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Ion Selectivity of Colicin E1: III. Anion Permeability

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Abstract. The antibiotic protein colicin E1 forms ion channels in planar lipid bilayers that are capable of conducting monovalent organic cations having mean diameters of at least 9 Å. Polyvalent organic cations appear to be completely impermeant, regardless of size. All permeant ions, whether large or small, positively or negatively charged, are conducted by this channel at very slow rates. We have examined the permeability of colicin E1 channels to anionic probes having a variety of sizes, shapes, and charge distributions. In contrast to the behavior of cations, polyvalent as well as monovalent organic anions were found to permeate the colicin E1 channel. Inorganic sulfate was able to permeate the channel only when the pH was 4 or less, conditions under which the colicin E1 protein is predominantly in an anion-preferring conformational state. The less selective state(s) of the colicin E1 channel, observed when the pH was 5 or greater, was not permeable to inorganic sulfate. The sulfate salt of the impermeant cation Bis-T6 (N,N,N′,N′-tetramethyl-1,6-hexanediamine) had no effect on the single channel conductance of colicin E1 channels exposed to solutions containing 1 M NaCl at pH 5. The complete lack of blocking activity by either of these two impermeant ions indicates that both are excluded from the channel lumen. These results are consistent with our hypothesis that there is but a single location in the lumen of the colicin E1 channel where positively charged groups can be effectively hydrated. This site may coincide with the location of the energetic barrier which impedes the movement of anions.

Key words: Colicin — Ion selectivity — Lipid bilayers — Electrostatic interactions — Anion channels

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Introduction

The idea that membranes may contain pores so narrow that they act as sieves for solute molecules was proposed in the first half of the nineteenth century to explain the phenomenon of osmosis. It is thus fair to say that channel theory ranks as one of the earliest attempts to understand a biological function on the basis of structures having molecular dimensions. In the succeeding one hundred fifty years, we have come to understand that membranes pose a barrier to the movement of polar substances because they are composed of a fluid, bimolecular leaflet of amphipathic lipids, and that the pores which provide pathways through them are constructed of specialized proteins that are embedded in this fluid matrix. This knowledge has allowed us to frame structural questions more precisely, since they inevitably lead directly to problems of protein folding. The functional questions, on the other hand, have become more complex than those originally posed. It is now clear that channels have the ability to discriminate between various solutes in ways that are more complicated than simple sieving on the basis of molecular size. Furthermore, it is now appreciated that channels are not static structures. Not only do they spontaneously open and close, but extrinsic signals, like membrane voltage and ligand binding, can control the opening and closing processes. These two phenomena, known as selectivity and gating, represent the fundamental molecular questions regarding ion channel function. Channels formed in planar lipid bilayers by a group of proteins known as colicins are particularly interesting objects for the study of these processes [7, 9, 14, 33, 36, 44, 45]. The selectivity properties of these channels has been of particular interest to us because they have challenged many of our naive notions about the ways channels tell one ion from another. The pores formed by these proteins are wide, at least 9 Å in diameter at the narrowest point [11, 37]. Assuming that the

ions behave in the channel as they do in bulk solution, the conductance of a simple pore filled with 1 M NaCl having this diameter and a length of 60 Å can be calculated to be 1500 pS [4]. The single channel conductance of colicin E1 in 1 M NaCl, on the other hand, is only 20 pS. It is not at all obvious what might slow the movement of small ions through such a wide conduit to a rate nearly one 100-fold less than what would be expected on the basis of a simple macroscopic calculation. Our aim has been to understand the nature of these impediments in terms of the mutual interactions between the transported ions, the structure of the channel, and the water contained within it.

The colicins are antibiotics produced by certain strains of *Escherichia coli.* Because they are water soluble proteins which can be readily purified in very large amounts, a wide variety of physical techniques have been used to study their properties. Functional studies can be performed in vivo with intact bacteria or spheroplasts, or by reconstitution in either planar or vesicular bilayer membranes. Conveniently, the proteins are encoded on plasmids, and site-directed mutants have been produced to extend the reach of structure-function studies of these molecules [3, 20, 21, 38–40]. The domains of the colicin E1 and A molecules which actually form channels have been shown to lie at the carboxyl terminal ends of the proteins, and all six of the channel-forming colicins show high degrees of homology in the corresponding regions [12, 22, 24, 29, 37, 43, 47]. Previously, 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. In the present study, we have extended this work to include anionic compounds. 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 Megohm-cm) was used for all solutions. In addition to the sodium salt of the anion under test, working solutions contained each of the following compounds at concentrations of 3 mM: calcium glutarate, pK_a = 4.13, 5.03; 2-(N-morpholino)ethanesulfonic acid (MES), pK_a = 6.15; 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-tris propane), pK_a = 6.8, 9.0. In our previous work [11], hexamethonium (Bis-Q6) and N,N,N′,N′-tetramethyl-1,6-hexanediamine (Bis-T6) were shown to be impermeant cations. These compounds were obtained from Sigma Chemical (St. Louis, MO) and Aldrich Chemical (Milwaukee, WI) respectively. Bis-T6 sulfate was prepared by neutralizing a solution containing a known amount of H_2SO_4 with the free base form of Bis-T6. Asolectin type IV-S (Sigma, 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).

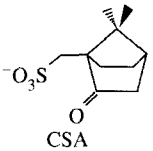
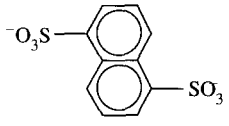
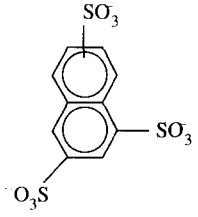
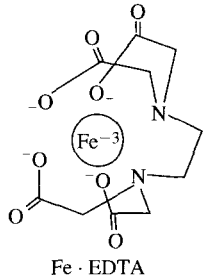
The following compounds, used as test anions, were obtained from Aldrich (Milwaukee, WI): *dl*-10-camphorsulfonic acid (CSA), 1,5-naphthalenedisulfonic acid (NDSA), and 1,3,(6 or 7)-naphthalenetrisulfonic acid (NTSA). Eastman Laboratory Chemicals (Rochester, NY) supplied methanesulfonic acid (MSA) and ethanedisulfonic acid (EDSA). Ferric ethylenediaminetetraacetic acid (FeEDTA) was obtained from Sigma (St. Louis, MO). The structures, formula weights, and molecular dimensions of these compounds are shown in Table 1. The dimensions given are the maximum overall length and minimum overall width as determined from CPK® models (Harvard Apparatus, South Natick, MA). This is equivalent to the size of the rectangular slot whose width is the narrowest space through which the molecule can squeeze and whose length is the longest distance over which the molecule can stretch. In all cases, the measurements were made on the fully extended conformation of the ion.

The compounds CSA, NDSA, and NTSA yielded slightly colored solutions which were decolorized with carbon prior to use. In addition, NDSA and NTSA were found to be contaminated with significant amounts of chloride and EDSA with bromide. These compounds were recrystallized twice from water and dried over $CaSO_4$. Other compounds were used without further purified. The halide concentrations of the working solutions of EDSA and NDSA, determined using specific ion electrodes, were found to be less than 0.1 mM. The residual chloride in the working solution of NTSA, which has a much higher water solubility than either EDSA or NDSA, was approximately 1 mM. EDSA, Fe(EDTA), NTSA were available only as sodium salts and not as free acids. CSA was used to adjust the pH of solutions of EDSA, and NDSA was used for solutions of Fe(EDTA) and NTSA. NDSA and Fe(EDTA) were used at concentrations of 0.30 M and 0.03 M, the former concentration being very close to the limits of solubility of these two compounds. As noted above, Na_3NTSA was quite soluble in water and not easily separated from NaCl by crystallization in water. This compound was used at formal concentrations of ⅓ and 1/30 Molar, so that the corresponding sodium concentrations were 1 and 0.1 Molar. All other compounds were used at formal concentrations of at 1.0 and 0.1 M. None of these compounds was found to permeate, destabilize, or disrupt the planar membranes used in this study. In addition, none of the compounds exhibited measurable activity as protonophores when tested in pH 4 versus pH 5 gradients, or had pharmacological effects upon the colicin E1 channel, such as that described for glutathione [34]. The selectivity of the colicin E1 channel for a particular anion was determined as the value of the zero current potential of the conductance induced by the protein in a membrane exposed to a tenfold gradient of the sodium salt of that anion. An anion was judged to be impermeant if its zero current potential was equal to the sodium equilibrium potential (E_{Na}) of the experimental solutions used to produce the tenfold gradient. Ion selective electrodes in combination with a pH/ion meter (Orion Research, Cambridge, MA) were used to determine the values of E_{Na} shown in Table 1. A double junction reference electrode filled with 10% KNO_3 was used to avoid contamination of the solutions by additional chloride. Using complexation and acid dissociation constants for EDTA [23], the concentration of uncomplexed ferric ion in the 0.3 M solution of Fe(EDTA) at pH 4 was calculated to be 2.5 nM. Calcium was omitted from the working solutions of Fe(EDTA).

Membrane Conductance Measurements

Planar phospholipid bilayer membranes of the solvent-free type [28] were formed across apertures in teflon septa as previously described [6]. The volumes of aqueous solution bathing each side of the membrane were approximately 1.5 ml. The apertures used in these experiments were either 100 μm or 150 μm in diameter.

Table 1. Properties of test anions

Anion	FW (Da)	$d_{max} \times d_{min}$ [a] (Å)	E_{Na} [b] (mV)
Cl^- Chloride	35.45	3.6	+56
$CH_3–SO_3^-$ MSA methanesulfonate	95.10	5.2 × 4.6	+56
 CSA camphorsulfonate	208.30	10.2 × 6.6	+57
$^-O_3S–CH_2–CH_2–SO_3^-$ EDSA ethanedisulfonate	188.16	9.3 × 4.9	+51
 NDSA 1,5-naphthalenedisulfonate	286.36	11.6 × 4.9	+51
 NTSA 1,3,(6 or 7)- naphthalenetrisulfonate	365.31	13.0 × 4.9	+48
 Fe · EDTA ferric ethylenediaminetetraacetate	344.1	10.6 × 7.4	+48
SO_4^{-2} Sulfate	96.08	5.1 × 4.5	+47

[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] Sodium equilibrium potentials (E_{Na}) were measured across tenfold concentration gradients of each of the test anions as sodium salts. The gradients were 0.3 *vs.* 0.03 M for NDSA and FeEDTA, ⅓ *vs.* 1/30 M for NTSA, and 1 *vs.* 0.1 M for all other compounds. All solutions also contained 3 mM calcium glutarate and 3 mM MES, pH 5.0.

Voltage clamp conditions were established across the bilayers by means of a Burr Brown 3528L 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 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 eight 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, as previously described [8]. As a matter of experimental convenience, stock solutions were prepared to contain both detergent and protein at a 10 to 1 weight ratio. Only membranes exhibiting a high resistance (>10^8 Ohm-cm) and 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 NaOH or the appropriate test anion as the free acid. The concentration of the titrants were chosen to match those of the primary electrolytes, so that only the buffers were subjected to cumulative concentration changes in the course of these experiments. In some cases, as noted above, the test anion was not available as the free acid and other anions were substituted. The concentrations of these exogenous titrants, then, increased by approximately 3 mM each time the pH was cycled in the course of the experiments. If these anions were to be permeant, their accumulation would produce apparent decreases in selectivity of the colicin E1 channel. Decreases in the absolute value of the zero current potential might also be caused by the development of nonselective leak conductances in the bilayer. To control these sources of error, decreases in selectivity that proved to be irreversible were excluded from the data reported and resulted in the termination of those experiments. Addition of NaOH to solutions of Fe(EDTA) resulted in the precipitation of the poorly soluble hydroxide of the ferric ion. For this ion, the pH was adjusted by perfusing the chamber with solutions whose pH had been previously adjusted, as described previously [7]. Note that perfusion is not effective in removing colicin E1 protein from bilayer chambers [7, 9].

Abbreviations

Bis-Q6: hexamethonium. Bis-T6: N,N,N′,N′-tetraamethyl-1,6-hexanediamine. Bis-tris propane: 1,3-bis[tris(hydroxymethyl)meth-

ylamino]propane. CSA: *dl*-10-camphorsulfonic acid. EDSA: ethanedisulfonic acid. Fe(EDTA): ferric ethylenediaminetetraacetic acid. MES: 2-(N-morpholino)ethanesulfonic acid. MSA: methanesulfonic acid. NDSA: 1,5-naphthalenedisulfonic acid. NTSA: 1,3, (6 or 7)-naphthalenetrisulfonic acid. PE: bacterial phosphatidylethanolamine.

Results

Determination of Selectivity

Channels formed by colicin E1 have been reported to be significantly permeable to a wide variety of both cations and anions [34]. In the preceding paper of this series [11], we demonstrated that while monovalent cations having mean diameters of 9 Å were measurably permeant, cations with more than one positively charged site were unable to permeate these channels. In the present study we have exposed planar membranes containing colicin E1 to tenfold gradients of the sodium salts of a series of anions. The zero current potential exhibited by the channels under these conditions is a measure of their permeability to the test anion relative to the permeant cation sodium. The conventions, limitations, and artifacts associated with this kind of selectivity study are described below.

In cellular electrophysiology, the sidedness of the membrane is clear (inside and outside) and consistent sign conventions for voltage and current have been adopted. The simple physical distinction in a planar bilayer system (front and back) is of no intrinsic significance, so that these assignments are completely arbitrary and no consistent convention has been followed in the literature. In defining the zero current potential, we have followed the convention adopted in the preceding paper of this series [11]. The ground potential, or zero reference point, was defined as the potential of the compartment containing the higher concentration of salt, without regard to the side of the gradient to which protein had been added. Thus, positive values of the zero current potential always indicate a preference for the sodium and negative values a preference for the anion. Note that in discussing the voltage dependence of the opening and closing of these channels, it is the orientation of the protein rather than that of the concentration gradient which is of primary importance. In this context, the potential of the compartment containing the protein (the *cis* compartment) is more commonly defined as the reference or ground potential. The results of a typical selectivity determination are shown in Fig. 1. Colicin E1 protein was added to the 0.1 molar side of a Na(CSA) gradient across a PE membrane. In response to the application of a *trans* negative potential, several hundred channels 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 one millivolt differences in applied potential are easily distinguished. Note that because the higher salt concentration was on the *cis* side of the membrane, the sign of the zero current potential from this record indicated that the colicin E1 was cation selective under these conditions and was reported as a positive value.

Selectivity scales for various ion channels can be conveniently defined relative to suitably chosen permeant reference ions. Most channels are permeable either to cations or anions but not both, so that the charge of the reference ion has the same sign as that of the test ion. In these cases, relative selectivity can be expressed as the ratio of the single channel conductance of a channel bathed by a solution of test ion to that associated with the same concentration of the reference ion, g_{TEST}/g_{REF}. The capability of such measurements to discriminate between slightly permeant and completely impermeant ions is limited by the size of the single channel currents that can be resolved. Alternatively, selectivity can be measured as a Goldman-Hodgkin-Katz permeability ratio under biionic conditions, $P_{TEST}/P_{REF} = exp(-V_0F/RT)$. In principle, the zero current potential, V_0, should go to infinity as the channel's permeability to the test ion approaches zero. Practical limitations are imposed on this method by the background conductance of the membrane and the range of voltages that can be applied to membrane without causing damage. For those channels which are permeable to ions of both signs, a class which includes all of the colicins, replacing a permeant anion with an impermeant one will not eliminate the flow of current, since permeant cations must necessarily be present on both sides of the membrane. Currents, therefore, never approach zero and potentials never tend toward infinity. Instead, the criterion for impermeance must be based 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 record shown in Fig. 1, the measurement of zero current potential fell 10 mV short of the sodium equilibrium potential of the CSA working solutions, suggesting that this anion is somewhat permeant. Because we had no way to measure the equilibrium potential of the test anion, E_{CSA}, we have not converted the measurement of zero current potential into a permeability or conductance ratio. Note that rather large currents passed through the channel, carried mainly by sodium ion, in the presence of the much less permeant CSA anion. This behavior was observed for all the compounds tested and is the same result as that obtained in our study of cation selectivity; in no case did impermeant or poorly permeant test ions appear to block the movement of permeant counterions through channels formed by colicin E1. Another general feature of colicin behavior is that a higher rate of channel formation was observed when the protein is introduced on the low concentration side of the salt gradient. This tendency was particularly pronounced in the case of CSA.

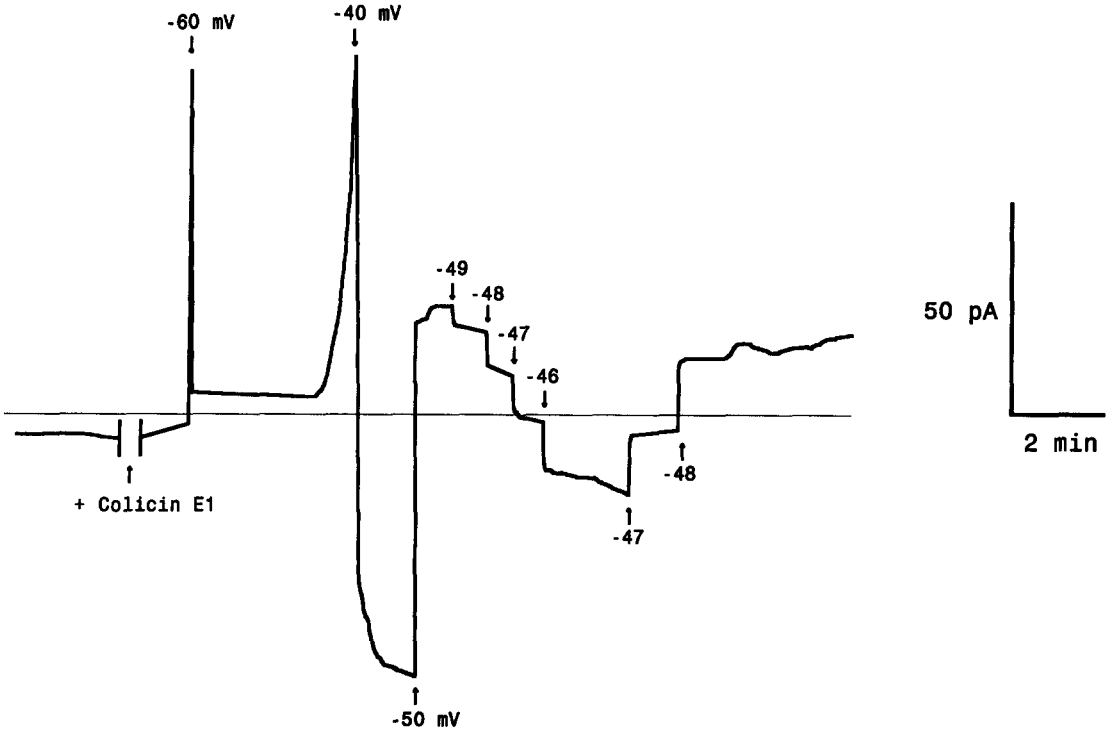


Fig. 1. Determination of zero current potential in a bacterial phosphatidyl ethanolamine membrane. This membrane was formed on a 150 μm diameter hole and was bathed by solutions containing 1.0 and 0.1 M Na(CSA). The potentials indicated are defined with respect to the *cis* of the membrane, which in this case was exposed to the lower salt concentration. The initial applied potential was 0 mV. Colicin E1 at a final concentration of 1 μg/ml was added to the 0.1 M salt side of the gradient at the time indicated, and the potential was clamped to –60 mV. Following a few minutes delay, which can be attributed to the diffusion of the protein through the unstirred layer adjacent to the bilayer, conductance of the membrane rose rapidly. The experimentally applied potential at which no current flowed across the membrane was found to be –47 mV. This corresponds to a zero current potential of +47 mV (cation selective). Both solutions contained 3 mM $CaCl_2$, 3 mM glutaric acid, 3 mM MES, and the pH was adjusted to 5.0.

As discussed in the earlier papers in this series [6, 11], selectivity measurements of this type are subject to a number of potentially troublesome artifacts, most tending to make the channel appear less selective than it really is. The first class of artifacts are associated with the bilayer system rather than the protein itself. There are, to begin, errors associated with the measurement of the equilibrium potentials using specific ion electrodes. For instance, we previously noted that using double junction electrodes rather than conventional calomel electrodes produced a 2 mV difference in the measurement of chloride equilibrium potentials of some, but not all salt solutions [11]. 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 potentials of tenfold gradients of sodium chloride and hexamethonium (Bis-Q6) chloride [11]. There was no evidence for osmotic polarization in membranes composed of PE. In addition, the working solutions contained buffers which may have been permeant, some of the titrants used appeared to be slightly permeant, solutions may have been contaminated by Cl^- or some other anionic impurity present in the test compounds, and KCl is known to leak from the calomel electrodes at a slow but appreciable rate. Permeant anionic contaminants produce their largest errors at low pH, where the colicin E1 channel has a strong preference for anions. The chloride to sodium permeability ratio calculated from the Goldman-Hodgkin-Katz equation at pH 5 is 1.5:1. Under these conditions, the presence of chloride, or some other anion equally as permeant, at a symmetrical concentration of 1 mM would cause the zero current potential of a completely impermeant test anion to be less than E_{Na} by approximately 0.5 mV. If present at 3 mM, the concentration of the buffering compounds, such contaminants would produce errors of approximately 1.1 mV.

The net effect of these system-associated errors is simply to blunt the precision of selectivity determinations by a few millivolts. Artifacts produced by less than ideal channel-forming behavior of the colicin E1 protein are more difficult to identify and measure. We [6, 11] and other [20, 34] have noted that a voltage-independent, nonselective leak pathway sometimes develops with time in membranes having high levels of colicin E1 conductance. When experiments are conducted at low pH in asolectin membranes, the voltage-dependent kinetics of colicin E1 are fast enough to allow selectivity measure-

ments to be corrected for this steady background conductance. Examples of this kind of zero slope potential, or reversal potential, experiment have been shown by us [11] and by Raymond et al. [34]. At higher values of pH, however, the gating of the channel becomes too slow to conduct this kind of experiment. In membranes composed of neutral lipid, furthermore, voltages in the range of possible zero current potentials are quite ineffective at gating the channel [8]. The behavior of the nonselective leak conductance in a more typical selectivity experiment can be seen in Fig. 4 of the preceding paper of this series [11]. In that experiment, the permeability of colicin E1 to the cation N-methylglucamine was followed for several hours on the same PE membrane as the pH was continually cycled between 4 and 6. The zero current potentials at all values of pH exhibited a slow creep towards less negative (i.e., less selective) values. When the membrane was reformed, no colicin channels were present in the new membrane and its conductance was close to zero. When colicin E1 conductance was induced in the reformed membrane by application of an appropriate voltage, the zero current potentials had shifted back towards their original values. We attributed this shift to the elimination of the nonselective leak conductance and the residual offset to leakage of KCl from the reference electrodes. The nonselective leak, when it occurs, is associated with high levels of colicin conductance, not just high concentrations of colicin protein in the chamber (i.e., we have not observed the leak conductance by itself). In the present work, individual experiments were conducted using moderate levels of colicin conductance, and membranes were reformed frequently to check for drifts in measurements.

Several investigators have raised concerns that the colicin E1 may form atypical or aberrant channels under some conditions. Liu et al. [21] reported that a very short peptide derived from colicin E1 was capable of forming channels. While this finding would seem to have important implications regarding the three-dimensional structure of the pore formed by this protein, this group of investigators chose to ignore their own finding in later discussions of this problem [1]. Cramer and his associates have described unpublished studies of a very similar peptide in a recent review [16]. While the macroscopic currents induced by this peptide resembled those characteristic of the intact protein, the single channels formed by the peptide were much more heterogeneous in size and in kinetics than those of the parent molecule. It was not determined whether the channels having larger conductances also were less selective than the smaller channels. In the long course of the studies of colicin E1 in our laboratory, we have made two kinds of observations which also may bear on this question. First, we routinely check the selectivity of our colicin protein under standard conditions (asolectin membranes, pH 5.0, 1 *vs.* 0.1 M NaCl). Zero current potentials near +25 mV (*cf.* ref. [11]) are routinely observed, though the concentration of protein in the chamber may range from 0.3 μg/ml to 2.0 μg/ml, and the conductance levels from 2 to 100 nS. It is very rare for freshly prepared protein solutions to exhibit less than normal selectivity; but in addition to the expected overall loss of channel-forming activity, stocks that have been stored on ice for more than two weeks sometimes showed a decreased selectivity. In a few instances, we have also had occasion to observe that single channels formed from such older protein stocks exhibited kinetics and unitary conductances which were different from those of the fresh protein. Cramer et al. [16], suggested that the heterogeneity of the channels formed by the shortened colicin E1 peptides was the result of varying states of aggregation. We find no evidence for such aggregation in the case of the intact protein, but do concur with the conclusion that products of colicin E1 proteolysis may form channels with altered properties.

A different sort of aberrant behavior was observed by Raymond et al. [34] in experiments designed to determine the selectivity of colicin E1 using the zero slope technique. They reported that at potentials near the reversal potential, the time course of activation of the colicin-mediated current sometimes did not have a simple slope but instead appeared to be biphasic. We have observed behavior in asolectin membranes at pH $\leq$ 4 using NaCl as the electrolyte which we also would describe as biphasic. To explain the appearance of their records, Raymond et al. suggested that when colicin E1 channels are opened in response to an applied potential, their average selectivity declines slightly as time progresses. This kind of current record could also be produced if a small, nonselective, but voltage-activated conductance were present in parallel with the slow, linear increase in conductance which is characteristic of colicin E1. While it is not possible to distinguish between these two possibilities on the basis of the scant description in the literature or our incidental observations, we prefer the latter explanation. In our laboratory, biphasic currents were seen only intermittently, and colicin E1 sometimes reverted to normal behavior upon reforming the membrane. Besides being reminiscent of the slowly developing leak conductance described above, these characteristics are typical of other artifactual conductances which are occasionally observed in planar bilayers and usually attributed to ionophorous contaminants. Regardless of its origin, this phenomenon produces only very small shifts in selectivity measurements, ±3 mV according to Raymond et al. [34].

The results of zero current determinations carried out in PE bilayers are summarized in Table 2. All of the test ions were clearly much less permeant than chloride. With the exception of sulfate, however, the differences

Table 2. Anion permeability of colicin E1 channels in PE bilayers

Anion	Zero current potential (mV)		E_{Na} (mV)
	Colicin added to 1-M-side	Colicin added to 0.1-M-side	
Cl^-	-2 ± 8 (39)	ND	+56
MSA^-	$+37 \pm 4$ (16)	$+33 \pm 2$ (8)	+56
CSA^-	$+43 \pm 3$ (10)	$+47 \pm 1$ (13)	+57
$EDSA^{-2}$	$+39 \pm 4$ (20)	$+39 \pm 2$ (3)	+51
SO_4^{-2}	$+43 \pm 2$ (14)	$+42 \pm 2$ (10)	+47

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

between the zero current potentials and the E_{Na} values for these salts are too large to be attributed to cumulative errors produced by the artifacts described above. Sulfate, therefore, is the only anion among those tested which appeared to be impermeant within experimental error. In the cases of CSA and MSA, the selectivity of colicin E1 exhibited a small but statistically significant dependence on the side of the gradient to which the protein was added. As noted above, the activity of colicin E1 was much lower when added to the 1 M sides of CSA gradients than when added to the 0.1 M sides. Because nonselective leaks produce proportionally larger errors when the colicin activity is low, it is not surprising that the channel appeared to be less selective when the protein was exposed to the higher salt concentration. The dependence of colicin E1 selectivity on salt concentration in MSA gradients was in the opposite direction, and we have no ready explanation for this behavior.

The pH Dependence of Anion Permeability

In our previous investigations of the cation selectivity of colicin E1, we identified a large number of compounds whose zero current potentials in PE membranes fell a scant 3–4 mV short of the corresponding E_{Cl} measurements. The question of their permeance was resolved by examining the dependence of the zero current potentials on the pH of the solution on the *cis* side of the membrane. The results of two such experiments are reproduced from reference [11] in Fig. 2. In a tenfold gradient of NaCl, the zero current potential of colicin E1 shifted from −38 to +25 mV as the pH on the *cis* compartment was varied from 3.0 to 8.0 while the *trans* pH was held constant at 5.0. Given the experimentally determined values E_{Na} = +56 mV and E_{Cl} = −47 mV, these zero current potentials correspond to permeability ratios (P_{Cl}:P_{Na}) of 16.2:1 and 1:2.8. By contrast, the zero current potential of colicin E1 measured in a tenfold gradient of (Bis-Q6)Cl_2 did not change when the pH was raised or lowered. If the 2 mV deviation of the zero current potential from E_{Cl} were the result of some slight permeation of Bis-Q6, making the channel itself less selective for chloride by raising the pH would have increased the deviation. The observation that the zero current potential of Bis-Q6 was independent of pH was interpreted as strong evidence that this cation is quite impermeant and that the deviation of the zero current potential from E_{Cl} was artifactual.

Also displayed in Fig. 2 are the results of applying a similar protocol to characterize the permeation of MSA and CSA. Lowering the pH increased the permeability of colicin E1 to both of these monovalent ions, just as had been observed for chloride. Both the pH dependence and the absolute values of the zero current potentials of these two ions indicate that they are permeant. We caution that this criterion for permeance is somewhat more prone to error when applied to anions rather than cations. Because of the strong preference of colicin E1 to anions at low pH, contamination by the equivalent of 1 mM chloride, which would shift the zero current potential by only 0.5 mV at pH 5, would produce a 2 mV error at pH 4. At contaminant concentrations of 3 mM, the calculated error rises to 4.5 mV. In view of the apparent permeance of MSA or CSA, the glutaric acid used as a buffer in this system might be expected to be permeant and produce such a pH-dependent error. The observed pH dependence of the selectivity for these two ions is, however, much too large to be attributed to this kind of artifact.

From a variety of evidence, we were previously able to conclude that the colicin E1 channel is impermeable to cations with more than one positively charged group. As can be seen from the data in Fig. 3, this simple rule does not apply to anions. The zero current potential in EDSA gradients was 8 mV short of E_{Na} at pH 7 and the gap widened by an additional 14 mV as the pH was lowered to 4. On the basis of the data shown in Table 2, sulfate appears to be a good candidate for an impermeant anion, and indeed as the pH was raised above 5.0, the zero current potential did not shift. Lowering the pH, on the other hand, produced a marked shift away from E_{Na}, indicating a significant permeability to sulfate under these conditions.

In an attempt to examine the effect of the steric size of polyvalent anions on permeability, the zero current potentials of three larger but less soluble ions were measured. The complex between ferric ion and EDTA has a net charge of −1 and was the largest anion tested. Its geometric mean diameter is 8.9 Å, comparable to that of the largest permeable cations previously identified [11].

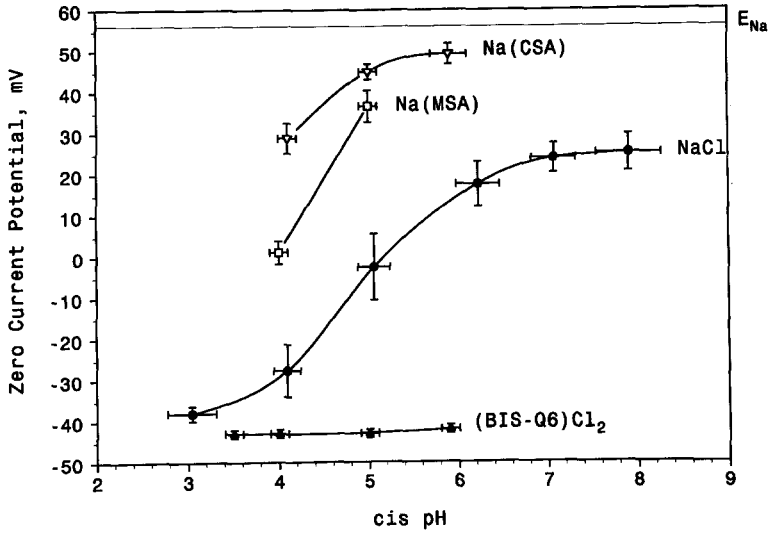


Fig. 2. The pH dependence of the permeability of colicin E1 in PE membranes to monovalent anions. Membranes were exposed to 1.0-*vs.* 0.1-M gradient of the salts indicated. Colicin E1 was added to the 1.0-M side of the gradient and the zero current potential determined. Individual membranes were subjected 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. Data for NaCl and (Bis-Q6)Cl_2 are reproduced from [11]. At pH 3, colicin E1 is quite selective for chloride over sodium (10.4:1), whereas at pH 8, this channel is moderately selective for sodium over chloride (2.3:1). The zero current potential in (Bis-Q6)Cl_2 was found to be near E_{Cl} and independent of pH, indicating that the Bis-$Q6^{+2}$ cation is impermeant. By these criteria, both MSA and CSA appear to be permeant. All solutions contained 3 mM calcium glutarate, 3 mM MES, and 3 mM bis-tris propane. Data are displayed as mean ± SD. The measured values of E_{Na} for all four of these salts were within 1 mV of the value displayed.

Naphthalene disulfonic acid (NDSA), like EDSA, has two negatively charged sulfonic acid groups. Naphthalene, however, is a larger parent molecule than is ethylene, and the charged groups are separated by a greater distance (11.6 *vs.* 9.3 Å). The trisulfonic acid of naphthalene, NTSA, was the only trivalent compound tested. Our initial finding was that we could observe no colicin activity whatsoever in PE membranes exposed to the sodium salts of either Fe(EDTA) or NDSA. Colicin activity was observed in the case of NTSA, but the currents were small, even at high protein concentrations. When examined in membranes composed of asolectin, however, the colicin activity was quite high in gradients of each of these salts. The results of these experiments, shown in Fig. 4, demonstrate that the selectivity of colicin E1 for these three compounds is virtually uniform. All appeared to be impermeant near pH 6, but anion permeability increased dramatically as the pH was lowered. Data for NaCl and (Bis-Q6)Cl_2 in asolectin membranes have been reproduced from reference [11].

Ion-ion Interactions

It is clear that none of the impermeant or poorly permeant test ions blocked the channel completely, since substantial currents carried by permeant counterions have always been observed in these studies. From these observations alone, however, it is not possible to conclude that impermeant ions have no effect of any kind on the movements of counterions. The fact that colicin E1 channels appear to be impermeable to an ion, furthermore, provides no information whatever about the effects of that ion on the movement of permeable ions of the same sign. Because colicin E1 conductance does not reach a steady state in planar membranes, even after a long time, effects of added compounds on channel conductance must be assessed on the single channel level. We examined a combination of two apparently impermeant ions, the anion sulfate and the cation Bis-T6, on the single channel conductance of colicin E1. In these experiments, colicin E1 was incorporated into PE membranes bathed in symmetrical solutions containing 1 M NaCl and buffered at pH 5.0. Single channel openings could be observed upon applying *trans* negative potentials of 80 mV or more. Under these conditions, closing events were rarely observed, even after prolonged application of *trans* positive voltages. For this reason, it was necessary to repeatedly reform the bilayer in order to accumulate channel opening data. The average conductance of the most prominent single channel state was found to be 9.8 ± 0.8 pS ($n = 53$) at −80 mV. In addition, six channels having conductances in the range from 2 to 7 pS were observed. Addition of Bis-T6 sulfate to both sides of the membrane had no effect on the single channel behavior. With Bis-T6 sulfate present at a concentration of 100 mM, the single channel conductance of colicin E1 was 9.6 ± 0.5 pS ($n = 60$). Four additional channels with conductances in the range of 2 to 5.5 pS were also observed. The results of these experiments

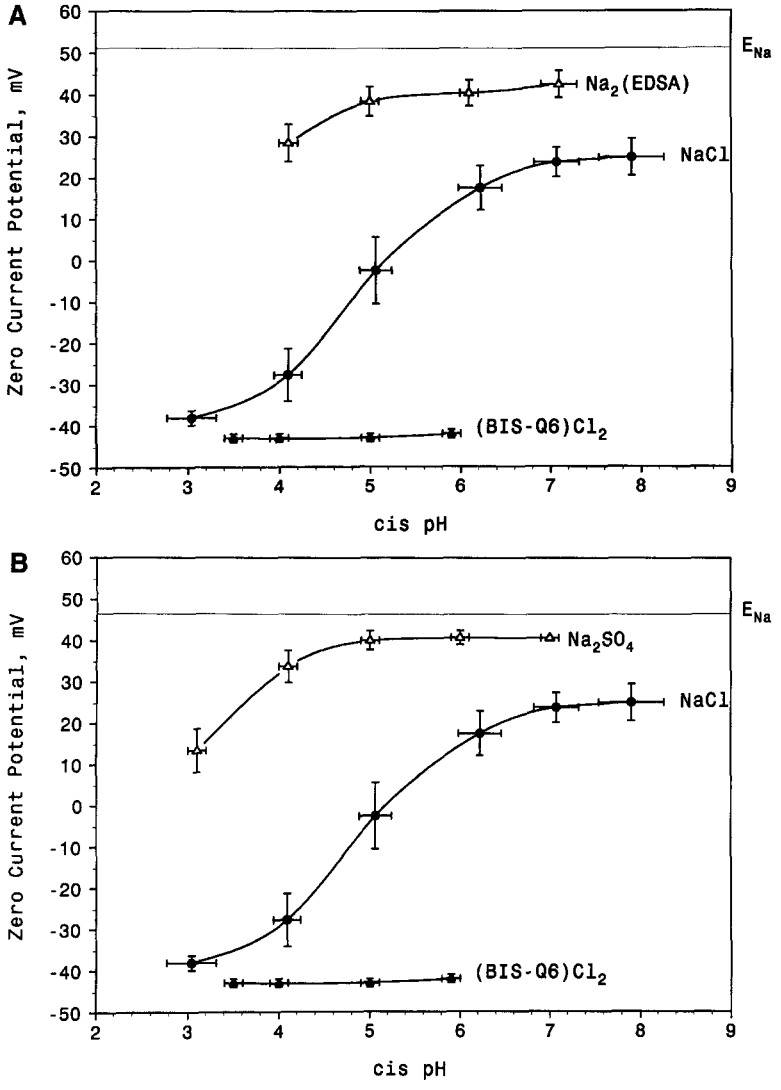


Fig. 3. The pH dependence of the permeability of colicin E1 in PE membranes to divalent anions. Membranes were exposed to 1.0- *vs.* 0.1-M gradient of the $Na_2(EDSA)$ in *A* and Na_2SO_4 in *B* as indicated. The displayed values of E_{Na} correspond to experimental measurements for these two test anions. Colicin E1 was added to the 1.0-M side of the gradient and the zero current potential 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. As in the previous figure, data for NaCl and $(Bis\text{-}Q6)Cl_2$ are reproduced from [11]. All solutions contained 3 mM calcium glutarate, 3 mM MES, and 3 mM bis-tris propane. Data are displayed as mean ± SD.

show that Bis-T6 sulfate is an inert electrolyte; that is, neither the cation, Bis-T6, nor the anion, sulfate, interfere with the movement of either Na^+ or Cl^- ions through the channel formed by colicin E1. It is important to note that if we had found that Bis-T6 sulfate reduced the single channel conductance of colicin E1, there would have been no way to distinguish which impermeant ion, Bis-T6 or sulfate, had the blocking activity, and which permeant ion, sodium or chloride, was being blocked.

Discussion

Identifying Permeant Species

The use of organic cations of different sizes as dimensional probes of channel structure is well established [2, 15, 17, 19, 27]. To be useful as a probe, an ion must be water soluble, must not possess detergent activity that might produce aggregation in aqueous solution or instability in membranes, and must be impermeant with respect to bare lipid bilayers. The preference of colicin channels for low pH conditions imposes an additional constraint on the choice of anion probes: they must be very strong acids which remain unprotonated down to pH 3. Phosphodiesters, including the phospholipids composing the bilayer, generally have pK_as in the range of 0–2 and thus are acceptable for use in these experiments. Because the secondary pK_a of the phosphate group is in the range from 5 to 7, monoesters of phosphoric acid, such as glucose-1-phosphate, could not be used in these studies. The use of carboxylic acids, which have pK_as in the region from 3 to 5, was also precluded. For this same reason, phosphatidyl serine could not be used as a membrane constituent. Asolectin has not been found to contain measurable amounts of phosphatidyl serine [18].

Sulfonic acids constitute a large group of useful compounds. Martell and Smith [23] cite a pK_a value for methane sulfonic acid of 1.92 at infinite dilution and

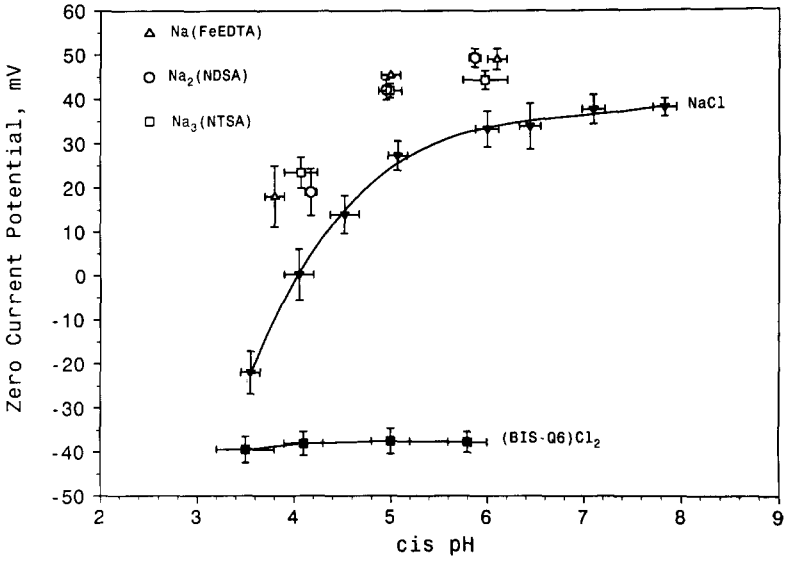


Fig. 4. The pH dependence of the permeability of colicin E1 in asolectin membranes to the divalent anion NDSA. Individual membranes were subjected to repeated titration of the *cis* pH over the entire range indicated, while the pH of the *trans* compartment was held at 5.0. Data for NaCl and (Bis-Q6)Cl_2 are reproduced from [11]. In the case of these two salts, membranes were exposed to 1.0- *vs.* 0.1-M gradients, and the zero current potential determined after adding colicin E1 to the 1-M side. Because the anionic lipids contained in asolectin favor the permeation of cations, these curves are shifted upwards when compared with those obtained in membranes composed of the neutral lipid, PE. For Na (FeEDTA) and Na_2(NDSA) the gradient was 0.30 versus 0.03 Molar, and for Na_3(NTSA) the gradient was ⅓ versus 1/30 Molar. For all three of these salts, the colicin E1 was added to the low concentration side of the gradient. All solutions contained 3 mM calcium glutarate, 3 mM MES, and 3 mM bis-tris propane. Data are displayed as mean ± SD. The experimentally determined values of E_{Na} were as follows: NaCl, E_{Na} = +56 mV; NaFe(EDTA), E_{Na} = +48 mV; Na_2NDSA, E_{Na} = +51 mV; Na_3(NTSA), E_{Na} = +48 mV.

25°C. This value is expected to decrease substantially as concentration is increased, typically by 1 pH unit at 1 molar. Electron donating substituent groups also serve to increase acid strength. the pK_a values for ethane and propane sulfonic acids are 1.68 and 1.53 respectively, and the value for sulfamic acid (amidosulfate) is 0.99 [23]. Fe(EDTA) is nearly as strong an acid as the sulfonic acids, having a pK_a of 1.3 [23]. The value of the pK_a for bisulfate anion (HSO_4^-) is 1.10 at a concentration of 1 M [23]. Since we expect the effective pK_a values for all of the acids used in this study to be less than 1.5, protonation of these compounds should be negligible at pH $\geqslant$ 3. If, for some reason, the pK values were shifted to higher values than expected, a much larger volume of the free acid used as the titrant would have been required to lower the pH, and no such behavior was observed.

The measured permeabilities of colicin E1 to the anions MSA, CSA, and EDSA were too large to be attributable to known artifacts. In the cases of the monovalent cations, MSA and CSA, this permeability increased sharply as the pH was lowered. While the pH dependence of the permeability of the divalent EDSA anion was less steep than that for CSA, the small increases observed between pH 7 and pH 5 were consistent and reversible. There is little room for doubt that colicin E1 channels are permeable to these three anions over the entire range of pH. Sulfate presents a different picture. At pH 5 and above, this anion appeared to be quite impermeant, since the measurements of zero current potential and E_{Na} were equal to within experimental error, and the size of the small gap between these two measurements was independent of pH. A large increase in anion permeability occurred only in the pH range between 3 and 4. As in the cases of the sulfonic acids, we have no reason to expect appreciable amounts to the monoprotonated species to be present at pH 3, and conclude that sulfate itself is able to permeate the colicin E1 channel, but only under conditions of low pH. The existence of special conditions in or near the channel which favor the formation of HSO_4^- remains a possibility, but the need for such a contrivance does not seem compelling.

The selectivity curves for the three test anions shown in Fig. 4 superficially resemble that of sulfate. Interpretation of this data is, however, less straightforward because of the complicating effects of the anionic lipid constituents of asolectin. This lipid mixture is an extract of soybeans containing 82.5% phospholipid, the remainder being made up of neutral lipids and glycolipids [18]. Phosphatidyl inositol and phosphatidic acid, which together make up 22.1% of the total mixture, impart a substantial fixed negative surface charge to asolectin membranes. As a result of electrostatic interactions with this fixed charge, the mobile anions of the bulk solution become depleted in the region near the membrane surface, and the cations become more concen-

trated. The cation selectivity of colicin E1 will therefore appear to be higher when the protein is incorporated into asolectin membranes than when it is in membranes composed of PE. Note that the NaCl selectivity curve for colicin E1 in asolectin membranes, shown in Fig. 4, appears to be shifted upwards in comparison with the data from PE membranes shown in the previous figures. The shift is not a simple translation. Zero current potentials cannot be shifted beyond the corresponding equilibrium potential for sodium, since doing so would violate thermodynamic constraints. The apparent agreement of the zero current and sodium equilibrium potentials at pH 6 is almost certainly the result of this limitation rather than lack of permeability. Even with this upward shift, these ions are clearly quite permeant at lower values of pH. The situation is further complicated by the effect of surface charge on the surface pH. Since protons are also cations, the actual values of the pH at both membrane surfaces will be lower than the values measured in bulk solution. On the *cis* surface of the membrane, this effect simply shifts the selectivity curves to the right. On the *trans* side of the membrane, on the other hand, reducing the pH increases the anion selectivity of colicin E1, so that negative surface charge produces a downward shift of the curves. As we have pointed out in previous publications [10, 11], attempts to account quantitatively for all these effects through the application of double layer theory have been unrewarding. It is not useful, therefore, to compare the properties of selectivity curves shown in Fig. 4 with those of the compounds tested in PE membranes. What is striking about the data of Fig. 4 is that the permeation properties of these three anions appear to be virtually identical to each other. While these sizes of these compounds are also approximately equal, their valences range from -1 to -3. Differences in the surface charge effects resulting from the unequal ionic strengths of the working solutions of these salts would not be large enough to mask any significant dependence of anion selectivity on the number of charged groups.

Not all of the effects of surface charge on pH are disadvantageous. As discussed above, asolectin contains significant amounts of phosphatidic acid. Thanks to the effects of surface charge, the bulk pH must be raised into the range from 8 to 9 in order to remove a second proton from the phosphate moiety of this lipid. Finally, we note that Raymond et al. [34] have previously reported that colicin E1 is permeable to NAD^{-1} at pH 4 in asolectin membranes. The identity of the actual permeating species is not completely clear, however. This pH may be sufficiently low to protonate the amino group on the adenine residue, forming significant amounts of neutral zwitterion. In addition, the identity of the acid used for pH adjustment was not identified, and the compound is known to be less stable at low pH. Because of these interpretational difficulties, along with the considerable expense of these experiments, we did not attempt to confirm this earlier finding.

All of permeability data are consistent in suggesting that the pore formed by colicin E1 is at least 9 Å in diameter at its narrowest point. In several other ways, however, the pattern of anion permeation contrasts sharply with that previously described for cations. Regardless of their size, cations of any size were impermeant if they carried more than one positively charged group. Polyvalent anions, on the other hand, were quite permeant. Calcium, which is approximately the same size as sodium, was much less permeant than sodium but more permeant than most of the large organic cations tested. Sulfate, which is only slightly larger than chloride, was nevertheless the only anion tested that appeared to be impermeant under any conditions. The implications of these patterns for the electrostatic environment within the channel are discussed below.

The Electrostatic Basis of Colicin Selectivity

As we mentioned in our Introduction, the greatest challenge presented by the ion conducting properties of colicin E1 lies not in explaining why such large ions are able to traverse the channel, but rather in explaining why small ions such as sodium and chloride cross it so slowly. In our previous study [11], we suggested that the limited cation conductance of colicin E1 is the result of severe restrictions on the number of locations within the channels at which ions can be effectively hydrated. According to this view, polyvalent cations are not so much impermeant as they are insoluble in the channel lumen; if one charged group were to occupy a site where it is effectively hydrated, all accessible locations for the other charge would be prohibitively high in energy. Polyvalent cations, then, should be excluded from the channel and, as a result, be completely inert. This behavior was observed in the case of Bis-T6. Another prediction of this model is that if the charged groups of a divalent cation were separated by a large enough distance, one charge could occupy a hydration site within the lumen while the other was allowed to remain outside the channel. Such compounds should block the movement of permeant cations such as sodium (*cf.* ref. 27). Since Bis-T6 exhibited no blocking activity, it appears that this critical distance is longer than six methylene groups. It is also possible that there is more than one cation hydration site within the colicin E1 channel. In this case, divalent ions whose charges can match the positions of the hydration sites should also block sodium movement. A systematic study of the blocking activity of ions having a wide ranger of chain lengths must be conducted in order to test these predictions.

Our present studies indicate that the movement of

anions through channels formed by colicin E1 is not subject to this sort of restriction. Neither the number of negatively charged groups nor the distance separating them had any clear influence on the permeance of the anions we examined. This is not particularly surprising, since the colicin E1 channel is, generally speaking, an anion-preferring channel. If the charge and dipole distributions within the channel are energetically favorable for cations only in a very few locations, one might expect anions to be fairly happy in most of the rest of the channel. The only ion included in this study that appeared to be impermeant under any conditions was inorganic sulfate. Since it was also the smallest probe tested, steric restrictions are clearly not the cause of sulfate's impermeance. Sulfate differs from the other anions of this study in that its two negative charges on this ion are delocalized among its four oxygen atoms. The high field strength of this ion greatly increases the electrostatic contributions to its free energy. If the colicin E1 channel were to contain an anion binding site (energy well), sulfate would be held much more tightly and transported much more slowly than an ion carrying a singly charged sulfonate group. Our finding that sulfate has no effect on the movement of either sodium or chloride suggests that the opposite is the case. Sulfate seems to be excluded from the channel because it encounters unfavorable and unavoidable electrostatic interactions along its route. Just as we have no evidence for more than one hydration site for cations, the existence of a single energy barrier along the reaction coordinate for anion transport would be sufficient to explain both sulfate's impermeance and its lack of blocking activity from either end of the channel. It is tempting to speculate that the anion barrier and the cation hydration site are mirror image effects of the electrostatic conditions at a single location in the channel. If the physical locations of these two functional features of the channel coincide, this specifically implies that anions cannot pass through this part of the channel when a cation is present in the site. In other words, the binding/barrier site and its associated water molecules must take up the entire cross section of the channel so that sodium and chloride, for instance, must take turns moving through it. Now that (Bis-T6)SO_4 has been identified as an inert electrolyte, it is possible to examine ion movements in the colicin E1 channel for such deviations from independence. Using Na_2SO_4 and (Bis-T6)Cl_2 as electrolytes, the sodium and chloride conductances of the colicin E1 channel can be measured separately. If sodium and chloride movements are independent, the total conductance of the channel to NaCl should simply be the sum of the conductances to sodium and chloride. If the ions must take turns going in opposite directions through a common site, the total conductance should be less than the sum. Should the total conductance be greater than the sum of two single ion conductances, it would indicate that the presence of sodium in the cation site lowers the barrier to chloride permeation and suggest that chloride can slip past sodium within the channel. Were it not for the low solubility of its sulfate salt, calcium would also be an interesting point of comparison.

From an experimental point of view, the porins of the outer bacterial membranes of gram-negative bacteria form a useful point of comparison with the colicins [31]. The porins are one of only two classes of membrane proteins for which high resolution x-ray diffraction structures have been obtained from ordered three-dimensional crystals [32]. The steric sizes of these pores determined from structure determinations are in good agreement with those obtained from sieving experiments. As discussed at length in our previous paper, simple theoretical considerations suggest that the presence of charged groups on the wall of a water-filled pore of this size will do little to slow the rate of ion transport or to confer selectivity. This assertion is supported by the structure and function of the porin channels. In the narrowest part of the lumen of the OmpF porin there are four basic and two acidic side chains arranged in a rigid ring structure. Measurements of the single channel conductances of the porins are nevertheless in good agreement with those calculated for featureless pores using simple macroscopic equations and experimentally determined dimensions. Porins, furthermore, exhibit only modest cation/anion selectivity [4]. The single channel conductance of the PhoE porin, which has two additional positive charges in the ring, is virtually identical to that of OmpF. The difference in cation/anion selectivity produced by these two additional charges is considerably less than the wide range exhibited by colicin E1 as a function of pH. Small ions appear to be able to pass down the center of porin channels, where they are insulated from effects of the charges on the walls by the dipoles of the intervening water molecules. The implications for colicins is that the arrangement of the water molecules within the channel inhibits the movement of small ions, or perhaps even forces them into a closer contact with the channel wall. We have previously pointed out that if the ion were to approach closely enough to the wall, charges, salt bridges, and dipoles buried within the channel structure could provide sufficient energy for effective hydration sites [11].

The general features of this picture are now supported by other evidence. Using saturation site directed mutagenesis of the prominent COOH terminal hydrophobic region of colicin E1, Song et al. [39] showed that this domain spans the bilayer twice as a helical hairpin loop. Because substitution of charged residues at particular locations in and near this loop altered the selectivity of colicin E1, these investigators have argued that this hydrophobic hairpin forms part of the channel lumen. A second helical hairpin, this one containing polar residues, has been shown to confer voltage dependence on the

channel and is also believed to be a part of the pore structure [1, 26]. Based all available data, including their own protease accessibility studies, Zhang and Cramer have concluded that there is no evidence that any of the other helical segments of the colicin E1 molecule contribute to the pore itself [48]. If this four helix model is correct, up to half of the surface of the lumen may be greasy. Not only would the nonpolar structure of the channel wall be unable to substitute for waters of hydration, but the water in contact with this hydrophobic surface would tend to take up a more extensively hydrogen bonded structure. It is easy to imagine that transported ions would migrate mostly along the wet side of the channel. Treating this problem theoretically will be very difficult, but perhaps not hopeless. There have been several attempts lately to use the Poisson-Boltzmann equation to perform a more sophisticated analysis of ion conduction through complex channel geometries [13, 30]. The differing hydration conditions within the channel can be modelled in such a formalism by allowing the dielectric constant to take on different values in different regions. If a more complete three dimensional structure for the pore becomes available, the problem may be approached more directly using molecular dynamics simulations. Unfortunately, it does not appear likely that a consensus structure will emerge in the near future. It is far from clear how any channel having an inside diameter of 9 Å can be assembled from a single colicin E1 molecule, even by allowing up to six helical segments to be involved.

Modulation of Selectivity by pH

We have previously established that colicin E1 can exist in at least two distinct conformational states having different selectivity properties, and that the distribution of the protein among these different states is influenced by the pH [6]. The major piece of evidence supporting this picture is that colicin E1 can be trapped in a distinct anion preferring conformation when incorporated into membranes composed of phosphatidyl choline. The selectivity effects produced by many site directed mutations may also be the result of altered protein folding [5, 20, 38–40, 42]. From our studies of the conditions under which conformational trapping occurs, we concluded that several different titratable groups located outside the channel lumen contribute to the stability of these conformational states. In addition, the selectivity of the anion-preferring form of channel is itself somewhat pH sensitive, probably as a result of acidic groups within or near to the ion conduction pathway [6]. Thus, while there may be only two distinct conformational states for colicin E1, the effect of pH on selectivity is not the result of a simple titration. Many acidic residues in different places on the protein influence the average conformational distribution and the average selectivity of each conformation. This model suggests a simple explanation for the behavior of sulfate: the anion-preferring state of colicin E1 channel is permeable to sulfate, but the other state or states are not able to conduct this ion.

It was hoped that the single channel behavior of colicin E1 might give some additional hints about the nature of this conformational modulation of selectivity, but the experimental observations are in dispute. In an early study, the single channel conductance of colicin E1 was reported to be independent of pH [34], leading those investigators to posit that the selectivity and the ion conduction rate of the channel were determined by different parts of the protein. A subsequent and more detailed study, however, reported opposite findings and came to the opposite conclusion [5]. While the single channel conductance data of this study comprised a broad distribution, the mean conductance at pH 4 was clearly reduced by approximately 40% from the mean value at pH 6. Chemical modification of the two histidine residues of the channel-forming region of the protein, furthermore, had the same effect on both selectivity and conductance as lowering the pH. These results suggest that low pH and histidine modification reduce the movement of sodium while leaving chloride movements relatively unaffected. It is possible to imagine that conformational changes or chemical modifications which disrupt the integrity of specific cation hydration (binding) sites within the lumen might produce this kind of results. If the cation site coincides with the barrier to anion movement, on the other hand, disrupting the structure should simultaneously decrease cation conductance and increase the conductance to anions. This possibility can now be examined directly using (Bis-T6)Cl_2 as an electrolyte to measure the chloride conductance of colicin E1 as a function of pH.

The Mechanism of Channel Formation

Under the influence of the membrane electric field, the colicin E1 protein is converted from a globular aqueous protein to a membrane-spanning channel structure. While a description of the conformational changes which accomplish this transformation is not the primary object of the present study, several of our findings bear indirectly on this problem. As we noted in our studies of cations, we consistently observed a much higher rate of channel formation by colicin E1 when the protein was added to the side of the membrane containing the lower concentration of salt. It is generally understood that conditions of high ionic strength tend to "salt in" polar groups and "salt out" apolar groups from aqueous solution. In sum, high salt conditions generally favor tightly folded structures. Since the colicin E1 molecule must become at least partially unfolded in order to be

inserted into the membrane, higher salt conditions might be expected to slow the rate of channel formation by stabilizing the aqueous form of the molecule relative to its translocation competent state. In symmetrical salt conditions, however, Cohen (*personal communication*) has observed higher rates of channel formation at higher ionic strengths. The behavior we observe in salt gradients suggests that this increase in the rate of channel formation is an effect of the high ionic strength on the *trans* side of the membrane, perhaps the result of stabilizing a cytoplasmic domain of this protein. Raymond and her colleagues have shown that in planar bilayers a large portion of the colicin E1 protein not directly involved in the channel structure crosses the membrane when the channel is formed [35, 41]. If this is the origin of the ionic strength effect, it should be greatly reduced in the channel forming C-terminal peptide of colicin E1.

In addition to this general effect of ionic strength, we have noted two effects on channel formation which appear to be specific to certain salts. First, the difference in colicin activity in high and low salt concentrations was markedly greater than usual in the case of CSA. When colicin E1 was added to 1 molar side of the gradient, activity was very low, so low that it became difficult to measure the zero current potential. One possible explanation for this behavior is that CSA acts as a denaturant, similar in action to guanidinium chloride. Such an effect could weaken the hydrophobic bonds involved in binding the protein to the membrane prior to its insertion. Even more puzzling was the behavior of colicin E1 in NDSA and Fe(EDTA) solutions. In PE membranes bathed with solutions of these salts, we were not able to observe any channel formation at all; high concentrations of protein failed to induce formation even of single channels. Our initial hypothesis was that these ions blocked the channel, and we were surprised to find that the rate of channel formation was extremely high when asolectin was used to form the bilayers. What these two salts have in common is their low water solubility; the 0.3 M working solutions were nearly saturated. NTSA is somewhat more soluble and we were able to observe colicin activity in PE membranes bathed with solutions of this salt. The activity, however, was too low to produce reliable values for the zero current potential as a function of pH. It is possible that the combination of these sparingly soluble salts and PE produces bilayers that are simply refractory to the insertion of extrinsic molecules.

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