

Cationic Selectivity and Competition at the Sodium Entry Site in Frog Skin

DALE J. BENOS, LAZARO J. MANDEL, and SIDNEY A. SIMON

From the Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710, and the Department of Physiology, Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT The cation selectivity of the Na entry mechanism located in the outer membrane of the bullfrog (*Rana catesbeiana*) skin epithelium was studied. This selectivity was determined by measuring the short-circuit current when all of the external sodium was replaced by another cation and, also, by noting the relative degree of inhibition that the alkali metal cations produced on Na influx. The ability of the Group Ia cations to permeate the apical membrane was determined from the tracer uptake experiments. The results demonstrate that (a) only Li and Na are actively transported through the epithelium; (b) the alkali cations K, Rb, and Cs do not enter the epithelium through the apical border and, therefore, Na and Li are the only alkali cations translocated through this membrane; (c) these impermeable cations are competitive inhibitors of Na entry; (d) the cations NH₄ and Tl exhibit more complex behavior but, under well-defined conditions, also inhibit Na entry; and (e) the selectivity of the cation binding site is in the sequence Li \approx Na > Tl > NH₄ \approx K > Rb > Cs, which corresponds to a high field strength site with tetrahedral symmetry.

INTRODUCTION

The determination of ion selectivity sequences for electrolyte permeation pathways through biological membranes has proved valuable for deducing certain stereochemical characteristics of ligands involved in ion translocation (Hille, 1972 and 1975; Szabo et al., 1973; Moreno and Diamond, 1974; Wright and Diamond, 1977). Knowledge of the chemical constituents of a particular transport entity can provide insight into the molecular process of ion translocation. Recently, much research has been devoted to elucidating the molecular mechanism of the facilitated entry step of sodium across the apical membrane of epithelial tissues, in particular, isolated frog skin. This entry process for sodium displays saturation kinetics and is specifically inhibited by the diuretic compound amiloride (Benos et al., 1976).

A striking property of the Na-entry mechanism in frog skin is its apparent high degree of selectivity, as reflected in its permeability to Na, and its seeming impermeability to K. Lindley and Hoshiko (1964) first addressed the problem of cation selectivity in frog skin by measuring the changes in open circuit (zero current) transepithelial potential as a function of ionic composition in the

outer medium. The underlying assumption was the same one made by Koefoed-Johnsen and Ussing (1958), namely, that changes in ionic composition in one solution elicit changes in the potential of the membrane facing that solution only. These authors concluded that the selectivity sequence was $\text{Na} > \text{Li} > \text{Rb} > \text{K} > \text{Cs}$ for the outer surface of the bullfrog skin. In the ensuing years, numerous investigators questioned the basic assumption of this study because ionic substitutions of the outer solution lead to complex changes in the potential distribution across the skin under open circuit conditions (Finn, 1974; Reuss and Finn, 1975; Nagel, 1976 and 1977 *b*). Given these complications, we decided to reinvestigate the cation selectivity of the Na-entry site by a more direct method. The cation selectivity of the Na-entry pathway was assessed in two ways: (*a*) by comparing the short-circuit currents (I_{sc}), measured when all of the external sodium was replaced by another cation, and (*b*) by comparing the I_{sc} and net Na fluxes at various $[\text{Na}]_o$, using various replacement cations to maintain the ionic strength. We found that of the Group Ia cations, only Li and Na can enter the cells through the apical entry site. Also, K, Rb, Cs, and, under certain conditions, NH_4 and Tl, are competitive inhibitors of Na entry, with a selectivity order commensurate with binding to a high field strength site.

MATERIALS AND METHODS

Ventral skins obtained from the bullfrog (*Rana catesbeiana*) were mounted and equilibrated in Ussing chambers under short-circuit conditions. Transepithelial open-circuit potential and zero-potential (short circuit) current (I_{sc}) were measured with a four-electrode, automatic voltage-clamping device. Dilution potentials were compensated as described by Mandel and Curran (1973). The bullfrogs were obtained from Jacques Weil Co., Rayne, La.; only male frogs were used.

The composition of the solution bathing the inner surface of the preparation was 110 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl_2 , and 5.0 mM Tris, buffered to pH 8.4. The inner surface of the frog skin was always exposed to this solution. The control solution (110 mM Na), which bathed the outside surface of the skin, was identical in all respects, except that it did not contain potassium. Solutions of choline, cesium, rubidium, potassium, ammonium, lithium, and thallium were made up by substituting equimolar concentrations of the chloride salts of these cations for NaCl (in the case of Tl, however, nitrate was the replacement anion in both bathing solutions). Solution changes were made on the outside only. All solutions were continuously bubbled with air and had a pH of 8.4 at room temperature (20°C). In the partial Na replacement experiments, each skin was sequentially exposed to four solutions per test cation, each solution containing a different ratio of $[\text{Na}]:[\text{Test Cation}]$, (in mM): 110:0; 20:90; 6:104; and 0:110. The I_{sc} observed at every $[\text{Na}]$ was then normalized to that observed when $[\text{Na}]_o = 110$ mM. The results are presented as the mean value \pm one standard error of the mean.

Transepithelial ^{22}Na influxes were measured under conditions in which 90 mM of the external Na was replaced by choline, potassium, cesium, ammonium, or thallium. The influx determinations were performed as described elsewhere (Benos et al., 1976).

The distribution of sodium (^{22}Na), potassium (^{42}K), rubidium (^{86}Rb), and cesium (^{137}Cs) within the skin after exposure from the external solution was measured in comparison to that of [^3H]mannitol, a known extracellular marker for frog skin (Biber et al., 1972; Cala et al., 1978). These experiments were performed in the presence of

either 110 mM of the replacement cation or 55 mM Na and 55 mM replacement cation in the external solution. After equilibration in the appropriate external solution, 1 μCi of the cationic tracer and 4 μCi of [^3H]mannitol were added to the external medium. After a 15-min exposure, the external solution was removed, the apical surface of the skin was briefly (<5 s) flooded with tracer-free medium, the chamber was rapidly dismantled, and the skin was removed. The exposed area of the skin was quickly excised with a metal punch, blotted, and placed directly in a scintillation vial. The piece of tissue was then dissolved in 1 M NaOH (1 ml) for 24–48 h, neutralized with HCl, and counted after the addition of 10 ml of scintillation fluid. Before chamber dismantling, the samples were removed from both bathing media for counting. The distribution spaces for each of the tracers were calculated according to the method described by Cala et al. (1978). All isotopes were purchased from New England Nuclear, Boston, Mass.

RESULTS

Cationic Selectivity for Translocation

The cationic selectivity of the skin was evaluated by transepithelial I_{sc} and Na flux measurements. Due to the complex morphology of the tissue, it was essential initially to establish that all ionic selectivity measurements pertained to properties of the same transport step. This necessity is illustrated by the results from experiments in which all external sodium was replaced by a test cation (Table I). The I_{sc} 's measured under these conditions were normalized to that observed during previous equilibration in external 110 mM sodium. In skins obtained from 28 animals, the absolute value of I_{sc} averaged $46.0 \pm 3.3 \mu\text{A}/\text{cm}^2$. As can be seen from Table I, only external lithium is actively transported across this epithelium at a rate comparable to Na, as others have found (Zerahn, 1955; Candia and Chiarandini, 1973). The other alkali metal cations abolish I_{sc} . The small remaining I_{sc} is amiloride insensitive and not different from what it is in the presence of external choline or even in distilled water.

Which step or barrier in the transepithelial cationic active transport system does not permit the transport of these alkali metal cations? Active transepithelial Na transport in frog skin may be understood as a process that involves two steps: passive Na entry through the apical border into epithelial cells followed by active extrusion through the basolateral side (Koefoed-Johnsen and Ussing, 1958). Therefore, the question is, can these alkali metal cations not be transported because they are unable to permeate the apical border or because they inhibit the Na pump once they enter the epithelial cells?

The second series of experiments measured the distribution space of each of the alkali metal cations (except lithium) when it was present in the external solution. The spaces were determined under conditions of either total cationic replacement or partial replacement of 55 mM NaCl by the appropriate alkali salt. Simultaneously, [^3H]mannitol spaces were determined as a measure of extracellular space (Biber et al., 1972; Cala et al., 1978). The results, shown in Table II, demonstrate that Cs, Rb, and K have distribution spaces not significantly different from the external extracellular space; these spaces do not change when active Na transport occurs, as shown in the partial replace-

ment experiments. In contrast, Na has a much larger distribution space, as expected of a substance that penetrates the cellular compartment. These results demonstrate that the I_{sc} is inhibited in the presence of total external replacement by Cs, Rb, and K because these cations cannot permeate the apical border of bullfrog skin.

TABLE I
THE EFFECT OF TOTAL REPLACEMENT OF
EXTERNAL SODIUM WITH VARIOUS CATIONS
ON I_{sc} OF ISOLATED BULLFROG SKIN

External condition	<i>n</i>	Normalized I_{sc} (\pm SEM)
		%
Sodium		100
Lithium	20	113 \pm 11
Potassium	28	3.7 \pm 0.5
Rubidium	28	3.3 \pm 0.4
Cesium	28	3.8 \pm 0.5
Choline	28	3.1 \pm 0.4
Distilled H ₂ O	16	3.2 \pm 0.6
Ammonium*	14	3.1 \pm 0.2
Ammonium‡	6	27 \pm 3
Thallium	8	72 \pm 17

Measurements were made ~5 min after solution change.

* Population A.

‡ Population B.

TABLE II
DISTRIBUTION SPACES OF ALKALI METAL CATIONS AND [³H]MANNITOL
WHEN APPLIED TO THE EXTERNAL SURFACE OF THE BULLFROG SKIN FOR
15 MIN

External solution	Cationic isotope	Cationic space	Mannitol space	<i>P</i>
		μ l/cm ²	μ l/cm ²	
110 mM CsCl	¹³⁷ Cs	0.33 \pm 0.05	0.34 \pm 0.09	>0.9
55 mM CsCl + 55 mM NaCl	¹³⁷ Cs	0.38 \pm 0.09	0.25 \pm 0.02	>0.2
110 mM RbCl	⁸⁶ Rb	0.29 \pm 0.05	0.28 \pm 0.04	>0.8
55 mM RbCl + 55 mM NaCl	⁸⁶ Rb	0.24 \pm 0.05	0.27 \pm 0.04	>0.6
110 mM KCl	⁴² K	0.23 \pm 0.10	0.28 \pm 0.13	>0.8
55 mM KCl + 55 mM NaCl	⁴² K	0.25 \pm 0.08	0.17 \pm 0.05	>0.3
110 mM NaCl	²² Na	1.18 \pm 0.24	0.42 \pm 0.07	<0.01

Each set of experiments was performed in eight skins.

In the study of active transepithelial Na transport in frog skin, several investigators have presented evidence that the entry step into the epithelial cells is rate-limiting (Biber, 1971; Mandel and Curran, 1973; Helman and Fisher, 1977; Mandel, 1978). Therefore, the I_{sc} reflects the Na transport properties of that barrier, and alterations of these transport properties will be

reflected in changes in the I_{sc} . The permeation of Li through the entry site was studied by Morel and Leblanc (1975), who found the rate to be similar to that for Na. Therefore, we can write the selectivity order of the Group Ia cations for translocation through the apical pathway as $Li \approx Na \gg K, Rb,$ and Cs. Because our techniques can differentiate transport at the 1% level (Table I), Na and Li must be at least 100 times more permeant than K, Rb, and Cs.

Cationic Selectivity for Inhibition of Na Entry

Inasmuch as only Na and Li can be transported by the apical entry mechanism, measurements were made to assess the ability of other monovalent cations to inhibit this translocation process. This method compared the currents observed at 20 and 6 mM $[Na]_o$ using various test cations as replacement ions. Initial experiments were performed to establish that the I_{sc} represented active Na transport under these experimental conditions. Trans-epithelial ^{22}Na influxes were measured under short circuit conditions in the presence of 20 mM external Na, using choline, K, and Cs as replacement cations. These fluxes were measured on the same frogs in the sequence indicated above. As shown in Table III, the Na influxes are not significantly different from the I_{sc} , demonstrating that the I_{sc} measures active Na transport under all these conditions. The magnitude of the I_{sc} in the presence of K and Cs as compared with that in choline should not be interpreted quantitatively because the I_{sc} decays as a function of time in prolonged flux experiments of this sort; the relevant parameter is the comparison between the simultaneously measured fluxes and currents. These measurements were not performed in the presence of Rb. We assume that the same considerations apply to Rb as a replacement cation.

Experiments were next performed to quantitatively evaluate the effects of these replacement cations on I_{sc} in the presence of constant external Na concentrations of 20 and 6 mM. The experiments, summarized in Table IV, were initiated with 110 mM Na in the external solution to serve as the reference I_{sc} . The I_{sc} observed at 20 mM Na ($66.1 \pm 1.0\%$), with choline substitution for sodium, represent the control values of Na current to which the currents observed with the other cations are compared. This is predicted upon the observation that the I_{sc} measured at varying $[Na]_o$ is the same when determined either at constant ionic strength (with choline) or at varying ionic strength (Mandel and Curran, 1973). We have verified this finding for *R. catesbeiana* skin (Fig. 1). From Table IV, it may be observed that the other alkali metal ions inhibit the I_{sc} . When the percent inhibition of I_{sc} is calculated with respect to choline, a sequence of inhibitory action may be determined, which shows that Cs inhibits the least and K inhibits the most.

Because it has been shown that external K is a competitive inhibitor of Na entry (Rotunno et al., 1970; Mandel and Curran, 1973; Benos et al., 1979), we assumed that K competes with Na for binding at a common site. The same assumption may be made with respect to Rb and Cs, since these cations also inhibit Na entry without themselves entering into the cytoplasm from the external solution. Competitive inhibition may be treated quantitatively with

the use of Michaelis-Menten kinetics, the resulting equation being (Segel, 1975)

$$I_{sc} = \frac{I_{max}[Na]_o}{K_t \left(1 + \frac{[I]_o}{K_i} \right) + [Na]_o}, \quad (1)$$

TABLE III
TRANSEPTHELIAL ^{22}Na INFLUXES ACROSS
ISOLATED BULLFROG SKIN IN THE PRESENCE OF
VARIOUS EXTERNAL REPLACEMENT CATIONS

Replacement cation (90 mM)	<i>n</i>	\bar{J}_{Na} (\pm SEM) $\mu\text{eq}/\text{h}\cdot\text{cm}^2$	I_{sc} (\pm SEM) $\mu\text{eq}/\text{h}\cdot\text{cm}^2$
Choline	8	0.94 \pm 0.14	0.94 \pm 0.18
Potassium	8	0.40 \pm 0.05	0.42 \pm 0.05
Cesium	8	0.41 \pm 0.07	0.41 \pm 0.07
Ammonium*	4	0.04 \pm 0.002	0.04 \pm 0.004
Ammonium‡	4	0.07 \pm 0.01	0.12 \pm 0.02
Thallium	8	0.15 \pm 0.01	0.39 \pm 0.04

In all of these experiments the external bathing solution contained 20 mM Na.

* Population A. See text for details.

‡ Population B. See text for details.

TABLE IV
THE EFFECT OF EXTERNAL CATION SUBSTITUTION ON I_{sc} OF ISOLATED
BULLFROG SKIN

Replacement cation	<i>n</i>	[Na] _o	Normalized I_{sc} (\pm SEM)	Inhibition of I_{sc} (\pm SEM)	K_t or K_i (\pm SEM)	ΔG_j°
		<i>mM</i>	%	%	<i>mM</i>	<i>kcal/mol</i>
Choline	8	20	66.1 \pm 1.1	0	10 \pm 1	
		6	31.4 \pm 1.0	0	13 \pm 1	-2.6*
Cesium	8	20	53.1 \pm 1.9	20 \pm 3	168 \pm 45	
		6	22.4 \pm 1.4	29 \pm 6	131 \pm 27	-1.1
Rubidium	8	20	44.4 \pm 1.4	33 \pm 2	76 \pm 9	
		6	16.0 \pm 0.9	49 \pm 3	61 \pm 6	-1.6
Potassium	8	20	34.6 \pm 1.2	48 \pm 2	39 \pm 3	
		6	11.6 \pm 0.8	63 \pm 3	36 \pm 3	-1.9
Ammonium‡	4	20	32.6 \pm 1.1	52 \pm 2	35 \pm 3	-1.9
Thallium	8	20	25.8 \pm 2.2	61 \pm 3	22 \pm 4	-2.2

Measurements were made 5 min after each solution change was completed.

* This is the ΔG_j° for Na, not choline.

‡ Population A. See text for details.

where I_{max} is the maximal I_{sc} observed at high $[Na]_o$, K_t is the $[Na]_o$ at which $I_{sc} = 0.5 I_{max}$ in the absence of an inhibitor, $[I]_o$ is the external concentration of the inhibitor, and K_i is the concentration of inhibitor that decreases I_{sc} to one-half of its original, uninhibited, value. In Table IV, values for K_t calculated for 20 and 6 mM Na in the presence of choline (absence of inhibitors) are 10

± 1 mM and 13 ± 1 mM, respectively. Utilizing the average of these two values ($K_t = 11.5$ mM) in Eq. 1, we can calculate values of K_i for each of the other replacement cations at both external Na concentrations. If the replacement cations are indeed competitive with sodium, the value of K_i should be independent of the external Na concentration, as is the case within experimental error (Table IV).

Since the translocation of Na is rate determining, K_t and K_i represent equilibrium dissociation constants for the Na entry site (Mandel, 1978). The Gibbs free energy of binding for each cation may be calculated from

$$\Delta G_j^{\circ} = RT \ln (K_j), \quad (2)$$

where R is the gas constant, T the absolute temperature, and K_j is the equilibrium dissociation constant for ion j . The ΔG_j° values calculated from

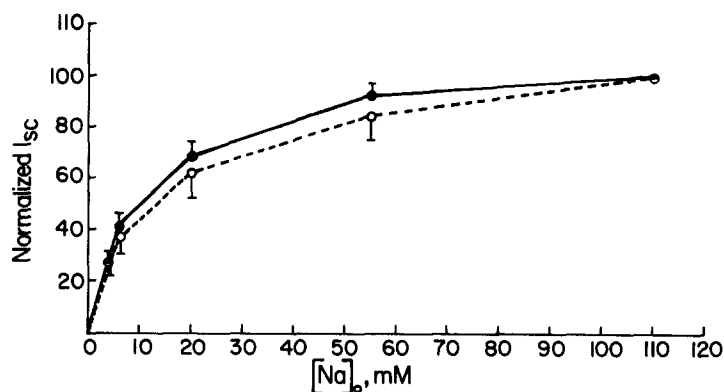


FIGURE 1. Plot of normalized I_{sc} vs. external Na concentration under conditions of choline substitution (closed circles) and dilute Na without substitution (no maintenance of external ion strength, open circles). Recasting these data as Eadie plots allowed the calculation of $K_t = 9.2 \pm 1.9$ mM and $I_{max} = 106 \pm 2$ μ A ($r^2 = 0.98$) for the choline substituted skins and $K_i = 8.9 \pm 1.4$ mM and $I_{max} = 99.1 \pm 5.6$ μ A ($r^2 = 0.94$) for the skins in dilute Na. These experiments, performed on eight skins of *R. catesbeiana*, show that choline substitution does not significantly affect the I_{sc} or K_t in this preparation.

Eq. 2 provide a relative selectivity sequence on the basis of binding free energy. Thus, the binding sequence determined from Table IV for the Group Ia cations is $Na > K > Rb > Cs$. The other alkali metal ion, lithium, is not amenable to this treatment because this ion can itself be transported.

We also attempted to determine the position of ammonium (NH_4) and thallium (Tl) in the selectivity sequence to derive additional information concerning ligand type and symmetry within the apical entry site. Experiments similar to those performed with the alkali metal ions were performed with NH_4 and Tl with mixed results, as is shown below. In 14 of the 20 skins in which all of the external Na was replaced with NH_4 (Table I), the resultant current was identical to that observed when the impermeant cations were present (3.1%), suggesting that NH_4 was not transported through this popu-

lation of skins (denoted population A). The normalized I_{sc} of the six other skins averaged 27% (range, 32.1–18.6%) under identical conditions, indicating that NH_4 was transported through this population of skins (denoted population B). The presence of two populations of frog skin was again evident as the unidirectional ^{22}Na influx (J_{Na}^i) was measured when 90 mM external Na was replaced with NH_4 (Table III). In four of eight skins, J_{Na}^i ($3.1 \pm 0.2 \mu\text{A}/\text{cm}^2$) equaled I_{sc} ($3.4 \pm 0.4 \mu\text{A}/\text{cm}^2$), and both J_{Na}^i and I_{sc} were reduced to zero with 10^{-4} M amiloride, demonstrating that all the I_{sc} was due to Na transport. The behavior of these skins may be equated to that of population A, since no NH_4 was transported. In these skins, the presence of NH_4 inhibited the I_{sc} to a normalized value of 32.6% (Table II). In the other four experiments with 20 mM Na and 90 mM NH_4 in the external solution, I_{sc} exceeded J_{Na}^i by $5.1 \pm 0.5 \mu\text{A}/\text{cm}^2$, indicating that 42% of the current was carried by other ions, most likely NH_4 . This behavior is similar to that of population B. It is noteworthy that the excess I_{sc} over J_{Na}^i was not amiloride sensitive, whereas J_{Na}^i itself was totally abolished by external amiloride. This result suggests that, whichever ions are transported under these conditions, this transport (population B) is amiloride insensitive and, thus, likely to occur through a pathway separate from the normal Na-entry pathway. The results obtained with this population are, therefore, not relevant to the present selectivity sequence. Conversely, the results obtained with population A, demonstrating inhibition of I_{sc} , with no transport of NH_4 , fulfill the requirements for interpretation on the basis of inhibitory ability. Using this population, we fit NH_4 into the sequence at the same level as K, i.e., $\text{Na} > \text{NH}_4 \approx \text{K} > \text{Rb} > \text{Cs}$.

The results obtained with Tl were unusual because the I_{sc} was 72.5% of the control value (Table I) when Tl completely replaced Na, whereas at 90 mM Tl (20 mM Na), Tl acted as an inhibitor of Na entry (Table II). Experiments measuring ^{22}Na influx revealed that replacement of 90 mM Na with Tl in the external solution produced a long-term (90-min) transient in the I_{sc} , initially inhibiting the current (these are the values reported in Table II) and, thereafter, steadily increasing it to 166.7% of the initial I_{sc} . J_{Na}^i could not be measured during the initial inhibition, but, after 30–60 min of exposure to 90 mM Tl–20 mM Na, I_{sc} exceeded J_{Na}^i by a factor of 2.6 ($32.6 \pm 3.3 \mu\text{A}/\text{cm}^2$ and $12.7 \pm 1.3 \mu\text{A}/\text{cm}^2$; $n = 8$, respectively, Table III). Under these conditions, even J_{Na}^i became amiloride insensitive. The unusual behavior of Tl in this tissue is not surprising in view of the multiple effects it has in other preparations (see Discussion). Despite these complex effects of Tl, it may still be possible to assign its location in the selectivity sequence for the apical entry step. This may be accomplished by assuming that the initial decrease in I_{sc} (Table II) is due to competitive inhibition of Na entry. This assumption was made because the initially inhibited current is amiloride sensitive. Thus, under these conditions, the I_{sc} provides an upper bound for Na current. Using this criterion, we obtain a selectivity sequence for competition with Na entry: $\text{Na} > \text{Tl} > \text{NH}_4 \approx \text{K} > \text{Rb} > \text{Cs}$.

DISCUSSION

The ability to discriminate among monovalent cations has been elegantly described by Eisenman (1962) in terms of the interplay between dehydration

energies and electrostatic interactions with the selectivity site. An important prerequisite for the application of this theory is that the selectivity properties that are actually measured pertain to a single site. Much of the present effort was devoted to ascertaining that the selectivity for transepithelial active Na transport resides at a single site within the apical border of the bullfrog skin. This analysis is predicated on three considerations: (a) only Na and Li permeate through the apical border; (b) the current measured in the presence of replacement cations remains a Na current; and (c) these cations are competitive inhibitors of Na transport.

In terms of the first consideration, the classical experiment of Koefoed-Johnsen and Ussing (1958) suggested that the apical border was permeable to Na but impermeable to K in *Rana temporaria*. Recent results from Hirschmann and Nagel (1978) and Zeiske and Van Driessche (1978) indicate that a small permeability to K is present in these species of frog in the absence of external Na; this K permeability is, however, not amiloride sensitive, indicating that it occurs through another pathway. No evidence for such a pathway was found in bullfrog. Our results (Table II) demonstrate that the distribution of K, Rb, and Cs is extracellular when these ions are present in the external solution. The total cationic replacement experiments (Table I) show that the I_{sc} is inhibited in external distilled H₂O and choline to the same extent that it is in K, Rb, or Cs. The small remaining current was not amiloride sensitive and, thus, was unlikely to have originated from residual Na transport. This current was probably not due to net ionic movements through the paracellular shunt pathway either, even though the skin was bathed in asymmetrical solutions. This possibility can be excluded because there is a net inward current when distilled H₂O is on the outside of the skin, and there is no difference in I_{sc} when the outside is bathed in 110 mM choline, Rb, K, or Cs, molecules of widely differing free-solution mobility and radii. The origin of this remaining I_{sc} is unknown. One possible explanation is that it is due to Cl leaving the epithelial cells through the apical border and residual cellular Na being transported through the basolateral side, since this current is abolished by serosal ouabain (results not shown).

The second consideration is clearly satisfied by the results shown in Table III, i.e., the measured Na influxes are not significantly different from the I_{sc} 's with any of the cationic replacements. The Na influxes do contain a component that transverses the paracellular pathway (Mandel and Curran, 1972); however, this component is small and probably hidden within the standard errors.

Finally, on the basis of the formalism of Michaelis-Menten kinetics, the other alkali metal ions have been shown to compete with Na for binding at its entry site (Table IV). Various laboratories have demonstrated that Na entry saturates as a function of external Na concentration (Kirschner, 1955; Cerejido et al., 1964; Rotunno et al., 1970; Biber and Curran, 1970; Moreno et al., 1973; Erlij and Smith, 1973; Rick et al., 1975; Mandel, 1978) in a manner describable by Michaelis-Menten kinetics. The application of this kinetic description and its justification for use on Na transport in frog skin have been discussed at length in several publications from our laboratory (Benos et al., 1976 and 1979; Mandel, 1978).

Two major types of molecular interpretations have been advanced to explain the observed saturation behavior of sodium entry on frog skin. In the first of these, saturation occurs via a direct interaction of Na and its entry site, whether this site be a carrier (Biber and Sanders, 1973) or a pore (Heckmann et al., 1972). The second interpretation is that the saturation behavior of Na entry occurs by the self-inhibition of Na transport at a modifier site (Lindemann and Voute, 1976; Fuchs et al., 1977). The latter authors have suggested that saturation was not a property of the individual Na entry units themselves but, rather, a result of Na interacting with another site in such a way as to close the entry pathways.

Our experiments cannot differentiate between these two molecular interpretations. However, the kinetic interaction between Na and amiloride has been shown to be different in *Rana temporaria* and *Rana esculenta* (Lindemann and Voute, 1976; Fuchs et al., 1977) as compared with *Rana pipiens* and *R. catesbeiana* (Benos et al., 1979). In *R. temporaria* and *R. esculenta*, Na and amiloride are competitive. Because of this property, Lindemann and Voute (1976) have proposed that the modifier site is also the locus of amiloride's inhibitory action. In our epithelial preparations, amiloride and Na interact noncompetitively, suggesting that they act at separate sites (Benos et al., 1979). This conclusion has been further substantiated in recent studies using site-specific reagents (Benos et al., 1980). Although it is possible that Na acts at a modifier site distinct from the amiloride modifier site, the simplest explanation for these data is that amiloride acts at a modifier site and Na interacts directly with the Na translocation site. Thus, in *R. pipiens* and *R. catesbeiana*, saturation could occur by a direct interaction of Na with the entry sites. The observed competitive inhibition of Na entry by other monovalent cations can thus be interpreted as resulting from the direct interaction of these cations at the Na-translocation site.

The concept of inhibition may be easily understood in terms of the binding energy of the site for the other cations. The selectivity of inhibition is identical to the binding selectivity, that is, the tighter the binding, the more effective the inhibition is. Binding of a competing cation prevents the binding of sodium to its site. These effects of the competing cations manifest themselves in an increased effective $K'_t = K_t (1 + [I]_o/K_i)$, which cause a decrease in the average binding energy for sodium.

The observed selectivity for the Group Ia cations to the Na binding site ($\text{Na} > \text{K} > \text{Rb} > \text{Cs}$) is that of either sequence X or XI of Eisenman (1962), depending on the position of Li. As shown in Table I, Li is itself transported through the apical border. However, Leblanc (1972) and Nagel (1977 *a*) found that its presence in the external solution causes Li accumulation in the epithelial cytoplasm that is often accompanied by the presence of large transients in the I_{sc} and decreases in intracellular potential (Nagel, 1977 *a*). Thus, although an inhibitory K_i has been calculated for Li on Na entry (Biber and Curran, 1970), its interpretation has been questioned by Nagel (1977 *a*). It may be argued that Li binds to the entry site with comparable energy to that of Na, since their rates of transport into the cell are similar (Morel and

Leblanc, 1975). On this basis, we tentatively assign the following place to Li on the selectivity sequence for the binding site: $\text{Li} \approx \text{Na} > \text{K} > \text{Rb} > \text{Cs}$, which falls between sequences X and XI. Either sequence describes a high field strength site which presumably causes partial or total dehydration of the cations. It should be noted that this selectivity sequence is inversely related to ionic radius, and, therefore, the selectivity may also be the result of steric hindrance rather than electrostatic interactions.

Somewhat more information regarding the molecular architecture of this site may be obtained from the behavior of NH_4 and Tl. To investigate whether the selectivity of this site strictly follows the inverse of the ionic radius, it is informative to plot the binding energy vs. ionic radius for all the replacement cations, as done by Moreno and Diamond (1974). As shown in Fig. 2, the binding energy for the alkali metal ions does vary inversely with the ionic

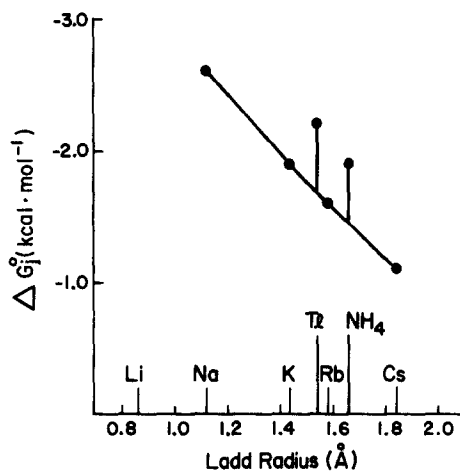


FIGURE 2. Free energy of binding as a function of Ladd radius (Ladd, 1968) for monovalent cations. Note that for both Tl and NH_4 the negative free energies are larger than expected for alkali cations of the same size.

radius; however, both NH_4 and Tl possess larger binding energies than would be expected from their ionic radii. For ammonium, this type of behavior is usually interpreted as indicating the presence of tetrahedral symmetry at the site (Eisenman and Krasne, 1973; Moreno and Diamond, 1974). The positive charge in NH_4 is distributed over all five atoms rather than being concentrated on the central nitrogen and, hence, its ability to hydrogen-bond with appropriately placed ligands is enhanced. Since NH_4 has a tetrahedral configuration, a similar array at the site would cause tighter binding. For Tl, additional energy terms are contributed by its electronic polarizability, partial electron transfer, and promotion of electrons to higher energy orbitals (Moreno and Diamond, 1974). Thus, its interaction energies with a variety of binding sites are larger than would be expected from its ionic radius by comparison with

alkali metal ions. Therefore, the results with NH_4 and Tl emphasize the importance of molecular binding in addition to electrostatic interaction and/or steric hindrance in determining the selectivity of a site. The more complete cationic selectivity of this site would then be: $\text{Li} \approx \text{Na} > \text{Tl} > \text{NH}_4 \approx \text{K} > \text{Rb} > \text{Cs}$.

From the results shown in Tables I and II, it appears that, of the alkali metal ions, only Na and Li are translocated. The results with NH_4 and Tl are, however, more difficult to interpret since these cations appear to contribute to the I_{sc} under certain conditions. It is noteworthy that their contributions to the I_{sc} are amiloride insensitive, which would suggest ionic movement through a pathway parallel to that of Na entry. It is somewhat baffling why NH_4 contributes to the I_{sc} in only part of the experiments. The complex behavior of Tl, on the other hand, is hardly surprising given the multiple (including toxic) effects it has in other preparations such as frog myelinated nerve (Hille, 1972), the squid axon (Landowne, 1975), starfish eggs (Hagiwara et al., 1977) and gramicidin A channels (Neher, 1975; Eisenman et al., 1978). It is possible that some of the observed effects of Tl are due to its ability to form neutral complexes with Cl and NO_3 , which may be permeable through the apical border. Once present in the cytoplasm, Tl could cause multiple effects. The initial amiloride-sensitive inhibition of I_{sc} caused by Tl has been utilized in the analysis of selectivity for the Na binding site; however, the transient increase in amiloride-insensitive I_{sc} does not provide any information regarding the properties of the actual translocation step. On the basis of these considerations, it will be assumed that the entry site allows the translocation of only Li and Na.

The sharper selectivity of translocation as compared with binding may represent the presence of a higher field strength site or a steric effect restricting entry to cations with dehydrated radii less than about 1.2 Å (Fig. 2). The observed translocation selectivity could also reflect a mechanism involving a conformational change occurring at the entry site when the appropriate ion is present.

It is important to evaluate the extent to which changes in membrane potential across the apical border might affect the I_{sc} in the total replacement experiments. Nagel (1977 *b*) found that the intracellular potential became more negative as external Na was substituted with either choline or K. This is the direction opposite that which would be expected if the inhibitory action of K were exerted through this potential. In addition, alterations in the potential would cause changes in the maximal translocation rate at high $[\text{Na}]_o$, which is not observed with K. Similar considerations apply to the other impermeant replacement cations, although intracellular potentials have reportedly not been measured with them in the external solution. Therefore, it is unlikely that membrane potential is important in evaluating this inhibitory action.

One of the consequences of a model which restricts the translocation site to only a fixed number of ions at a time is that it can explain the reported observations of Na transport inhibition by increased cytoplasmic Na concen-

tration in frog skin and other epithelia (Cuthbert and Shum, 1977; Turnheim et al., 1978). Postulating a binding site for Na from the cytoplasmic side would accomplish this, since Na binding to that internal site could inhibit Na entry.

We thank Ms. Kathie Collatos for her excellent technical assistance. We also thank Dr. Olaf Andersen for his critical reading of the manuscript.

This work was supported by National Institutes of Health grants AM-05624, AM-16024, HL-12157, and AM-25886.

Please address requests for reprints to Dr. Dale J. Benos, Department of Physiology, Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02115.

Received for publication 26 March 1979.

REFERENCES

- BENOS, D. J., L. J. MANDEL, and R. S. BALABAN. 1979. On the mechanism of the amiloride-sodium entry site interaction in anuran skin epithelia. *J. Gen. Physiol.* **73**:307-326.
- BENOS, D. J., L. J. MANDEL, and S. A. SIMON. 1980. The effects of chemical group specific reagents on sodium entry and the amiloride binding site in frog skin: evidence for separate sites. *J. Membr. Biol.* In press.
- BENOS, D. J., S. A. SIMON, L. J. MANDEL, and P. M. CALA. 1976. Effect of amiloride and some of its analogues on cation transport in isolated frog skin and thin lipid membranes. *J. Gen. Physiol.* **68**:43-63.
- BIBER, T. U. L. 1971. Effects of changes in transepithelial transport on the uptake of sodium at the outer surface of the frog skin. *J. Gen. Physiol.* **56**:83-99.
- BIBER, T. U. L., L. J. CRUZ, and P. F. CURRAN. 1972. Sodium influx at the outer surface of frog skin. *J. Membr. Biol.* **7**:365-376.
- BIBER, T. U. L., and P. F. CURRAN. 1970. Direct measurement of uptake of sodium at the outer surface of the frog skin. *J. Gen. Physiol.* **56**:83-89.
- BIBER, T. U. L., and M. L. SANDERS. 1973. Influence of transepithelial potential difference on the sodium uptake at the outer surface of the isolated frog skin. *J. Gen. Physiol.* **61**:529-551.
- CALA, P. M., N. COGSWELL, and L. J. MANDEL. 1978. Binding of [³H]ouabain to split frog skin: the role of the Na,K-ATPase in the generation of short circuit current. *J. Gen. Physiol.* **71**:347-367.
- CANDIA, O. A., and D. J. CHIARANDINI. 1973. Transport of lithium and rectification by frog skin. *Biochim. Biophys. Acta.* **307**:578-589.
- CEREIJIDO, M., F. HERRERA, W. J. FLANIGAN, and P. F. CURRAN. 1964. The influence of Na concentration on Na transport across frog skin. *J. Gen. Physiol.* **47**:879-893.
- CUTHBERT, A. W., and W. K. SHUM. 1977. Does intracellular sodium modify membrane permeability to sodium ions? *Nature (Lond.)*. **266**:468-469.
- EISENMAN, G. 1962. Cation selective glass electrodes and their mode of operation. *Biophys. J.* **2**(Pt. 2):259-323.
- EISENMAN, G., and S. KRASNE. 1973. The ion selectivity of carrier molecules, membranes, and enzymes. In *MTP International Review of Science*, Vol. 2. C. F. Fox, editor. Butterworth Publishers Inc., Woburn, Mass. 1-42.
- EISENMAN, G., J. SANDBLOM, and E. NEHER. 1978. Interactions in cation permeation through the gramicidin channel. *Biophys. J.* **22**:307-340.
- ERLIJ, D., and M. W. SMITH. 1973. Sodium uptake by frog skin and its modification by inhibitors of transepithelial sodium transport. *J. Physiol. (Lond.)*. **228**:221-239.

- FINN, A. L. 1974. Transepithelial potential difference in toad urinary bladder is not due to ionic diffusion. *Nature (Lond.)*. **250**:495-496.
- FUCHS, W., E. H. LARSEN, and B. LINDEMANN. 1977. Current-voltage curve of sodium channels and concentration dependence of sodium permeability in frog skin. *J. Physiol. (Lond.)*. **267**: 2137-166.
- HAGIWARA, S., S. MIYAZAKI, S. KRASNE, and S. CIANI. 1977. Anomalous permeabilities of the egg cell membrane of a starfish in K^+ - Tl^+ mixtures. *J. Gen. Physiol.* **70**:269-281.
- HECKMANN, K., B. LINDEMANN, and J. SCHNAKENBERG. 1972. Current-voltage curves of porous membranes in the presence of pore-blocking ions. *Biophys. J.* **12**:683-702.
- HELMAN, S. I., and R. S. FISCHER. 1977. Microelectrode studies of the active Na transport pathway of frog skin. *J. Gen. Physiol.* **69**:571-604.
- HILLE, B. 1972. The permeability of the sodium channel to metal cations in myelinated nerve. *J. Gen. Physiol.* **59**:637-658.
- HILLE, B. 1975. Ionic selectivity, saturation, and block in sodium channels. A four-barrier model. *J. Gen. Physiol.* **66**:535-560.
- HIRSCHMANN, W., and W. NAGEL. 1978. The outer membrane of frog skin: impermeable to K^+ ? *Pfluegers Arch. Eur. J. Physiol.* **373**(Suppl.):R48.
- KIRSCHNER, L. B. 1955. On the mechanism of active sodium transport across the frog skin. *J. Cell. Comp. Physiol.* **45**:61-87.
- KOEFOD-JOHNSEN, V., and H. H. USSING. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298-308.
- LADD, M. F. C. 1968. The radii of spherical ions. *Theoretica Chimica Acta (Berlin)*. **12**:333-336.
- LANDOWNE, D. 1975. A comparison of radioactive thallium and potassium fluxes in the giant axon of the squid. *J. Physiol. (Lond.)*. **252**:79-96.
- LEBLANC, G. 1972. The mechanism of lithium accumulation in the isolated frog skin epithelium. *Pfluegers Arch. Eur. J. Physiol.* **337**:1-18.
- LINDEMANN, B., and C. VOUTE. 1976. Structure and function of the epidermis. In *Frog Neurobiology*. R. Llinas and W. Precht, editors. Springer-Verlag, Berlin. 169-210.
- LINDLEY, B. D., and T. HOSHICO. 1964. The effects of alkali metal cations and common anions in the frog skin potential. *J. Gen. Physiol.* **47**:749-771.
- MANDEL, L. J. 1978. Effects of pH, Ca, ADH, and theophylline on kinetics of Na entry in frog skin. *Am. J. Physiol.* **235**:C35-C48.
- MANDEL, L. J., and P. F. CURRAN. 1972. Response of the frog skin to steady-state voltage clamping. I. The shunt pathway. *J. Gen. Physiol.* **59**:503-518.
- MANDEL, L. J., and P. F. CURRAN. 1973. Response of the frog skin to steady-state voltage clamping. II. The active pathway. *J. Gen. Physiol.* **62**:1-24.
- MOREL, F., and G. LEBLANC. 1975. Transient current changes and Na compartmentalization in frog skin epithelium. *Pfluegers Arch. Eur. J. Physiol.* **358**:135-157.
- MORENO, J. H., and J. M. DIAMOND. 1974. Discrimination of monovalent inorganic cations by "tight" junctions of gallbladder epithelium. *J. Membr. Biol.* **15**:277-318.
- MORENO, J. H., I. L. REISIN, E. RODRIGUEZ-BOULAN, C. A. ROTUNNO, and M. CEREJIDO. 1973. Barriers to sodium movement across frog skin. *J. Membr. Biol.* **11**:99-115.
- NAGEL, W. 1976. The intracellular electrical potential profile of the frog skin epithelium. *Pfluegers Arch. Eur. J. Physiol.* **365**:135-143.
- NAGEL, W. 1977 a. Influence of lithium upon the intracellular potential of frog skin epithelium. *J. Membr. Biol.* **37**:347-359.
- NAGEL, W. 1977 b. The dependence of the electrical potentials across the membranes of the frog skin upon the concentrations of sodium in the mucosal solution. *J. Physiol. (Lond.)*. **269**:777-796.

- NEHER, E. 1975. Ionic specificity of the gramicidin channel and the thallos ion. *Biochim. Biophys. Acta.* **401**:540-544.
- REUSS, L., and A. L. FINN. 1975. Effects of changes in the composition of the mucosal solution on the electrical properties of the toad urinary bladder epithelium. *J. Membr. Biol.* **20**:191-204.
- RICK, R., A. DORGE, and W. NAGEL. 1975. Influx and efflux of sodium at the outer surface of frog skin. *J. Membr. Biol.* **22**:183-196.
- ROTUNNO, C. A., F. A. VILALLONGA, M. FERNANDEZ, and M. CEREJIDO. 1970. The penetration of sodium into the epithelium of the frog skin. *J. Gen. Physiol.* **55**:716-735.
- SEGEL, I. H. 1975. *Enzyme Kinetics*. Wiley-Interscience, New York.
- SZABO, G., G. EISENMAN, R. LAPRADE, S. M. CIANI, and S. KRASNE. 1973. Observed effects of carrier molecules in lipid bilayer membranes. In *Membranes*, Vol. 2. G. Eisenman, editor. Marcel Dekker, Inc., New York. 179-328.
- TURNHEIM, K., R. A. FRIZZELL, and S. G. SCHULTZ. 1978. Interaction between cell sodium and the amiloride-sensitive sodium entry step in rabbit colon. *J. Membr. Biol.* **39**:233-256.
- WRIGHT, E. M., and J. M. DIAMOND. 1977. Anion selectivity in biological systems. *Physiol. Rev.* **57**:109-156.
- ZEISKE, W., and W. VAN DRIESSCHE. 1978. K^+ -uptake across the outer border of frog skin (*R. temporaria*) and its inhibition by Cs-ions. *Pfluegers Arch. Eur. J. Physiol.* **373**(Suppl):R48.
- ZERAHN, K. 1955. Studies on the active transport of lithium in the isolated frog skin. *Acta Physiol. Scand.* **33**:347-358.