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IONIC SELECTIVITY OF PORES FORMED BY THE MATRIX PROTEIN (PORIN) OF *ESCHERICHIA COLI*

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Summary

Incorporation of the matrix protein (porin) from the outer membrane of *Escherichia coli* into black lipid films results in the formation of ion-permeable pores with a single-pore conductance of the order of 2 nS (in 1 M KCl). Information on the structure of this pore has been obtained by determining the selectivity for various ion species differing in charge and size. From the permeability of the pore for large organic ions ($Tris^+$, $glucosamine^+$, $Hepes^-$) a minimum pore diameter of 0.8 nm is estimated. At neutral pH the pore is two to four times more permeable for alkali ions than for chloride. On the basis of the observed pH dependence of permeability, this cationic selectivity is explained by the assumption that the pore contains fixed negative charges.

Introduction

The outer membrane of gram-negative bacteria, such as *Escherichia coli*, is permeable to sugars and other hydrophilic solutes up to a molecular weight of 500—600 [1—5]. This passive permeability seems to depend on the presence of a class of major membrane proteins [3]. One of them, called protein I [6], matrix protein [7], protein 1 [8], and porin [9] has been isolated and shown to consist of a single polypeptide chain of molecular weigh 36 500 [6]. Incorporation of purified porin into phospholipid vesicles rendered the vesicle membrane permeable to hydrophilic solutes up to a molecular weight of about 550 [9]. Recently it has been demonstrated that incorporation of detergent-

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulphate.

solubilized porin into planar lipid bilayer membrane results in the formation of ion-permeable pores with a high electrical conductance (of the order of 1—2 nS in 1 M alkali chloride solutions) [10]. The electrical properties of these pores were found to be independent whether sodium dodecyl sulphate or cholate was used as a detergent. Furthermore, detergent-free porin, isolated from the shock fluid of *E. coli*, gave pores of identical properties [11]. Porin pores of a similar size have recently been obtained using a different incorporation technique [12].

Although the magnitude of the unit conductance increment and the voltage-independent behavior of the conductance are consistent with the notion that porin forms large water-filled pores in the lipid bilayer membrane, the structure of these pores is largely unknown. If it is assumed that the single conductance step corresponds to the formation of a single pore of circular cross-section and that the pore is filled with an aqueous solution of the same conductance as the external phase, then the diameter of the pore is estimated to be about 0.9 nm. On the other hand, the conductive unit could consist of a bundle of parallel pores of smaller size which are formed at once. Another unsolved problem concerns the broad distribution of channel conductances which is found in the bilayer experiment [10,11]. This distribution could result from a heterogeneity in channel diameters and/or from leakage pathways created by a disturbance of the lipid layer after incorporation of the pore. Information bearing on these questions may be obtained by studying the ionic selectivity of the pore. In the following we describe experiments in which the selectivity has been studied by measuring the pore conductance in a series of electrolyte solutions with cations and anions of different size, as well as by determining the membrane potential in the presence of a salt concentration difference. In addition, the pH dependence of the pore conductance was studied. The results of these experiments argue against the concept of a bundle of parallel pores; they are consistent, however, with the notion that the single conductive unit is a pore of large diameter.

Materials and Methods

Black lipid bilayer membranes were obtained in the usual way [13] from a 1—2% (w/v) solution of the lipids in *n*-decane or *n*-hexadecane (Fluka, Buchs, Switzerland, purum). The cell used for bilayer formation was made from Teflon; the circular holes in the wall between the two aqueous compartments had an area of either 2 mm^2 (for the macroscopic conductance measurements) or 0.1 mm^2 (for the single-channel experiments). The temperature was kept constant at 25°C throughout all experiments.

All salts, besides *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (Sigma, analytical grade), tetramethylammoniumhydroxide and tetraethylammoniumhydroxide (Fluka, purum) were obtained from Merck (Darmstadt, F.R.G., analytical grade). The pH of the aqueous salt solutions was adjusted to the values given in the text by adding the corresponding hydroxide or acid. In the case of the salts with large ions the natural pH of the salt was used. In the experiments on the pH dependence of conductance the aqueous salt solutions were buffered with 10^{-3} M citrate or 10^{-3} M Tris. In separate

experiments it was tested that citrate or Tris in such concentrations did not have an influence on the results. Aqueous solutions with a pH around 6 were used without buffering. The specific conductance of the various salt solution were measured with a conductometer (Metrohm, Herisau, Switzerland).

Porin was isolated from the outer membranes of *E. coli* K 12, strain pop 1730, by sodium dodecyl sulphate (SDS) solubilization following trypsin treatment of SDS-extracted membranes [10]. Strain pop 1730 has a deletion in *lam B* and lacks the λ-receptor [14]. The porin fraction of K 12 strains of *E. coli* bacteria contains two different proteins (Ia and Ib) with almost identical amino acid sequence and molecular weight [16]. The isolated protein gave a single band on SDS-polyacrylamide gel electrophoresis corresponding to a molecular weight of about 37 000 which is in close agreement to the value given in the literature [7]. The protein was stored in lyophilized form at −25°C. In this form it remained active in membrane experiments for at least one year. For membrane experiments a stock solution was prepared containing 0.2 mg/ml protein, 0.1% SDS, 5 mM Tris-HCl, pH 8, and 3 mM NaN_3. This stock solution remained active in membrane experiments two to three months if it was stored in the refrigerator at 4°C. At high ionic strength ($1 \cdot 10^{-2}$—3 M) and in high dilution (1 μg/ml SDS), however, the protein became inactive overnight. Therefore fresh solutions were prepared prior to the experiments from small aliquots of the stock solution.

In order to avoid the trypsin treatment two alternative methods were used. First a salt extraction method with 0.5 M NaCl in the presence of EDTA. Second a method which uses lysozyme instead of trypsin to destroy the peptidoglycan layer [5]. However, both porin preparations were found to be completely inactive in membrane experiments, although SDS-polyacrylamide gel electrophoresis of the proteins showed for protein obtained from all three procedures one single band corresponding to the same molecular weight of about 37 000. The reason for the inactive porin obtained by the lysozyme preparation is not clear so far. In the case of the salt extraction method, however, it may be mentioned that SDS-porin [10] as well as porin from the shock fluids [11] were found to be completely inactivated in solutions of high ionic strength overnight (20 h)

It is interesting to note that the porin isolated by the salt extraction method as well as the porin obtained by the lysozyme treatment of the cell wall showed the same immunological cross-reaction as porin obtained by trypsin treatment [11]. In the Ouchterlony immunodiffusion test no precipitin lines were found within the gel. Precipitation with the antibodies occurred only on the border of the porin containing well, possibly caused by the strong aggregation of the porin. The largely different behavior of the porins obtained by three different methods with lipid bilayers indicates that the experiments are very powerful studying the structural integrity of the protein.

An interesting mechanical effect on the membrane conductance was observed during the experiments in presence of porin. If the aqueous solution in the membrane cell was intensively stirred or if the membrane was subjected to mechanical shock in parallel to its plane the membrane conductance decreased. In single channel experiments the pores could be switched off by a similar procedure. The origin of this behaviour is not clear so far, although it cannot be

excluded that at least a part of this effect may be explained by the formation of new membrane from the torus during mechanical disturbance of the membrane.

Two different lipids were used for membrane formation: Monoolein (Nu Check Prep. Elysian, Minn. U.S.A.) and oxidized cholesterol. The latter lipid was prepared by boiling a 4% (w/v) suspension of cholesterol (Eastman, reagent grade) in *n*-octane for 4 h under reflux and bubbling oxygen through the suspension [15]. For the electrical measurements. Ag/AgCl or platinized platinum electrodes were inserted in the aqueous compartments on both sides of the membrane. For the conductance-fluctuation experiments a Keithly 427 preamplifier was used. The amplified signal was monitored with a Tectronix 5111/5A22 storage oscilloscope and recorded with a stripchart recorder. The rise time of the single steps in the fluctuation measurements was limited by the band-width of the measuring circuit and was between 0.3 and 3 ms, depending on the amplification. Zero-current membrane potentials were measured with a Keithley 610 C electrometer, using Ag/AgCl electrodes which were connected via agar bridges to the membrane cell.

Results

Pore conductance

If small amounts of porin (of the order of 0.1—1 ng/ml) are added to one or both aqueous compartments of the membrane cell, the conductance of the black film starts to increase in a stepwise fashion [10]. From records of the membrane current over long periods of time the average conductance increment $\overline{\Lambda}$ is obtained by counting a sufficient number of single events. Values of $\overline{\Lambda}$ for various electrolytes differing in the charge and the size of cation and anion are listed in Table I. In addition to $\overline{\Lambda}$, the specific conductance σ of the aqueous phase, as well as the ratio $\overline{\Lambda}/\sigma$ are given in Table I. It is seen that, despite a variation of $\overline{\Lambda}$ by a factor of about 70, the ratio $\overline{\Lambda}/\sigma$ varies only by a factor of 4. This follows more or less the mobility sequence in water. This is also reflected in Fig. 1. The points for the different salts can be fitted by a straight line with the slope one. Even ions as large as $N(CH_2CH_3)_4^+$ or $Hepes^-$ seem to be permeable; $N(CH_2CH_3)_4^+$ has a nearly spherical shape with a diameter of 0.75 nm, $Hepes^-$ may be represented as an ellipsoid with axes of 1.4, 0.6, and 0.5 nm (Table II). If it is assumed that the pore is a cylinder with spherical cross-section and is filled with an aqueous solution of the same conductance as the external phases, the $\overline{\Lambda}$ value measured in 1 M KCl gives an average pore diameter of about 0.93 nm [10]. The finding that ions like $Hepes^-$ are permeable is consistent with this estimate; on the other hand, the observed values of $\overline{\Lambda}$ make a model consisting of a bundle of parallel pores of smaller size rather unlikely.

Two examples for the distribution of Λ values are shown in Fig. 2. As reported earlier [10] the observed conductance increments are spread over a considerable range. The distribution is somewhat narrower in the case of $Tris^+$ $Hepes^-$ as compared with KCl. A possible reason for this difference in the width of the distribution may be that with $Tris^+$ $Hepes^-$ as electrolyte the pores at the lower end of the size distribution do no longer contribute to the conductance.

TABLE I

AVERAGE CONDUCTANCE INCREMENT $\overline{\Lambda}$ IN DIFFERENT SALT SOLUTIONS OF CONCENTRATION c

The solution contained 0.5 ng/ml porin and 2.5 ng/ml SDS; the pH was between 6.0 and 7.0, if not otherwise stated. The membranes were made from oxidized cholesterol in n-decane (1–2%, w/v), T = 25°C. The voltage was 50 mV. $\overline{\Lambda}$ was determined by recording a large number n of conductance steps and averaging over the distribution of Λ values (Fig. 1). σ is the specific conductance of the aqueous phase at 25°C. Data marked with an asterisk have been taken from ref. 10.

Salt	c (M)	Λ (nS)	σ (mS · cm^{-1})	$\overline{\Lambda}/\sigma$ (10^{-8} cm)	n
LiCl *	1.0	0.72	71	1.01	418
NaCl *	1.0	1.2	84	1.43	206
KCl *	1.0	1.9	112	1.70	321
NH_4Cl *	1.0	2.0	112	1.79	213
RbCl *	1.0	2.1	115	1.88	251
CsCl	1.0	2.0	115	1.74	128
$MgCl_2$	0.5	0.43	64	0.67	219
$CaCl_2$	0.5	0.44	78	0.56	426
$BaCl_2$	0.5	0.43	78	0.55	199
K_2SO_4	0.5	0.96	76	1.26	208
$MgSO_4$	0.5	0.24	33	0.73	115
Na^+Hepes^- (pH 9.0)	0.5	0.24	18	1.33	119
$Tris^+Cl^-$	0.5	0.30	30	1.00	203
$Tris^+Hepes^-$ (pH 8.0)	0.5	0.064	7.2	0.89	301
$Glucosamine^+Cl^-$ (pH 3.0)	1.0	0.46	45	1.02	208
$N(CH_3)_4^+Cl^-$	1.0	0.67	71	0.94	144
$N(CH_3)_4^+Hepes^-$ (pH 8.5)	0.5	0.087	15	0.58	321
$N(C_2H_5)^+Hepes^-$	0.5	0.032	4.8	0.67	151

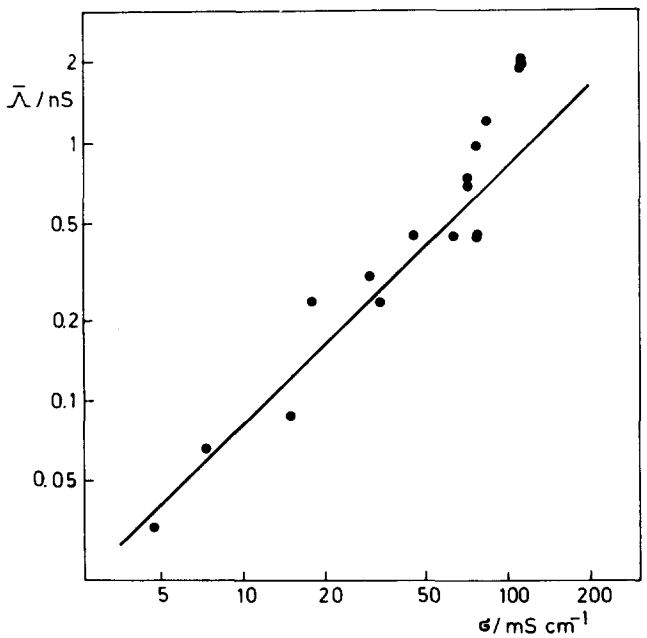

Fig. 1. Average pore conductivity $\overline{\Lambda}$ of the porin pore for different salts given as a function of the specific conductance of the corresponding aqueous salt solutions. T = 25°C. The data were taken from Table I.

TABLE II

APPROXIMATE SIZE OF THE ORGANIC IONS USED IN THE CONDUCTANCE EXPERIMENTS (DETERMINED FROM SPACE-FILLING MODELS)

In the case of glucosamine$^+$ and Hepes$^-$ the length of the axes of the equivalent rotational ellipsoid are given.

	$N(CH_3)_4^+$	$N(C_2H_5)_4^+$	$Tris^+$	Glucosamine$^+$	Hepes$^-$
Diameter (nm)	0.60	0.75	0.67	0.8/0.7	1.4/0.6/0.5

Zero-current membrane potentials

The conclusions drawn from conductance experiments are supported by measurements of zero-current membrane potentials V_m in the presence of different concentrations of the same electrolyte on both sides of the membrane. These measurements were carried out in the following way. The membrane was formed in a 10^{-2} M salt solution. After the membrane was in the optically 'black' state, the conductance was measured applying a voltage of 10 mV. In all experiments reported here the conductance of the undoped membrane was below 10 nS · cm^{-2}. After addition of porin from a concentrated stock solution to a final concentration of 0.2 μg/ml, the conductance increased to $\lambda \approx 20$ μS · cm^{-2} in the case of the alkali chlorides. (In the case of the other salts the conductance was lower, corresponding to the $\bar{\Lambda}$ values given in Table I.) Higher values of λ were avoided in order to minimize diffusion polarization in the unstirred layers adjacent to the membrane. The salt concentration on one side of the membrane was raised by adding a small amount of concentrated solution under stirring an equal volume of the original solution

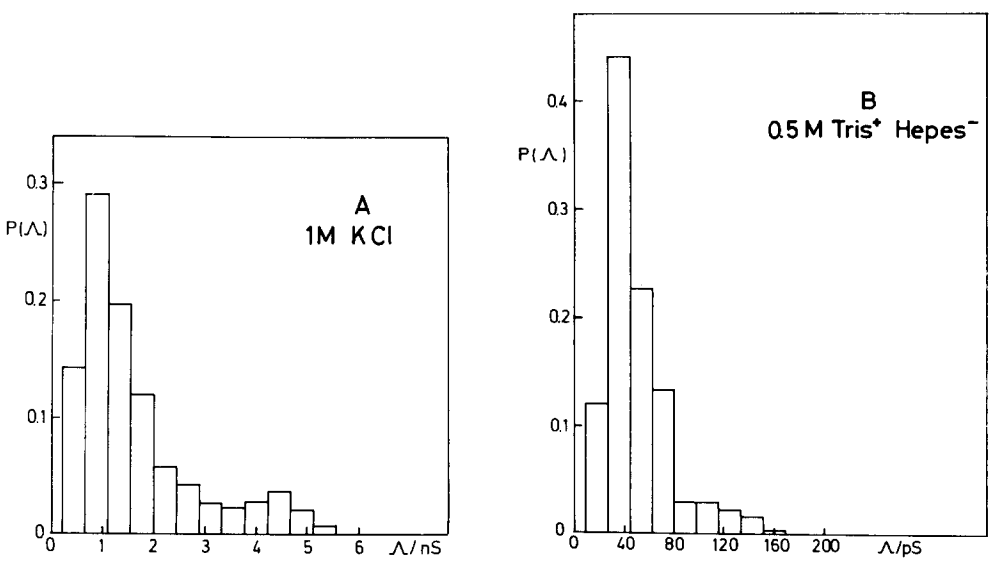

Fig. 2. Probability P (Λ) of the occurrence of a conductance step of magnitude Λ. P (Λ) is the number of observed steps within an interval of width $\Delta\Lambda = \pm 220$ pS (A) or $\Delta\Lambda = \pm 8.9$ pS (B) centered at Λ, divided by the total number n of steps. The membranes were made from 2% (w/v) oxidized cholesterol in n-decane. Besides the electrolyte the aqueous phase contained 0.5 ng/ml porin and 2.5 ng/ml SDS; $T = 25°C$. The applied voltage was 50 mV. (A) 1 M KCl, 10^{-3} M citrate, pH 3, $\bar{\Lambda} = 1.6$ nS ($n = 142$); (B) 0.5 M $Tris^+$ $Hepes^-$, pH 8, $\bar{\Lambda} = 64$ pS ($n = 301$).

was added to the other side. The membrane potential reached its final value within about 10—15 min.

The observed values of the membrane potential V_m are given in Fig. 3 and Table III. From V_m and the salt concentrations c' and c'' in the left-hand and right-hand solution the ratio of the permeabilities P_c (cation) and P_a (anion) was determined according to the Goldman-Hodgkin-Katz equation:

$$V_m = \psi' - \psi'' = \frac{RT}{F} \ln \frac{P_c c'' + P_a c'}{P_c c' + P_a c''} \quad (1)$$

(R is the gas constant, T the absolute temperature and F the Faraday constant). It is seen from Fig. 2 and Table III that single monovalent cations have a somewhat higher permeability than chloride. This slight cationic selectivity may result from the presence of negative charges in or near the pore (see below). Otherwise the discrimination even between ions of rather dissimilar size such as $Tris^+$ and Cl^- or Na^+ and $Hepes^-$ is rather poor, in accordance with the findings from the single-pore conductance experiments.

pH dependence of permeability

In order to test the hypothesis that the ionic selectivity of the pore is influenced by the presence of fixed charges, the pH dependence of the permeability was studied. In Table IV the values of the average pore conductance $\overline{\Lambda}$ is given for different systems at three different pH values (3, 6 and 9). From pH 3 to pH 9 $\overline{\Lambda}$ increases by a factor ranging between 1.25 (1 M KCl, oxidized cholesterol membranes) and 1.6 (1 M NaCl, oxidized cholesterol membranes). The finding that the pH dependence of $\overline{\Lambda}$ is similar for monoolein and oxidized

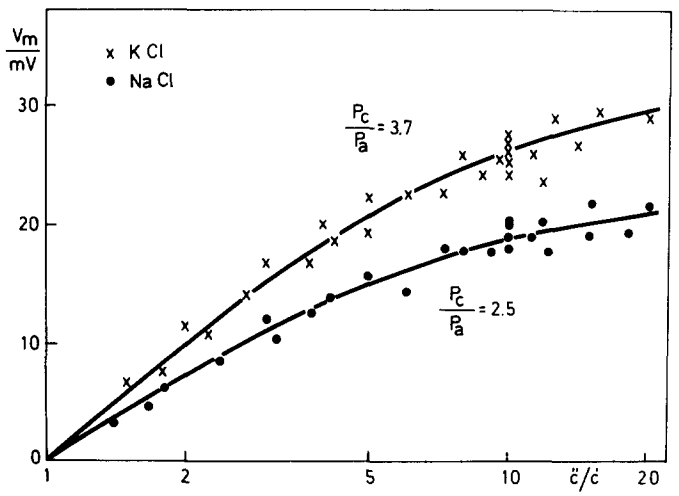

Fig. 3. Zero-current membranes potentials $V_m = \psi' - \psi''$ as a function of the ratio c''/c' of the salt concentrations on both sides of the membrane. c' was maintained at 10^{-2} M and c'' was varied by adding increasing amounts of a concentrated solution under stirring. In addition to the salt (NaCl or KCl) the aqueous phase contained 200 ng/ml porin and 1 μg/ml SDS; T = 25°C. The membranes were made from 1—2% (w/v) oxidized cholesterol in *n*-decane. V_m was positive on the more dilute side. In order to check the reproducibility of the measurements, the membrane potential was measured with four different membranes under the condition $c''/c' = 10$. The other V_m values were obtained from the same membrane in the two sets of experiments. The lines have been drawn according to Eqn. 1 with the specified values of the permeability ratio P_c/P_a (the subscripts c and a refer to the cation and anion, respectively).

TABLE III

ZERO-CURRENT MEMBRANE POTENTIALS V_m IN THE PRESENCE OF A 10-FOLD CONCENTRATION GRADIENT OF THE SALT

$c' = 10^{-2}$ M; $c'' = 10^{-1}$ M; V_m is the electrical potential of the dilute side minus the potential of the concentrated side. The membranes were formed either from oxidized cholesterol or from monoolein in *n*-decane. In order to avoid diffusion polarization, the amount of porin added to the system was kept low so that the conductance (in 10^{-2} molar salt) was of the order of 10 $\mu S \cdot cm^{-2}$ or less. The aqueous solutions were unbuffered and had a pH of about 6, if not otherwise stated; $T = 25°C$. The ratio of the permeabilities P_c (cation) and P_a (anion) was calculated according to Eqn. 1. The values of V_m were obtained from at least four different membranes.

Salt	V_m (mV)	P_c/P_a
Oxidized cholesterol membranes		
NaCl	19 ± 2	2.5 ± 0.2
KCl	26 ± 3	3.7 ± 0.5
CsCl	27 ± 3	3.8 ± 0.5
Na^+Hepes^- (pH 9)	21 ± 2	2.8 ± 0.3
$Tris^+Cl^-$	17 ± 3	2.3 ± 0.3
$Tris^+Hepes^-$ (pH 8)	19 ± 2	2.5 ± 0.3
$Glucosamine^+Cl^-$ (pH 3)	0 ± 3	1.0 ± 0.2
Monoolein membranes		
NaCl	18 ± 2	2.4 ± 0.2
KCl	27 ± 3	3.8 ± 0.5
NH_4Cl	25 ± 3	3.6 ± 0.5

cholesterol membranes suggests that the negative charges on the oxidized cholesterol do not appreciably influence the pH effect on $\overline{\Lambda}$.

The distribution of the conductance increments Λ was not much changed by variation of the pH , although the histograms were somewhat narrower at pH 9. The influence of pH on the most frequently assumed Λ value (which is in general by 20—40% lower than $\overline{\Lambda}$) was stronger than the influence on $\overline{\Lambda}$. For instance, in 1 M KCl the most frequently assumed Λ value was 0.9 nS at pH 3 and 1.8 nS at pH 9. This increase by a factor of 2 may be compared with an increase of $\overline{\Lambda}$ of only 25% in the same pH interval.

The pH dependence of the permeability ratio P_{Na}/P_{Cl}, as determined from membrane potential measurements (Eqn. 1) is represented in Fig. 4. The membrane is virtually unselective for Na^+ and Cl^- at pH 2 ($P_{Na} \approx P_{Cl}$), but becomes increasingly cation selective at the higher pH values up to a permeability ratio P_{Na}/P_{Cl} of about 3.

TABLE IV

AVERAGE CONDUCTANCE INCREMENT $\overline{\Lambda}$ AS A FUNCTION OF pH

The values for pH 6.0 were taken from ref. 10. In the experiments at pH 3.0 and 9.0 the solution was buffered with 1 mM citrate or 1 mM Tris, respectively. The aqueous solutions contained 0.5 ng/ml porin and 2.5 ng/ml SDS. The lipids were dissolved in *n*-decane (1—2%, w/v); $T = 25°C$. A voltage of 50 mV was applied. The number *n* of events used for the evaluation of $\overline{\Lambda}$ ranged between 100 and 320.

Lipid	Salt	$\overline{\Lambda}$ (ns)		
		pH 3	pH 6	pH 9
Oxidized cholesterol	1 M NaCl	0.80	1.2	1.3
Oxidized cholesterol	1 M KCl	1.6	1.9	2.0
Monoolein	1 M NaCl	0.90	1.1	1.3
Monoolein	1 M KCl	—	1.9	—

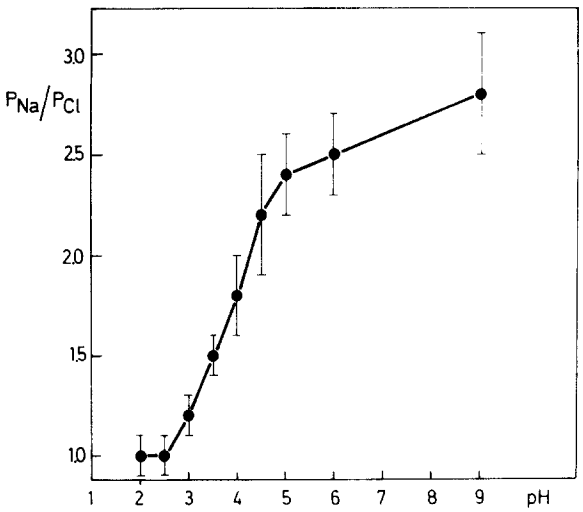

Fig. 4. Permeability ratio P_{Na}/P_{Cl} as a function of pH, as obtained from membrane-potential measurements in the presence of a 10-fold differences of NaCl concentration ($c' = 10^{-2}$ M, $c'' = 10^{-1}$ M). P_{Na}/P_{Cl} was calculated from Eqn. 1. In addition to the salt, the aqueous phase contained 0.2 μg/ml porin and 1 μg/ml SDS. The solution was buffered with 1 mM citrate in the pH range 2—4.5 and with 1 mM Tris at pH 9. The membranes were formed from oxidized cholesterol in *n*-decane (1—2%, w/v).

Discussion

The main result of the selectivity studies described here is the finding that the porin-induced ion channel is permeable to ions as large as $Hepes^-$ or $glucosamine^+$. From the dimensions of these ions a minimum pore diameter of about 0.8 nm is estimated. This value is consistent with an estimated diameter of 0.9 nm obtained from the average conductance increment $\overline{\Lambda}$ in 1 M KCl on the basis of the assumption that the pore is filled with an aqueous solution of the same conductance as the external phase. In view of the relatively broad distribution of conductance increments, pores of larger and smaller size must also occur. In any case, the finding that $\overline{\Lambda}$ is proportional to the conductance of the aqueous phase for all salts studied here is consistent with the notion that porin forms large water-filled pores in the black film. Implicit in the calculation of the pore diameter from $\overline{\Lambda}$ is the assumption that the single conductance steps represent independent events and correspond to the formation of new pores. We cannot exclude the possibility, however, that some of the conductance steps arise from transitions of already existing pores to higher conductance states, e.g. by incorporation of additional monomers into the oligomeric structure.

At neutral pH the pore has a preference for small univalent cations over chloride. For a 0.9 nm wide pore specific interactions with the permeating ions are rather improbable; a more likely explanation for the observed cationic selectivity therefore consists in the assumption that fixed negative charges are present in (or near) the pore. The observed pH dependence of the permeability ratio P_{Na}/P_{Cl} supports this interpretation; P_{Na}/P_{Cl} is close to unity at pH 2 and increases with increasing pH. As the P_{Na}/P_{Cl} versus pH curve (Fig. 3) is less

steep than a single titration curve, it is likely that more than one kind of ionizable group participates in the pH effect. The charged groups responsible for the pH effect may be located on the pore molecule itself, for the following reasons. The pH dependence of permeability is observed in membranes made from a uncharged lipid (monoolein). On the other hand, SDS which may be adsorbed to the membrane has a pK below 1 and should be ionized in the whole pH range (Expt. 1). Furthermore, a cationic selectivity ($P_K/P_{Cl} \approx 3$) was also found with porin isolated from the shock fluid of *E. coli* in the absence of detergent (Benz, R., unpublished). It is therefore likely to assume that the pH dependence of permeability results from fixed negative charges located on the pore walls or at the mouth of the pore.

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