) and to the earlier findings with valinomycin-Rb^+ [3]. In these cases there exists almost no influence of the membrane thickness on the rate constant k_{MS}. This difference in behavior of the negatively and positively charged ions is not readily explained, although there are some differences in the kinetic behavior of the probes. For instance, it has been shown that the total voltage applied to the membrane is seen neither by PV-K^+ [5] nor by valinomycin [28], while DPA^- or tetraphenylborate see a much larger fraction of the voltage [1, 9]. This may mean that the adsorption planes of the two differently charged probes is different. The positively charged probes may be adsorbed at the inner side of the dipolar layer, whereas DPA^- or tetraphenylborate may be adsorbed more towards the aqueous side of the membrane interface. The findings with cholesterol/lipid mixtures [2] seem to support this hypothesis.

Another possible explanation has to do with the size of the molecules. PV-K^+ and valinomycin-Rb^+ are large molecules with a diameter of approximately 1.5 nm whereas tetraphenylborate and DPA^- are much smaller. The incorporation of the probe into the bilayer could lead to a structural perturbation which is different for the two probes. If this is the case the perturbation may increase with decreasing membrane thickness. Possibly the two opposite effects, the increase of k_{MS} due to Δw and its viscosity change due to increased perturbation could compensate each other. The surfaces of the PV-K^+ and valinomycin-Rb^+ complexes are presumably more lipophilic than the surface of tetraphenylborate and DPA^-. The different behavior of the two probes under certain conditions may also be influenced by this fact.

These considerations are supported by the finding that the positively charged lipophilic ions tetraphenylphosphonium and tetraphenylarsonium, which are structural analogs to the negatively charged tetraphenylborate, show a similar dependence for k_i on the membrane thickness as the negatively charged lipophilic ions DPA^- and tetraphenylborate (R. Benz and A. Pickar, *in preparation*).

Besides the membrane thickness, other structural properties of the membranes may be responsible for the observed changes of k_{MS} and k_i with changes in the structure of the lipids. From Table 2 and Eq. (9) it may be easily shown that the decrease of k_i with increasing chain length of the fatty acid residues of monoglycerides and lecithins cannot be explained in terms of thickness increase alone. Changes of viscosity, dielectric constant, and changes of the dipolar potential of the membranes may also influence the values of the translocation rate constants k_{MS} and k_i. The structure of the fatty acid residues of a lipid should not change the surface potential of the

Table 8. Values for $k_{MS} \cdot \beta$ (PV-K^+) and $k_i \cdot \beta$ (DPA$^-$) for different lipids dissolved in *n*-decane, $T = 25\,^{\circ}C$[a]

Lipid	PV-K^+ $k_{MS} \cdot \beta$ $(s\,cm)^{-1}$	DPA$^-$ $k_i \cdot \beta$ $(s\,cm)^{-1}$	d nm	V mV	Ref.
Glycerolmonooleate	14	1.8	4.8	320	[20]
Dioleoylphosphatidyl-choline	0.96	16	5.0	380, 440	[33, 20]
Dioleoylphosphatidyl-ethanolamine	0.088	28	5.0	420	[20]
Di-O-oleyl phos-phatidylethanolamine	3.42	5.6	5.2	310	[33]
1-Oleoyl-2 stearoyl-phosphatidylcholine	0.33	8.8	5.0	390	[33]
1-O-Oleyl-2-O-palmityl 3-phosphatidylcholine	2.8	0.74	5.3	290	[33]

[a] d is the thickness of the membranes as calculated from the specific capacitances and V the values of the surface potentials of monolayers from corresponding types of lipids.

membranes to a large extent. Therefore, the large influence of the number of carbon atoms and of the number of double bonds in the hydrocarbon backbone on k_{MS} (Tables 2 and 4) may be discussed on the basis of viscosity changes of the membranes. Similar considerations apply to the differences found in the kinetics of PV-K^+ between a lipid with branched or unbranched fatty acid residues. A comparison between the kinetics for PV-K^+ and for valinomycin-Rb^+ [3] shows a similar dependence on structural properties of the membranes such as thickness and lipid composition.

The negatively charged probe DPA$^-$ seems to be much less sensitive to viscosity effects of the membrane than the positively charged PV-K^+ system. The position of the double bond, the cis trans isomerism, the number of double bonds, and the difference between branched and straight chain also have a small influence on the rate constant k_i of translocation. The different behavior of the two transport systems with respect to the discussed structural differences is not clear. But as it was already mentioned above, the different sizes and surfaces of the probes may possibly play a role in this question.

Another approach to explain the differences in the DPA$^-$ and PV-K^+ data for the different systems considers the dipolar potential of the membranes. There is a strong evidence that, as a result of the existence of dipolar layers, the interior of lipid bilayer membranes is positive by several

hundred millivolts with respect to the aqueous phase [20] (*see also* [30] for a review). The existence of dipolar layers in the membrane surface may affect in principle the partition coefficient β as well as the rate constants of translocation. The relative effect on both may be dependent on the location of the adsorption plane of the probe. Therefore, the product $k_i \cdot \beta$ may be regarded as a measure of the magnitude and sign of the dipolar potential in the membrane surfaces. The existence of a dipolar potential seems to contradict the conclusion that PV-K^+ is located towards the interior of the membranes. But the hydrophobic interaction between the surface of the complex and the fatty acid chains may be strong enough to overcome the electrostatic repulsion.

Table 8 shows the value for $k \cdot \beta$ for the two systems DPA^- and PV-K^+ obtained at membranes with a similar thickness. The surface potentials for monolayers from the same type of lipids are also included in Table 8. It is seen that there is some qualitative agreement between the magnitude of the surface potential and the data for $k_i \cdot \beta$ and $k_{MS} \cdot \beta$. For higher surface potential (where the dipolar potential of a bilayer is also expected to be high) larger values for $k_i \cdot \beta$ as for $k_{MS} \cdot \beta$ were observed and vice versa for lower potentials. However, the quantitative agreement between the experimentally found and the expected (from the difference of the surface potentials) values is poor. From this deviation one may conclude that either the variation of the surface potential is not alone responsible for the differences between the individual lipids, or that changes in the surface potentials of monolayers are to some extent not comparable with those of the dipolar potentials in bilayer membranes. In the first case, which is more likely, hydrophobic interactions between probe and membranes and its structural properties may also play a role. The large difference between $k_{MS} \cdot \beta$ derived for membranes from dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine shows this very clearly.

Similar considerations apply to the results obtained from membranes composed from dioleoylphosphatidylcholine/cholesterol mixtures. For an increasing mole fraction of cholesterol between 0 and 0.8, the translocation rate constant k_{MS} of PV-K^+ decreases 40-fold, whereas the surface potential of monolayers does not change very much [20]. The strong influence on k_{MS} seems to be very similar to the observed decrease of nonelectrolyte permeability of liposomes in the presence of cholesterol, an effect which has been explained in terms of increasing viscosity of the membranes with increasing content of cholesterol [16]. The kinetics of DPA^- seems to be almost insensitive to this viscosity effect. The reason for this finding is not

yet clear, but it may be caused by the different size and/or location of the probes.

The transport kinetics of the two probes are almost insensitive to the other sterols. So far as epicholesterol is concerned, these findings are in agreement with studies with liposomes [16]. In the case of ergosterol a cholesterole-like, but less pronounced, effect on the permeability of liposomes has been found [15]. Although it seems not very likely, this may be explained by the assumption that ergosterol (and possibly also epicholesterol) are completely excluded from a bilayer. The concentration of sterols in the membrane is a critical point because it is not clear if the molar ratio lipid/sterol is the same in the bulk phase and in the black part of the membrane. It has been shown that this is not given in certain cases, especially at high mole fractions of cholesterol in the phosphatidylcholine/cholesterol mixtures [13, 32]. Similar considerations apply to membranes from monoolein/sterol mixtures, where the influence of sterols which are structural analogs of cholesterol on the transport kinetics of the probes is also rather small [2].

The action of cholesterol on the kinetics of PV-K^+ through membranes made from monoolein/cholesterol mixtures dissolved in *n*-hexadecane is much more complex compared with the action on DPA^-. It is seen from Table 7 that not only k_{MS} decreases with increasing cholesterol content, but also β shows a similar behavior. If only β is dependent on the change of the dipole potential and k_{MS} changes by viscosity changes of the membrane, the dependence of β on mole fraction x should be given by:

$$\beta = \beta^* \exp(-F x \Delta V_D / RT) \tag{10}$$

where β^* is the partition coefficient for $x = 0$ and ΔV_D is the total change of the dipolar potential V_D from a pure monoolein membrane to a pure cholesterol membrane. A reasonable fit of Eq. (10) is obtained with a value of $\Delta V_D = 70$ mV, which is close to the surface potential difference for monolayers of cholesterol ($V_D = 390$ mV) and monoolein ($V_D = 320$ mV) [20]. The significance of this finding is not obvious, as some of the assumptions which are implicit in Eq. (10), *viz.*, cholesterol and monoolein are ideally mixed and the membrane has the same composition as the bulk phase, seem to be doubtful. A similar consideration has been applied to the results obtained for the translocation rate constant k_i of DPA^- [2]. But for this transport system only k_i has been found to be dependent on the cholesterol content [2], whereas for PV-K^+ β and k_{MS} are affected. Because the effects on β for PV-K^+ is as large as the effect on k_i for DPA^-, the

additional effect on k_{MS} ($\Delta V_D = 120$ mV for the change of k_{MS} and β) may be caused by a viscosity change of the membranes in the presence of cholesterol. Similar conclusions have been derived for the influence of cholesterol on the rate constants of translocation k_{MS} and k_S in the case of valinomycin-mediated Rb^+ transport [3]. The findings with PV-K^+ and DPA^- support the hypotheses that DPA^- is adsorbed towards the aqueous side of the dipolar layer, whereas PV-K^+ is located towards its hydrocarbon side. The results obtained with cholesterol are in qualitative agreement with the data of G. Szabo [38, 39]. The deviations between his data and ours may be explained by the fact that his membranes, being made from lipid dissolved in *n*-decane, are thinned upon the inclusions of cholesterol [39] in *n*-decane, thus causing an additional increase of k_i.

From the data presented here and in previous publications [2, 3, 8] some conclusion can be drawn as to which of the different membrane parameters such as thickness, viscosity, and magnitude of dipole potential act on the two transport systems. The transport of the lipophilic ions seems to be mostly influenced by membrane thickness and the magnitude of the dipole potential. Changes in the dipole potential, as in the case of cholesterol [2] and phloretin [31], act only on the translocation rate constant. Thickness changes of the membrane have a neglectible influence on the translocation rate constants of carrier mediated ion transport, which in fact are limited to a large extent by the viscosity of the membrane. As has been pointed out already, the different behavior of the two systems may be caused by a different location of the adsorption planes of the two transport systems.

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