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Ion Selectivity of Colicin E1: II. Permeability to Organic Cations

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Summary. Channels formed by colicin E1 in planar lipid bilayers have large diameters and conduct both cations and anions. The rates at which ions are transported, however, are relatively slow, and the relative anion-to-cation selectivity is modulated over a wide range by the pH of the bathing solutions. We have examined the permeability of these channels to cationic probes having a variety of sizes, shapes, and charge distributions. All of the monovalent probes were found to be permeant, establishing a minimum diameter at the narrowest part of the pore of approximately 9 Å. In contrast to this behavior, all of the polyvalent organic cations were shown to be impermeant. This simple exclusionary rule is interpreted as evidence that, when steric restrictions require partial dehydration of an ion, the structure of the channel is able to provide a substitute electrostatic environment for only one charged group at time.

Key Words colicin · ion selectivity · lipid bilayers · electrostatic interactions

Introduction

The ability of ion channels to play functional roles in the membranes of particular cells is dependent on their capacity to discriminate among the various ions which may be present. As more kinds of channels have been made available for study and our knowledge of their molecular structures has increased, understanding the basis for the ion selectivity of channels has become a more challenging task. Channels formed in planar lipid bilayers by a group of proteins known as colicins are particularly interesting objects for the study of this process [7, 9, 13, 32, 35, 41, 42]. Not only are these pores permeable to cations and anions alike, they are apparently so wide that no ions have yet been identified which will not pass through them [34]. These channels are not simply featureless conduits, however. The absolute transport rates, even for the most permeant small ions, are quite slow. Furthermore, the relative preference of the channels for cations *versus* anions is modulated over a very wide range by the pH of the bathing medium [6, 34]. Our aim has been to understand how the interactions of the transported ions with the channel structure create this unexpected intricacy in its selectivity behavior.

The colicins have attracted the attention of investigators with a wide variety of interests. They are secreted by certain strains of *Escherichia coli* as aqueous proteins and are readily purified in large amounts. A variety of physical techniques have been used to study their properties [5, 26, 33, 42, 43], and site-directed mutants have been produced to examine structure-function relationships in these molecules [2, 19, 21, 37–39]. Because they are produced for the purpose of killing other strains of *E. coli,* these plasmid-encoded proteins can properly be considered toxins or antibiotics. A single channel formed by one of these molecules in the plasma membrane of the target cell produces a lethal depolarization. To gain access to the plasma membrane, other regions of the colicin molecules bind to specific receptors in the outer membrane and translocate the toxic region into the periplasmic space. The domains of the colicin E1 and A molecules which actually form channels have been shown to lie at the carboxy terminal ends of the proteins, and all six of the channel-forming colicins show high degrees of homology in the corresponding regions [11, 12, 22, 24, 29, 36, 40, 44]. Both the proteins and their C-terminal peptides have been used as model systems for the study of membrane assembly and protein export [15, 31, 33]. In the present study, we have explored the steric and electrostatic topology of the pore formed by colicin E1 in planar lipid membranes using cationic probes of varying size and charge distribution. A preliminary account of this work has appeared [10].

Materials and Methods

Chemicals and Biochemicals

Inorganic salts, buffer compounds, and solvents were of reagent grade and used without further purification. Conductivity-grade water (18 MΩ-cm) was used for all solutions. Working solutions

contained the chloride salt of the cation under test at a formal concentration of either 1.0 or 0.1 M. All solutions also contained 3 mM $CaCl_2$, to enhance membrane stability, and each of the following pH buffer compounds at concentrations of 3 mM: glutaric acid, $pK_a = 4.13, 5.03$; 2-(N-morpholino)ethanesulfonic acid (MES), $pK_a = 6.15$. Asolectin type IV-S (Sigma Chemical, St. Louis, MO) was washed in acetone, and bacterial phosphatidylethanolamine (PE) was obtained from Avanti Polar Lipids (Birmingham, AL). Colicin E1 protein was the generous gift of Dr. W.A. Cramer (Purdue University, West Lafayette, IN). Di(pentafluorophenyl)mercury $[(C_6H_5)_2Hg]$ was obtained from PCR, (Gainsville, FL) and was generously given to us by Dr. Alan Finkelstein (Albert Einstein College of Medicine, Bronx, NY).

The following compounds, used as test cations, were obtained from Aldrich Chemical (Milwaukee, WI): Tetramethylammonium (TMA); DL-3-amino-ε-caprolactam (ACL); 4-(2-hydroxyethyl)morpholine (ME-OL); Tropine; 1-benzyl-4-cyano-4-hydroxypiperidine (BCHP); 1-azabicyclo[2.2.2]octane (Quinuclidine); 1,4-bis(2-hydroxyethyl)piperazine (HEPE-OL); 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bis-Tris); N-methyl-D-glucamine (NMG); N,N,N′,N′-tetramethyl-1,3-propanediamine (Bis-T3); 1,3-bis[tris-(hydroxymethyl)-methylamino]propane (Bis-Tris Propane); 4,4′-bipiperidine (Bipiperidine); 1,1′, methylenedipiperidine (dipiperidinomethane); 4,4′-trimethylenedipiperidine (TMDP); N,N,N′,N′-tetramethyl-1,6-hexanediamine (bis-T6); and Hexamethonium (Bis-Q6). Spermine and Spermidine were obtained from Sigma Chemical (St. Louis, MO). The structures, formula weights, (FW), and molecular dimensions of these compounds are shown in Tables 1 and 2. The dimensions given are the maximum overall length and minimum overall width as determined from CPK models. This is equivalent to the size of the rectangular slot having the narrowest width and longest length which could be made to tightly enclose the molecule. In all cases, the measurements were made on the fully extended conformation of the ion.

The compounds we have abbreviated as BCHP and TMDP yielded highly colored solutions which were decolorized with carbon prior to use; all other compounds were used without further purification. Because BCHP was available only as the hydrochloride salt, HEPE-OL was used as a base to adjust the pH of the buffers in the working solutions. Similarly, Bis-Tris propane base was used with bipiperidine dihydrochloride and Bis-T6 base with Bis-Q6 dichloride. Aqueous titration was used to determine the formal concentration of the stock solutions of the test compounds and the pK_a values of compounds for which no reports could be found in the literature (*see* Tables 1 and 2). All of these compounds were soluble to concentrations of at least 1 M at room temperature and were impermeant with respect to bare bilayers. None was found to destabilize or disrupt the planar membranes used in this study. The selectivity of the colicin E1 channel for a particular cation was determined as the value of the zero current potential of the conductance induced by the protein in a membrane exposed to a 1.0- *versus* 0.1-M gradient of the cation as a chloride salt (*see below*). In order to assess the ability of the channel to transport a cation, its zero current potential was compared to the liquid junction potential ($E_{liq\ jnc}$) and chloride equilibrium potential (E_{Cl}) of the experimental solutions used to produce the 10-fold gradients. The values of $E_{liq\ jnc}$ shown in Tables 1 and 2 were determined using an agar bridge and a high impedance digital multimeter (Hewlett Packard). Ion-selective electrodes in combination with a pH/ion meter (Orion Research, Cambridge, MA) were used to determine E_{Cl}. A double junction reference electrode filled with 10% KNO_3 was used to avoid contamination of the solutions by additional chloride.

Membrane Conductance Measurements

Planar phospholipid bilayer membranes of the solvent-free type [28] were formed across apertures in Teflon septa as previously described [9]. The volume of aqueous solution bathing each side of the membrane was either 1.5 or 5 ml. The apertures used in these experiments were either 100 or 150 μm in diameter. The smaller sized holes offered considerable improvement in the stability of the bilayers, especially those composed of PE, but they required the use of higher protein concentrations to achieve the levels of colicin conductance necessary for the accurate determination of zero current potentials, as described below.

Voltage-clamp conditions were established across the bilayers by means of a Burr Brown 3523L operational amplifier configured as a current-to-voltage converter. Electrical contact with the two aqueous compartments was established by means of a single pair of miniature calomel electrodes, one of which was connected to the system ground and the other to the inverting input of the converter amplifier. Command signals were applied to the noninverting input of this amplifier and subtracted from its output using a unity gain differential amplifier (Burr Brown 3627BM). The resulting signal, which was proportional to transmembrane electric current, was electronically filtered at 3 Hz by an 8-pole, low-pass Bessel filter (Frequency Devices, Haverhill, MA) and monitored using an oscilloscope and a chart recorder. DC command voltages were supplied by mercury batteries in combination with digital voltage dividers (Digitran, Pasadena, CA). The aqueous compartments were magnetically stirred, so that only a few seconds were required for complete mixing in the bulk phase. All measurements were made at room temperature.

After a membrane had been formed, small aliquots of aqueous stock solutions of purified colicin E1 protein were added to one compartment, defined as the *cis* side of the membrane. Colicin E1 was present at final concentrations ranging from 0.5 to 5.0 μg protein/ml of aqueous solution. The extremely low activity exhibited by the colicins in bilayers composed of PE was enhanced by the addition to the *cis* compartment of the uncharged detergent octylglucoside at a final concentration of 24 μg/ml, as previously described [8]. Only membranes exhibiting a high resistance ($>10^8$ Ω-cm) and a low level of noise were considered suitable for the introduction of protein. Membranes which became unstable or noisy after the introduction of the protein were likewise discarded.

Measurement and Control of pH

The pH in the *cis* compartment was monitored by means of a miniature glass electrode (Model 407B, Microelectrodes, Londonderry, NH) and a small, battery-powered pH meter. The calomel electrode in contact with the solution in that compartment provided the reference potential for pH measurement as well as the ground return path for transmembrane currents. The pH of the *cis* compartment could be altered during the course of conductance measurements by titration with aliquots of solutions of HCl or the appropriate test cation as the free amine.

Results

Determination of Selectivity

Channels formed by colicin E1 have been reported to be significantly permeable to a wide variety of

Table 1. Properties of monovalent test cations

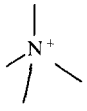
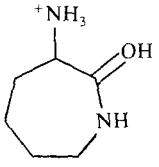
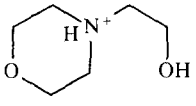
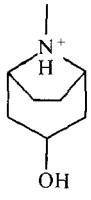
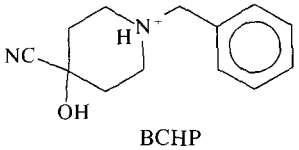
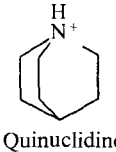
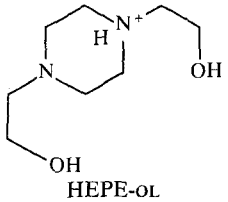
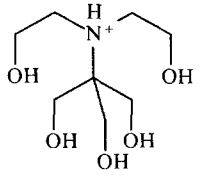
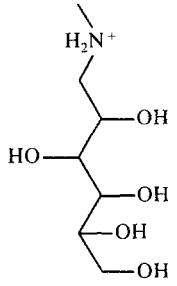
Cation	FW (Da)	d_{max}[a] × d_{min} (Å)	pK_a[b]	$E_{liq\ jnc}$[c] (mV)	E_{Cl}[c] (mV)
 Sodium	22.99	1.9	—	−8	−47
 TMA (tetramethylammonium)	74.14	6.0 × 5.4	—	−13	−47
 ACL (DL-3-amino-ε-caprolactam)	129.19	7.7 × 5.0	8.6	−21	−42
 ME-OL (4-(2-hydroxyethyl)morpholine)	132.19	9.8 × 4.6	6.9	−21	−46
 Tropine	142.22	8.2 × 6.1	>10	−23	−46
 BCHP (1-benzyl-4-cyano-4-hydroxypiperidine)	217.3	13.4 × 5.8	>9	−23	−45
 Quinuclidine (1-azabicyclo[2.2.2]octane)	112.20	6.6 × 6.2	11.1[MS]	−20	−44
 HEPE-OL (1,4-bis(2-hydroxyethyl)piperazine)	175.25	13.2 × 5.1	7.8 3.7	−27	−44

Table 1—Continued

Cation	FW (Da)	d_{max}[a] × d_{min} (Å)	pK_a[b]	$E_{liq\ jnc}$[c] (mV)	E_{Cl}[c] (mV)
 Bis-Tris (2,2-bis(hydroxymethyl)-2,2′,2″,-nitrilotriethanol)	210.25	11.0 × 7.6	6.6^{MS}	−26	−45
 NMG (N-methyl-D-glucamine)	196.23	13.3 × 5.5	9.6^{J}	−27	−42

[a] Dimensions of the rectangular slot having the longest length (d_{max}) and shortest width (d_{min}) which would just accommodate a CPK model of the test compound.

[b] Determined by aqueous titration. Values taken from literature: *MS*-Martell and Smith [23], *J*-Juvet [20].

[c] Chloride equilibrium potentials (E_{Cl}) and liquid junction potentials ($E_{liq\ jnc}$) were measured across 10-fold concentration gradients (1 *vs.* 0.1 M) of each of the test cations as chloride salts. All solutions also contained 3 mM $CaCl_2$, 3 mM glutaric acid, and 3 mM MES, pH 5.0.

both cations and anions [34], and no impermeant species of either sign have yet been identified. In the present study we have exposed planar membranes containing colicin E1 to 10-fold gradients of the chloride salts of a variety of organic cations. The zero current potentials exhibited by the channels under these conditions is a measure of their permeability to the test cation relative to the permeant anion chloride. In all cases, the membrane was bathed in solutions containing the cation at a formal concentration of 1.0 M on one side and 0.1 M on the other. In a departure from previous practice, the system ground, or zero reference point, was defined as the potential of the 1.0-M compartment, rather than the potential of the *cis* compartment. Thus, positive values of the zero current potential indicate a preference for the cation and negative values a preference for chloride, regardless of the side of the gradient to which the protein was added. The results of a typical selectivity determination are shown in Fig. 1. Colicin E1 protein was added to the 1-M side of a Bis-T3 gradient across a PE membrane. In response to the application of a negative potential, several hundred channels were opened. The voltage was then switched to determine the value at which the current was equal to zero. In this record, changes in the current produced by 1-mV differences in applied potential are easily distinguished.

If a channel does not allow passage of anions of any sort, the evaluation of the permeance of a particular cation is based on its ability to sustain a flow of current through that channel. The capability of such experiments to discriminate between slightly permeant and completely impermeant ions is limited by the size of the currents which can be detected, the range of voltage which is accessible, and the ability to assign currents to a specific channel. For those channels which are permeable to both ions of both signs, a class which includes all of the colicins, replacing a permeant cation with an impermeant one will not eliminate the flow of current, since the permeant anions must necessarily be present on both sides of the membrane. The criterion for impermeance must be based, not simply on the resolution of

Table 2. Properties of polyvalent test cations

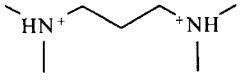
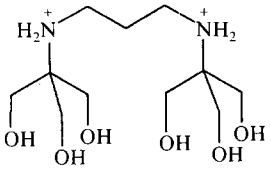
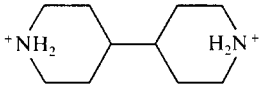
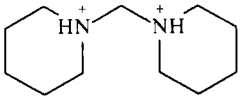
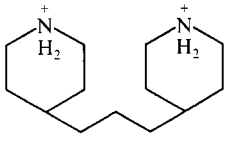
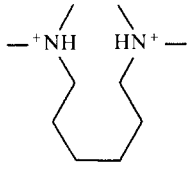
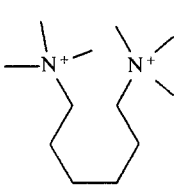
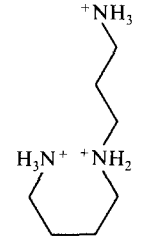
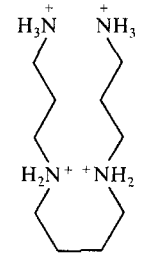
Cation	FW (Da)	d_{max}[a] × d_{min} (Å)	pK_a[b]	$E_{liq\ jnc}$[c] (mV)	E_{Cl}[c] (mV)
 Calcium	40.08	2.0	—	−18	−48
 Bis-T3 (N,N,N′,N′-tetramethyl-1,3-propanediamine)	132.26	10.7 × 4.5	9.8[MS] 7.8[MS]	−23	−43
 Bis-Tris propane (1,3-bis[tris-(hydroxymethyl)-methylamino]propane)	284.36	16.3 × 7.6	9.0 6.8	−27	−43
 Bipiperidine (4,4′-bipiperidine)	170.31	11.2 × 4.9	10.7[G] 9.5[G]	ND	−44
 Dipiperidinomethane (1,1′-methylenedipiperidine)	184.33	11.4 × 5.6	>9 6.3	−23	−42
 TMDP (4,4′-trimethylenedipiperidine)	212.39	14.8 × 4.9	10.6[G] 9.9[G]	ND	−43
 Bis-T6 (N,N,N′,N′-tetramethyl-1,6-hexanediamine)	174.34	14.8 × 4.7	>9	−25	−45
 Bis-Q6 (hexamethonium)	202.4	14.8 × 5.4	—	−25	−45

Table 2.—Continued

Cation	FW (Da)	d_{max}[a] × d_{min} (Å)	pK_a[b]	$E_{liq\ jnc}$[c] (mV)	E_{Cl}[c] (mV)
 Spermidine (1,5,10-triazadecane)	148.28	14.6 × 3.8	10.9^{MS} 9.8^{MS} 8.3^{MS}	−23	−42
 Spermine (1,5,10,14-tetraazatetradecane)	206.39	19.6 × 3.8	10.8^{MS} 10.0^{MS} 8.9^{MS} 8.0^{MS}	−27	−44

[a] Dimensions of the rectangular slot having the longest length (d_{max}) and shortest width (d_{min}) which would just accommodate a CPK model of the test compound.
[b] Determined by aqueous titration. Values taken from literature: *MS*-Martell and Smith [23], *G*-Gerzon et al. [17].
[c] Chloride equilibrium potentials (E_{Cl}) and liquid junction potentials ($E_{liq\ jnc}$) were measured across 10-fold concentration gradients (1 *vs.* 0.1 M) of each of the test cations as chloride salts. All solutions also contained 3 mM $CaCl_2$, 3 mM glutaric acid, and 3 mM MES, pH 5.0. ND indicates not determined.

small currents, but on the agreement of two independent experimental measurements: the zero current potential of the channel and the equilibrium potential of the permeant counterion. For the compound used in obtaining the record shown in Fig. 1, the measurements of zero current potential and chloride equilibrium potential were in close agreement, suggesting that Bis-T3 is relatively impermeant. Note, however, that the movement of chloride through the channel was not noticeably impeded by the presence of the impermeant cation. This behavior was observed for all the compounds tested; in no case did the test cations block current carried by the permeant counterion. Since the turn-off kinetics of colicin E1 are relatively slow at pH 5.0, it was also possible to determine zero current potentials when the protein was placed in the 0.1-M side of the gradient. In general, the channel-forming activity of colicin E1 was noticeably higher when the protein was introduced on the low concentration side of the salt gradient.

In a previous paper [6], we discussed the artifacts associated with this type of selectivity measurements at some length. The systematic errors to which the present studies are susceptible tend to reduce the magnitudes of the measured zero current potentials and cause the equilibrium potentials of the permeant counterion to be overestimated. Although permeation of the buffer compounds and calcium could account for an error of only 1–2 mV at most, it was possible that leakage of KCl from the reference electrodes could produce an offset of several millivolts in experiments conducted over a period of hours. To prevent this occurrence, individual experiments were limited to shorter time spans whenever possible. A stable, nonselective leak pathway which has been shown to develop with time in membranes containing large amounts of protein is the most serius potential source of error. Conducting experiments with moderate levels of colicin conductance lessened the likelihood that this phenomenon would develop, but except for terminating experiments

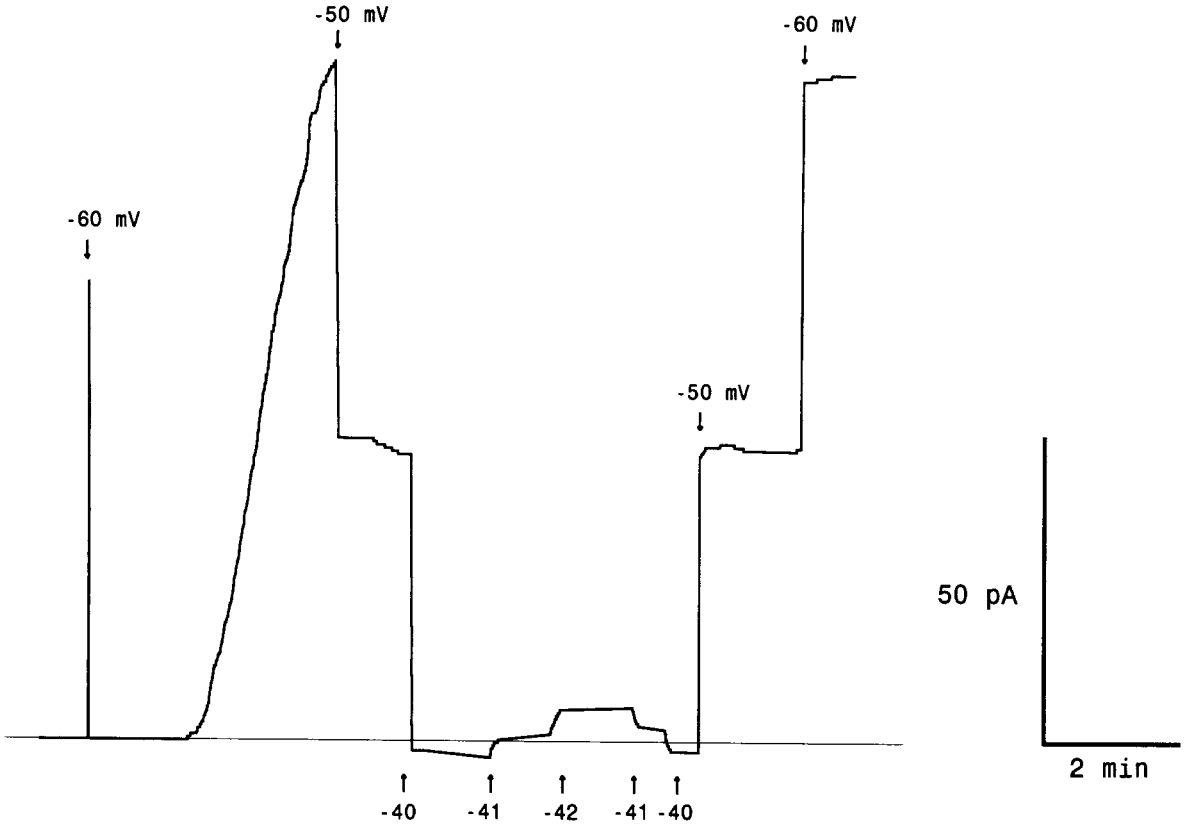


Fig. 1. Determination of zero current potential in a bacterial phosphatidylethanolamine (PE) membrane. This membrane was formed on a 150-μm diameter hole and was bathed by solutions containing 1.0 and 0.1 M Bis-T3 · $(HCl)_2$. Potentials are defined with respect to the 1.0-M side of the gradient. Colicin E1 at a final concentration of 1 μg/ml had been added to the high salt side of the gradient several minutes prior to the beginning of the record. Following the induction of channel formation by application of −60 mV, the zero current potential was found to be −41 mV. Both solutions contained 3 mM $CaCl_2$, 3 mM glutaric acid, and 3 mM MES, and the pH was adjusted to 5.0.

when repeated measurements of zero current potential were seen to decline in magnitude, there was no effective method to correct for or to prevent this error. We have also reported that, because of the relatively high water permeability of asolectin membranes, osmotic movement of water produces polarization amounting to a 4-mV shift in the chloride equilibrium potential of a 10-fold gradient of sodium chloride. There was no evidence for osmotic polarization in membranes composed either of PE or of PC. Note that for the polyvalent ions used in the present study, the osmotic gradients imposed across the membranes were significantly larger than those created by simple 1 : 1 electrolytes.

Permeability to Monovalent Cations in Asolectin Membranes

The relative cation-to-anion selectivities of channels become exaggerated when they are incorporated into membranes composed of asolectin because of the negative surface charge associated with the acidic components of this lipid mixture. As a result, the use of this lipid provides increased discrimination between sparingly permeant and impermeant cations. In addition, the activity of the colicin proteins was greatly enhanced by negatively charged lipids, making asolectin membranes experimentally convenient for examining the cation selectivity of these channels. The results of zero current potential measurements for nine different monovalent organic cations in comparison to the highly permeant ion, sodium, are shown in Table 3. All of these ions were significantly permeant. In fact, these zero current potentials are in much closer agreement with the corresponding liquid junction potentials than with the chloride potentials.

In all but two cases, the zero current potentials were independent of the side of the gradient to which the colicin protein was added. This suggests that the selectivity of colicin E1 was not strongly influenced by the ionic strength of the solutions bathing the

Table 3. Permeability of colicin E1 channels in asolectin bilayers to monovalent cations

Cation	Zero current potential (mV)		E_{Cl} (mV)
	Colicin added to 1-M side	Colicin added to 0.1-M side	
Na^+	+26 ± 2 (18)	+24 ± 2 (14)	−47
TMA^+	−26 ± 2 (10)	−27 ± 2 (11)	−47
ACL^+	−21 ± 2 (4)	−21 ± 1 (7)	−42
ME-OL$^+$	−23 ± 2 (5)	−22 ± 2 (10)	−45
Tropine$^+$	−22 ± 3 (8)*	−28 ± 2 (13)*	−46
$BCHP^+$	−40 ± 1 (5)*	−23 ± 2 (4)*	−45
Quinuclidine$^+$	−29 ± 2 (6)	−29 ± 2 (8)	−44
HEPE-OL$^+$	−30 ± 8 (5)	−30 ± 2 (7)	−47
NMG^+	−29 ± 3 (7)	−30 ± 3 (7)	−42
Bis-Tris$^+$	−29 ± 3 (5)	−30 ± 1 (5)	−45

Zero current potentials of conductance induced by colicin E1 in membranes exposed to 1- *vs.* 0.1-M gradients of the chloride salt of the indicated test cations. Values are reported as mean ± SD. The number of determinations is given in parentheses; each measurement was obtained from a different membrane. Negative values denote a preference for chloride, and positive values denote a preference for the test cation. All solutions contained 3 mM glutaric acid, 3 mM morpholineethanesulfonate (MES), and 3 mM $CaCl_2$ and were adjusted to pH 5.0.
*Denotes values for which the dependence of apparent selectivity upon the orientation of the gradient are significant at the 5% level.

Table 4. Permeability of colicin E1 channels in asolectin bilayers to polyvalent cations

Cation	Zero current potential (mV)		E_{Cl} (mV)
	Colicin added to 1-M side	Colicin added to 0.1-M side	
Ca^{+2}	−18 ± 2 (21)*	−14 ± 1 (17)*	−48
Bis-Tris propane^{+2}	−36 ± 2 (11)	−35 ± 1 (6)	−43
Bipiperidine^{+2}	−37 ± 3 (5)	−40 ± 2 (6)	−43
Dipiperidinomethane^{+2}	−38 ± 1 (6)	−38 ± 2 (6)	−42
$TMDP^{+2}$	−38 ± 2 (11)	−37 ± 3 (3)	−43
Bis-Q6^{+2}	−39 ± 2 (10)	−39 ± 2 (9)	−45
Spermidine^{+3}	−34 ± 1 (11)	−33 ± 1 (11)	−42
Spermine^{+4}	−35 ± 1 (6)	−37 ± 1 (10)	−44

Zero current potentials of conductance induced by colicin E1 in membranes exposed to 1- *vs.* 0.1-M gradients of the chloride salt of the indicated test cations. Values are reported as mean ± SD. The number of determinations is given in parentheses; each measurement was obtained from a different membrane. Negative values denote a preference for chloride, and positive values denote a preference for the test cation. All solutions contained 3 mM glutaric acid, 3 mM morpholineethanesulfonate (MES), and 3 mM $CaCl_2$ and were adjusted to pH 5.0.
*Denotes values for which the dependence of apparent selectivity upon the orientation of the gradient are significant at the 5% level.

membrane, at least not in this range. In experiments using $CaCl_2$, in which the ionic-strength gradient was approximately three times higher than in the cases of monovalent salts, a significant effect of the orientation of the gradient on selectivity was observed (*see* Table 4). We believe that the 6-mV difference found in the case of tropine may be artifactual. The activity of the protein was dramatically lower in those experiments in which the colicin was present on the high salt side of the gradient, so that a small leak conductance would have produced a proportionately greater error. The greater difference seen in the case of BCHP cannot be attributed to any such trivial artifact. This ion appeared to be nearly impermeant when the protein was added to the 1.0-M side of the gradient but quite permeant when added to the 0.1-M side. Because the commercially available product did not appear to be pure, even after treatment with decolorizing carbon, the presence of a contaminant which blocked the channel from the *cis* side is a possible alternative explanation for this phenomenon.

Permeability to Polyvalent Cations in Asolectin Membranes

In principle, if the dimensions of an ion exceed those of the transmembrane pore formed by colicin E1, that ion should be impermeant. In practice, however, other commercially available, large, singly charged organic cations proved to be unsuitable for this kind of study either because they were insoluble in water, were soluble in the bilayer itself, or were amphipathic and disrupted membranes via detergent effects. For this reason, we expanded our survey of organic cations to include some having more than one positive charge. The zero current potentials obtained from gradients of seven different polyvalent cations are shown in Table 4. Values for the divalent metal cation calcium are shown for comparison. While all of the polyvalent organic ions were significantly less permeant than any of the monovalent ions, their zero current potentials were 3–8 mV less negative than the corresponding values for E_{Cl}. There was no obvious relationship between the amount of this deviation and the structure of the ion; indeed, by this criterion the largest, most highly charged cation appeared to be the most permeant.

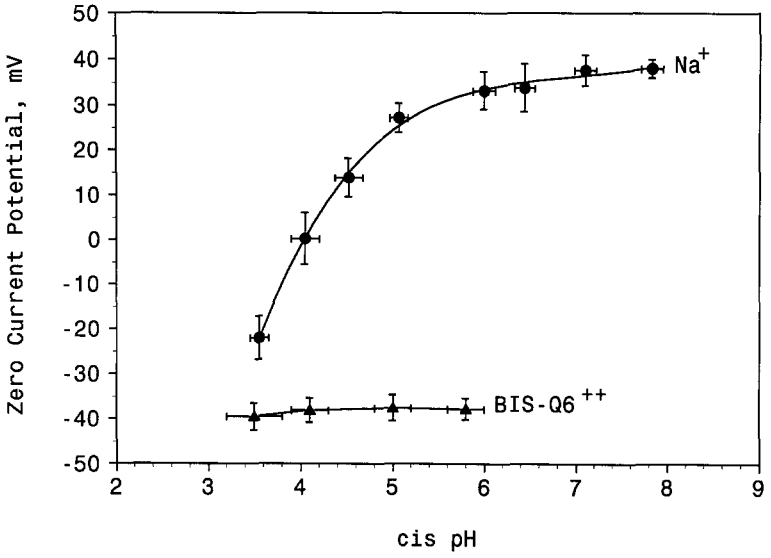


Fig. 2. Effects of *cis* pH on the selectivity of colicin E1 in asolectin bilayers. Membranes were exposed to 1.0- *versus* 0.1-M gradient of the chloride salt of either sodium or Bis-Q6 as indicated. Colicin E1 was added to the 1.0-M side of the gradient, and the zero current potential was determined. Individual membranes were subjected to repeated titration of the *cis* pH over the entire range indicated, while the pH of the *trans* (0.1-M) compartment was held at 5.0. The zero current potential in Bis-Q6 was found to be independent of pH, indicating that this ion is impermeant. Both solutions contained 3 mM $CaCl_2$, 3 mM glutaric acid, and 3 mM MES. Data are displayed as mean ± SD.

To determine whether the gap between the zero current potential and E_{Cl} was the result of actual permeation of the channel by the ions or the cumulative errors of experimental artifacts, the selectivity of a representative member of this group was examined as a function of pH. As shown in Fig. 2, the zero current potential of colicin E1 in NaCl gradients shifted by 55 mV as the pH on the *cis* compartment was varied from 3.5 to 6.0. By contrast, the zero current potential measured in a 10-fold gradient of Bis-Q6 did not change when the pH was raised or lowered over this range. If the apparent deviation of the zero current potential from E_{Cl} was the result of some slight permeation of Bis-Q6, making the channel itself less selective for chloride would have increased the deviation. The observation that the zero current potential of Bis-Q6 is independent of pH is, therefore, strong evidence that this cation is impermeant and that the deviation from ideality is artifactual.

Cation Permeability of Colicin E1 in PE Membranes

We have previously reported that, because of the presence of unstirred layers adjacent to the membranes, osmotic movement of water through asolectin bilayers exposed to 10-fold gradients of sodium chloride reduces transmembrane E_{Cl} to values below that measured in the bulk phases [6]. No such effects could be detected in membranes composed either of PC or PE. To determine whether osmotic polarization could account for the apparent permeation of the colicin E1 channels by polyvalent cations, the zero current potentials of several of these compounds were determined in membranes composed of PE. The results, shown in Table 5, indicate that all of the polyvalent organic amines tested were impermeant, since their zero current potentials were in close agreement to the corresponding chloride potentials. The removal of surface charge effects, however, also caused a shift of the zero current potentials of the monovalent ions Na^+ and NMG^+ to more negative potentials. The effect in NMG^+

Table 5. Cation permeability of colicin E1 channels in PE bilayers

Cation	Zero current potential (mV)		E_{Cl} (mV)
	Colicin added to 1-M side	Colicin added to 0.1-M side	
Na^+	−2 ± 8 (30)	ND	−47
NMG^+	−38 ± 2 (15)	ND	−42
Bis-T3^{+2}	−41 ± 2 (12)	ND	−43
Bis-Tris propane^{+2}	−40 ± 1 (8)	ND	−43
Bis-T6^{+2}	−42 ± 3 (11)	ND	−45
Bis-Q6^{+2}	−43 ± 1 (9)	−45 ± 2 (8)	−45
Spermidine^{+3}	−41 ± 2 (6)	ND	−42
Spermine^{+4}	−40 ± 2 (5)	ND	−44

Zero current potentials of conductance induced by colicin E1 in membranes exposed to 1- *vs.* 0.1-M gradients of the chloride salt of the indicated test cations. Values are reported as mean ± SD. The number of determinations is given in parentheses; each measurement was obtained from a different membrane. Negative values denote a preference for chloride, and positive values denote a preference for the test cation. All solutions contained 3 mM glutaric acid, 3 mM morpholineethanesulfonate (MES), and 3 mM $CaCl_2$ and were adjusted to pH 5.0. ND indicates not determined.

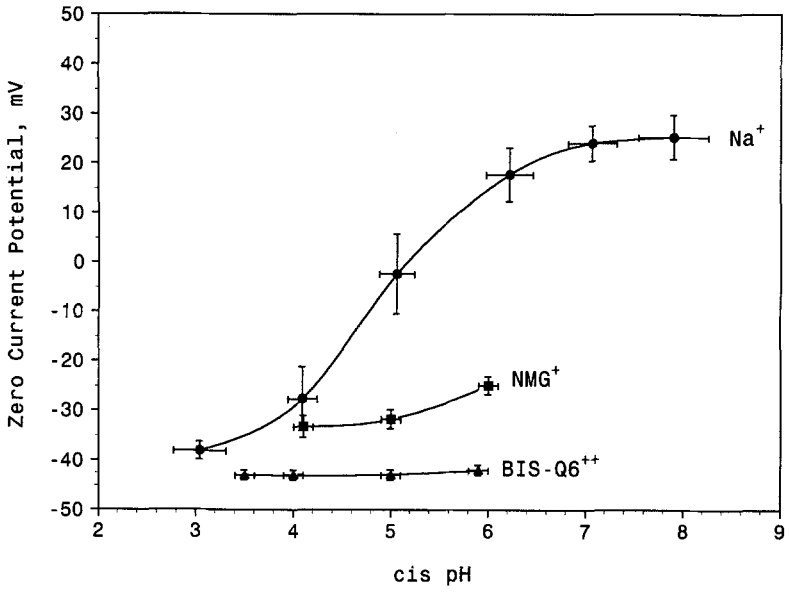


Fig. 3. Effects of *cis* pH on the selectivity of colicin E1 in PE bilayers. Titration of the *cis* compartment was carried out as described for Fig. 2. The pH dependence of the selectivity of colicin E1 in NMG solutions indicates that this ion is slightly permeant.

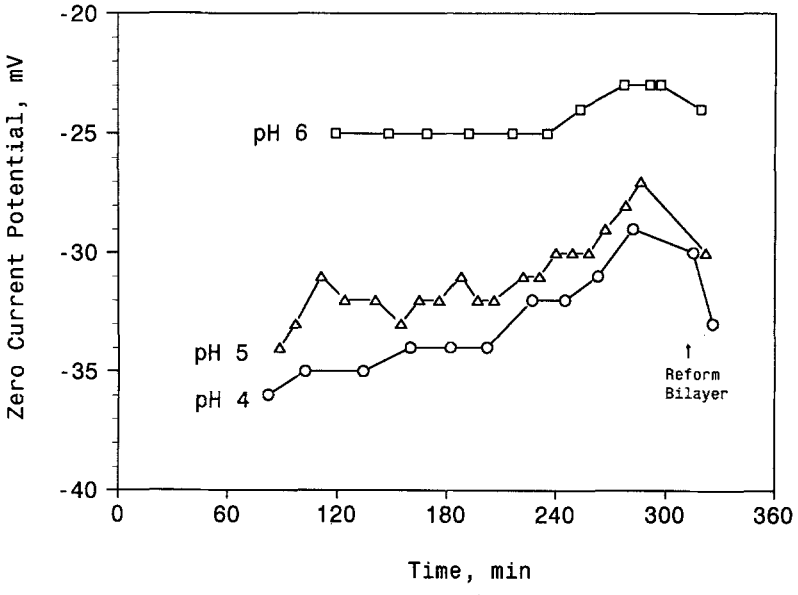


Fig. 4 The pH dependence of colicin E1 permeability to NMG. Measurements from one of the experiments used in compiling the data of Fig. 3 are displayed as a function of time elapsed from the first addition of protein. Each data point represents a single determination of the zero current potential in between adjustment of the *cis* pH by titration. The bilayer was reformed immediately prior to the earliest data point shown and again at the time indicated by the arrow. The selectivity was unambiguously dependent on pH throughout the entire experiment. The slow upward drift in the zero current potentials is attributable to the development of a nonselective leak conductance. Note that reforming the membrane partially restored the selectivity by reducing this leak.

solutions was strong enough to make it appear as though it is nearly as impermeant as the polyvalent ions under these conditions. That the permeance of NMG^+ persists in neutral membranes was confirmed by changing the *cis* pH. As shown in Fig. 3, the zero current potential of NMG · HCl became less negative as the intrinsic anion selectivity of the channel was decreased under the influence of the *cis* pH. The time course of a portion of one of the NMG^+ experiments is shown in Fig. 4. Each of the symbols corresponds to a single determination of the zero current potential following a change in the pH in the *cis* compartment. Two additions of colicin E1 were made during the first 60 min of the experiment to induce sufficient channel formation for the determinations, and the membrane was reformed immediately prior to first data point shown. When examined in this way, it is apparent that the error bars displayed in the previous figure are not simply the result of random scatter in the data. While the pH dependence of the selectivity was consistently observed throughout this experiment, the values at each pH showed a definite upward trend as the pH was cycled repeatedly over the range from 4 to 6. This trend was stronger for the data at pH 4 and 5 than that at pH 6, as would be expected if the drift were caused by the development of a nonselective leak conductance. Note that the opposite would be expected if

Table 6. Chloride equilibrium potentials

Salt	E_{Cl} (mV)		
	Asolectin[a] $(C_6H_5)_2Hg$	PE[a] $(C_6H_5)_2Hg$	Specific ion electrode[b]
NaCl	−43 ± 2	−47 ± 1	−47 ± 1
NMG · HCl	−42 ± 1	−44 ± 1	−44 ± 1
Bis-Q6 · Cl_2	−45 ± 2	−49 ± 1	−47 ± 1

[a] Zero current potentials of conductance induced by $(C_6H_5)_2Hg$ in bilayers composed of the lipid indicated. Membranes were exposed to 1- *vs.* 0.1-M gradients of the chloride salt of the test cations. Values are reported as mean ± SD. All solutions contained 3 mM glutaric acid, 3 mM morpholineethanesulfonate (MES), and 3 mM $CaCl_2$ and were adjusted to pH 5.0.

[b] Differences in potential measurements were obtained from same working solutions as those used with bilayers. Standard calomel electrodes rather than double-junction electrodes were used as references for these determinations. The two methods yielded slightly different values for NMG and Bis-Q6 but not for NaCl.

the cause had been leakage of KCl from the reference electrodes. That the drift is primarily associated with the membranes rather than the electrolyte solutions is also suggested by the fact that it was at least partially corrected by reforming the membrane at the time shown.

Although the zero current potentials for Bis-Q6 · Cl_2 were completely independent of pH in membranes composed either of PE or asolectin, the value in PE was significantly more negative. The contribution of osmotic polarization to the lipid dependence of the zero current potentials of the polyvalent ions was examined in the experiments reported in Table 6. Values of E_{Cl} determined using specific ion electrodes for gradients of NaCl, NMG · HCl and Bis-Q6 · Cl_2 are shown in comparison with values determined using the chloride carrier $(C_6H_5)_2Hg$ [3] in asolectin and PE membranes. In all cases, the value obtained in the PE membranes was more negative than that found for asolectin, consistent with our earlier conclusion that the water permeability of asolectin membranes is much higher than that of PE membranes. Since the osmotic gradient was larger for the Bis-Q6 gradient than that for NMG, the larger polarization produced by the former compound was expected. We would expect the corresponding effects for spermidine and spermine to be larger still. We were somewhat surprised by the apparently higher water permeability of asolectin membranes in gradients of NaCl as compared to NMG · HCl, and we speculate that they may be related to the differences in the bulk viscosities of the solutions. In the course of these studies it was found that, while the difference in the potentials produced by standard calomel electrodes and double junction reference electrodes did not vary in NaCl solutions, this value varied systematically with concentration in solutions of either NMG or Bis-Q6. All of the values given in Table 6 were obtained with calomel electrodes, since this was the type used in the bilayer measurements, whereas the entries in all the previous tables were obtained using double junction electrodes filled with KNO_3. In the case of Bis-Q6, the E_{Cl} value obtained using the specific ion electrodes was less negative than the value derived from the carrier in PE membranes. We would not have been surprised if the deviation had been in the opposite direction, since the larger osmotic driving force of the Bis-Q6 gradient might be expected to move enough water across PE membranes to produce a measurable polarization. Since we know of no artifact which could shift the zero current potential of the carrier to a value more negative than the chloride equilibrium potential, and organic amines have not been reported to interfere with determinations using chloride electrodes, we are unable to account for this behavior.

Although we have not been able to quantitatively account for all the artifacts associated with determining the permeance of NMG and Bis-Q6, the pH studies unambiguously established that the monovalent compound is permeant and the polyvalent one is impermeant. Since the apparent selectivities of the polyvalent ions, whether examined in membranes composed of asolectin or PE, did not differ systematically or significantly from each other, we are led to the conclusion that all of them are impermeant. Furthermore, they appear to be impermeant because they are polyvalent, since several members of this group are smaller in molecular weight and dimensions than some permeant monovalent cations.

Discussion

Colicin E1 as a Molecular Sieve

The definition of a channel as a water-filled, transmembrane pore implies a structure with definite dimensions. Organic cations of different sizes have been used to great advantage as dimensional probes of a variety of channels [1, 14, 16, 18]. At the simplest level of interpretation, ions which are larger than the narrowest part of a channel must be unable to pass through it. In addition, ions which cannot themselves traverse the channel but do interfere with the movement of permeant ions are taken to be small enough to enter the channel but too large to fit through the tight spots. The dimensional information

obtained about the colicin E1 channel through this kind of analysis has been limited. What we can say is that the channel, even at its narrowest point, must be at least as large as any of the monovalent ions tested. Bis-Tris, having a geometric mean diameter of 9.1 Å, could be considered our largest permeant probe. Because it is also fairly symmetrical and quite inflexible in structure, we believe it establishes a reasonable lower limit for the diameter of the pore formed by colicin E1. By ion channel standards, a 9.1-Å cross section is quite large, being able to accommodate at least 10 water molecules or a sodium or calcium ion complete with primary hydration shell. Because many of the other probes are quite asymmetrical and some are quite flexible, we cannot be sure how much larger than 9.1 Å or what shape the pore might be, since we have no basis for discerning what conformations the probes may take up within the channel.

Concerning the impermeant polyvalent probes, we have two important conclusions. First, they are not impermeant because of their size, since only Bis-Tris Propane has a geometric mean diameter greater than 9.1 Å. In addition, they do not block the movement of chloride through the pore. If the pore diameter were only slightly larger than these impermeants, one would expect anion movement to be impeded by their presence inside the channel. The absence of such an effect implies either that the pore is considerably wider than the impermeant probes in some locations or that the probes are excluded from the channel lumen altogether.

Electrostatic Sieving by Colicin E1

Exclusion of ions from channels on the basis of valency is not at all uncommon, as most channels can be classified as exclusively cation or anion conducting. Superficially, sprinkling a few fixed charges along the inside of the channel or around its entrances would seem to be all that is required to confer this kind of selectivity. Because electrostatic interactions do not exhibit such a sharp dependence on distance, however, they are not amenable to analysis by the simple "go/no-go" criteria which have proved advantageous for steric interactions; resort must be taken to some sort of primitive energetic calculations. The transport of a monovalent ion across a membrane under the influence of a 50-mV driving force corresponds to an overall energy drop of 1.1 kcal/mol. If the transport of a monovalent cation x^+ is considered as a single kinetic step, the single-channel conductance, γ, is given by

$$\gamma = \frac{FkT}{VN_Ah} \exp\left(\frac{-U + F(1-\delta)V}{RT}\right)(1 - e^{\frac{FV}{RT}})[x^+]$$

where F is Faraday's constant, k, Boltzmanns constant, h Planck's constant, N_A Avogadro's number, R the gas constant, T the absolute temperature, V the membrane voltage, U the free energy of activation, δ the fractional electrical distance of the barrier from the membrane surface, and $[x^+]$ the molar concentration of the monovalent cation. Assuming the free energy barrier to be in the center of the membrane ($\delta = 0.5$), a single-channel conductance of 20 pS in 1-M 1 : 1 electrolyte with 50-mV applied potential corresponds to an activation energy of 12.4 RT or 7.3 kcal/mol. The physical structure corresponding to this energy barrier is termed the selectivity filter [18]. According to the Equation, increasing the activation energy by 2.7 kcal/mol would decrease the conductance of the channel by a factor of 100, an effect sufficient to render the channel effectively impermeable. The task at hand, then, is to calculate the energy associated with the electrostatic interactions between the ion and the channel as the selectivity filter. In order to account for other characteristics of ion transport by channels, kinetic schemes often include some number of smaller barriers and intervening energy wells in addition to the large central selectivity barrier. The net effect on the conductance of the channel produced by changing the energy at any one location in these more complicated profiles will, however, be less than in the one-barrier case. The single-step formalism is thus convenient for examining the upper limits of the effects of electrostatic interactions on ion permeation.

It might be tempting to evaluate the interaction between the fixed charges of the channel structure and the mobile ions in solution using the familiar Gouy-Chapman formalism [25], in which both the fixed and mobile charges are treated as a continuous density distributions rather than discrete entities. This method works remarkably well when the fixed charge is borne on phospholipids which are spread out over the vast membrane surface, but the continuum assumption loses its utility when the crucial interactions occur between specific structures and take place over short distances. For instance, the concept of the counterion cloud of an ion in solution has no physical significance when the Debye length is greater than the average interion separation [4]. At concentrations above about 1 mM, therefore, ions in solution do not behave as though they were surrounded by a cloud of smeared-out charge; one must consider that there are discreet counterions nearby the central ion. In the case of channels, then, we must consider the interaction between a discreet fixed charge within a channel and an ion traveling through it. At the lowest order of approximation, the energy for such an interaction can be calculated as the coulombic potential of two point charges in a continuous high dielectric medium separated by a

distance equal to the radius of the channel. If a monovalent cation traveling down the center of a water-filled channel approached a fixed positive charge within a distance of 4.5 Å (the minimum radius of the colicin E1 channel), the interaction energy would amount to only 0.5 kcal/mol, corresponding to a 43% reduction of the channel conductance. Thus, placing positive charges in the channel lumen is remarkably ineffective in reducing the cation conductance of a wide, water-filled channel. On the basis of these considerations it is not so surprising that, as pointed out by Miller in a recent review [27], there are no negative charges in the pore lumen of the consensus structure for the highly cation-selective acetylcholine receptor channel. Since both cations and anions permeate the channels formed by colicin E1, we must consider that an added positive charge will also lower the energy barrier for anions by 0.5 kcal/mol, corresponding to an 2.3-fold increase in anion conductance. Overall, the anion-to-cation conductance ratio will be increased by a factor of 5.4 for each added positive charge. For colicin E1, this ratio varies 75-fold between pH 4.0 and 6.0 [6], much too great a variation to be attributable to this kind of simple electrostatic repulsion. The ability of this channel to discriminate between sodium and calcium is also much greater than the 43% allowed by this model.

Since the coulombic potential varies inversely with distance, the effectiveness of fixed charges in filtering mobile ions would be increased if they were forced to approach more closely. This situation can arise either when the channel is narrower, or, as in the present case, when the molecule bearing the ionic charge is larger. In an aqueous environment, however, the dielectric continuum approximation breaks down for those water molecules which are the nearest neighbors of dissolved ions. The critical distance is the ionic radius plus diameter of the water molecule (2.76 Å). Within this region, termed the primary hydration shell of the ion, we can calculate the ion-solvent interaction energy simply by considering a point charge and a point dipole separated by the sum of the crystallographic radii of the molecules or ions which bear them. Note, however, that this interaction must be considered to take place in a vacuum, since the dielectric constant used in the previous calculation was simply an approximate treatment of the behavior of the water molecules which we now treat explicitly. For a sodium ion, the interaction with the dipole moment of each of the water molecules in its primary hydration shell contributes 24 kcal/mol. Thus, the four nearest neighbor water molecules account for nearly 80% of the experimentally measured free energy of hydration of the sodium ion (*see* Chapter 2 of ref. [4] for a lucid discussion of this problem).

It was Mullins [30] who originally pointed out the implications of steric restrictions in channels which require that an ion shed any of its waters of hydration in order to traverse the membrane. Requiring the removal of even one of these waters of solvation would create an insurmountable barrier to ion movement. It is apparent that all of the formal charges in the system, whether fixed charges which are part of the channel structure or mobile ions which are being transported, must remain effectively solvated at all times. If water molecules are prevented from playing this role for steric reasons, then other charged or dipolar groups which are part of the protein structure must provide energetically favorable substitutes. The demands of the energy constraints are severe; a substitute interaction which results in a scant 8% reduction in ion-dipole energy would be sufficient to render an ion effectively impermeant.

So strong are the nearest neighbor electrostatic interactions within the lumen of an ion channel that changing them can easily change a high barrier into a deep well. For example, the interaction energy of a sodium ion in direct contact with a negatively charged oxygen atom is some 250 kcal/mol greater than the corresponding value for a water dipole. Thus, if a mobile ion formed an ion pair with the structure of the channel, it would be held so tightly that it could not be transported at a measurable rate. Formation of ion pairs between free ions in aqueous solutions does not occur to any great extent because ion lattices are even more stable. If the ions are forced closer together by raising their concentrations, the salts simply precipitate out of solution. A different and more complicated situation obtains when the ions are forced together by the structure of a channel. For instance, interactions involving the water molecules in the overlapping primary hydration spheres of the members of the pair must be considered, and we have yet to mention entropic or thermal disordering effects. Our simple coulombic analysis, however crude, does make it clear that the energies associated with direct ion-ion contacts are so large to disallow transport of any ionic species, regardless of valency. In order for selective ion transport to occur, opposing electrostatic forces having energies of hundreds of kcal/mol must sum to produce an energy profile along the reaction coordinate for ion movement whose features are only a few kcal/mole in magnitude. These electrostatic interactions between the ion and the channel wall are thus most properly regarded as subtle changes in solvation. If the ion stays far enough away from the channel wall so that solvation remains relatively undisturbed, the electrostatic forces are sharply attenuated by the presence of the intervening water dipoles, and ion transport will be affected only

weakly. If, on the other hand, solvation is seriously disrupted, the electrostatic energies are so large that transport will be blocked.

The limiting cases of ion-channel electrostatic interactions having been shown to be inapplicable to the behavior of colicin E1, we are left to speculate about a complex set of interactions between the channel, the water molecules within it, and the ion being transported. Two general characteristics of such systems must be recognized if specific hypotheses are to be framed in a useful way. First, the discreet charges and dipole moments of the channel structure which control ion transport need not be located on the lumenal surface of the channel. Any charged residues in a protein structure which do not lie on an aqueous surface seem certain to exist either as salt bridges or to be stabilized by polar groups in the protein structure. Because the effective dielectric constant in the interior of a folded protein is expected to be considerably less than that of water, such interactions can be strong enough to define the local energy minima required for the conformational stability of the protein. For the same reason, the influence of buried charges and permanent dipoles may be felt by mobile ions near the channel wall over proportionately longer distances. The question of the charge distribution of the deeper structure of channel proteins is especially interesting in the case of the acetylcholine receptor, since there appears to be a dearth of polar groups on this surface of the lumen of this highly cation-selective and conductive pore [27]. In a previous paper [6], we provided evidence that the dependence of the selectivity of colicin E1 on pH was the result of a conformational rearrangement of the protein and suggested that shifts in the positions of buried charges and dipoles may mediate this effect. A number of mutants have also been isolated in which the substitution of charged residues has, either by design or by accident, resulted in subtle alterations in the selectivity properties of the protein [19, 37, 38]. If such single amino acid substitutions were to occur within the channel wall, we would not expect the newly introduced charges to be able to form salt bridges. This suggests the possibility that the effects of these mutations, like those of pH, are the result of changes in protein conformation.

It should also be recognized that ions can be brought close to the walls of channels by factors other than steric restrictions. The colicin E1 channel is sterically wide enough to accommodate hydrated sodium, calcium, and chloride ions. If these relatively small species simply traveled down the center of the channel lumen, however, we have shown that it is difficult to explain the ability of the channel to discriminate between them, and, indeed, why the effective barrier to permeation of these small ions is so high. Instead, they appear to be steered away from the center and forced towards the walls of the channel. This behavior may be attributable to interactions between the channel wall and the solvent. These dipole-dipole interactions are much weaker than the ion-dipole interactions which define the hydration of ions. If structure of the channel is capable of hydrating the ion effectively, however, the electrostatic energy associated with the arrangement of the remaining water dipoles could easily be sufficient to favor a position of the ion next to the wall. The energy associated with the hydrogen bonding arrangement of these solvent molecules, while smaller in absolute magnitude, may also be important in regions of the channel where the wall is nonpolar and electrostatic interactions are thus weaker.

The present work suggests that, whatever specific structural features of the protein are responsible, the selectivity filter of the wild-type colicin E1 channel appears to be able to accommodate only one partially hydrated positive charge at a time. Any second charged center appears to be able to interact with the channel only in an energetically unfavorable way. In terms of the one-barrier model, this implies that the impermeant ions are excluded from the channel altogether. There is as yet no experimental evidence, such as saturation or block, suggesting the existence of more than one barrier in these channels. If any of these polyvalent cations could be shown to interfere with the movement of sodium, for instance, it would imply the existence of a cation binding site within the channel. Before this or other questions of the independence can be addressed, however, we must determine what selection rules apply to the permeation of anions.

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