On the Mechanism of the Amiloride-Sodium Entry Site Interaction in Anuran Skin Epithelia

DALE J. BENOS, LAZARO J. MANDEL, and ROBERT S. BALABAN

From the Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710. Dr. Benos' present address is Department of Physiology and Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02115.

ABSTRACT The steady-state transport kinetics of the interaction between external sodium and the diuretic drug, amiloride, was studied in isolated anuran skin epithelia. We also investigated the effect of calcium on the amiloride-induced inhibition of short-circuit current ($I_{sc}$) in these epithelial preparations. The major conclusions of this study are: (a) amiloride is a noncompetitive inhibitor of Na entry in bullfrog and grassfrog skin, but displays mixed inhibition in R. temporaria and the toad. A hypothesis which states that the interaction sites for amiloride and Na on the putative entry protein are spatially distinct in all of these species is proposed. (b) The stoichiometry of interaction between amiloride and the Na entry mechanism is not necessarily one-to-one. (c) The external Ca requirement for the inhibitory effect of amiloride is not absolute. Amiloride, at all concentrations, is equally effective in inhibiting $I_{sc}$ of bullfrog skin independently from the presence or absence of external Ca.

INTRODUCTION

Previous studies have suggested that the entry of sodium into isolated frog skin is not due to a simple diffusive-type process, but that the sodium ion must interact with some specific membrane component for translocation to occur (Cereijido and Rotunno, 1968; Biber and Curran, 1970; Biber, 1971; Mandel and Curran, 1973). The evidence for this contention is: first, sodium influx is not a linear function of external sodium concentration, but displays saturation kinetics; and second, both lithium and potassium ions competitively inhibit sodium influx (Rotunno, et al., 1970; Biber and Curran, 1970; Mandel and Curran, 1973). Further evidence supporting the notion that sodium entry involves some type of specific membrane interaction comes from studies with the diuretic drug amiloride (Biber, 1971; Cuthbert, 1974; Benos et al., 1976). This drug inhibits specifically, reversibly, and with high affinity, the saturable portion of the radiotracer sodium influx and, therefore, active sodium transport across the entire epithelium. Therefore, amiloride (and as a necessary corollary, sodium) interacts with specialized regions or sites located on the external membrane surface which facilitate sodium entry into the active transport system.
These sites have been described as constituents of either a Na-selective carrier molecule (Biber and Sanders, 1973) or a pore (Lindemann and Van Driessche, 1977).

We conducted the present study to investigate further the interactions of amiloride and sodium with these transport sites located on the outer surface of amphibian skin. Experiments were designed with several questions in mind: (a) Does amiloride inhibit sodium entry by directly competing with Na for binding at "receptor" sites at the mouth of the transport moiety? (b) What is the stoichiometry of the amiloride-receptor site interaction? and (c) How stringent is the divalent cation requirement of the inhibitory effects of amiloride? (Cuthbert and Wong, 1972; Benos et al., 1976). To answer these questions, we studied the effects of amiloride and Ca concentration on the kinetics of active Na transport as a function of Na concentration. A comparative approach, utilizing the isolated skin epithelia of bullfrog, grassfrog, toad, and Rana temporaria (Northern European frog) was used to clarify several aspects of the amiloride-Ca-Na interaction.

The results presented demonstrate that amiloride and sodium may interact noncompetitively or exhibit mixed inhibition, depending on the species. The most general single model which can account for this species-dependent behavior is one in which the receptor sites for Na and amiloride are distinct. It also appears that the stoichiometry of interaction between amiloride and the sodium entry site is not necessarily one-to-one. Furthermore, the external divalent cation requirement for the inhibitory action of amiloride on sodium entry is not absolute; this is demonstrated in bullfrog skin where amiloride is equally effective in the absence, as well as in the presence, of external calcium.

**M A T E R I A L S  A N D  M E T H O D S**

**Isolated Amphibian Skin Experiments**

The abdominal skin of the grassfrog, Rana pipiens, the bullfrog, Rana catesbeiana, the toad, Bufo marinus, or the Northern European frog, Rana temporaria, was mounted as a flat sheet (3.14 cm² in area) between Lucite chambers equipped with solution reservoirs similar to those originally described by Schultz and Zalusky (1964). The solutions in each chamber (10 ml each) were stirred and oxygenated by bubbling with room air. All experimentation was performed at ambient temperature (20°C), pH 8.4.

The open circuit potential across the skin was measured with calomel electrodes, and current was passed through the skin via Ag-AgCl electrodes. The outside bathing solution always served as reference. Both pairs of electrodes were connected to the solution reservoirs with 4% agar bridges having a composition identical to that of the bathing solution in the chambers. An automatic voltage clamp that compensated for the resistance of the solution between the agar bridges was used to pass the appropriate current through the skin to clamp the membrane potential to 0 mV. In all experiments (except those in which the external calcium concentration was desired to be zero), both sides of the skin were bathed with identical solutions. Under these conditions, the equivalence between the magnitude of the short circuit current (Isc) and the net active sodium transport, originally established by Ussing and Zerahn (1951) in R. temporaria, has also been proven for the species utilized in the present study (Cereijido et al., 1964; Rawlings et al., 1970; Candia and Reinach, 1977). We also performed ²²Na-influx
experiments under standard short-circuit conditions on skins obtained from eight bullfrogs to verify and confirm the equivalence of net sodium influx and $I_{sc}$ in this tissue. The flux experiments were performed as previously described (Benos et al., 1976). The $^{22}$Na influx was found to be $26.3 \pm 2.3 \mu A/cm^2$ while the simultaneously measured $I_{sc}$ was $22.8 \pm 2.9 \mu A/cm^2$. Because these numbers are not significantly different from one another ($P > 0.5$), we conclude that in $R. catesbeiana$, the short-circuit current is a good measure of active sodium transport. The validity of this relationship has also been demonstrated in grassfrog skin when the sodium concentration of both solutions was simultaneously varied (Cereijido et al., 1964). We assume here that the equivalence between $I_{sc}$ and net sodium influx (active transport) is also valid at lower sodium concentrations in the bullfrog and toad skin preparations.

Considerable variation in the absolute values of $I_{sc}$ and transepithelial potential ($V_m$) were observed under control conditions within each species of amphibian. This was probably due to the lack of homogeneity in the frog and toad populations used. These experiments were performed during different seasons and utilized different frog suppliers. The grassfrogs were obtained from Carolina Biological Supply Co., Burlington, N.C., the bullfrogs from Jacques Weil, Rayne, La., the Mexican toads from West Jersey Biological Supply Co., Wenonah, N.J., and $R. temporaria$ as a gift from Dr. W. Nagel, Munich, West Germany. In order to minimize experimental variation, care was taken to perform each series of experiments under a given set of conditions on one batch of frogs or toads. In addition, all experimental manipulations were performed utilizing the same skin as the control. These internal controls validate significant differences between each experiment and its control. Furthermore, all results have been normalized to the values obtained in the control skins, and expressed as the mean value plus or minus one standard error of the mean.

The composition of the regular Ringer solution used in all of the present experiments was as follows: 110 mM NaCl, 2.5 mM KHCO$_3$, and 1 mM CaCl$_2$. The pH of this solution, when gassed with room air at room temperature, was 8.4. The pH of all solutions was measured with a Radiometer pH meter (Radiometer, Copenhagen, Denmark). In experiments in which the sodium concentration was varied, the NaCl was replaced with an osmotically equivalent amount of recrystallized choline chloride (Sigma Chemical Co., St. Louis, Mo.) in both bathing solutions; the sodium concentrations used were 110, 55, 20, 6, and 3 mM. In those experiments in which the calcium concentration was desired to be zero, CaCl$_2$ was omitted from the Ringer solution, and 0.5 mM ethyleneglycol-bis-(beta-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) added. Calcium was removed from the external bathing solution only. Amiloride was obtained as a gift from Merck Sharp & Dohme Research Laboratories, West Point, Pa.

**Theoretical Treatment of Data**

This communication is concerned with the effects produced by variations in sodium, calcium, and amiloride concentrations on the steady-state transport kinetics of net sodium influx across amphibian skin. Treatment of steady-state kinetic transport data is often done utilizing the Michaelis-Menten formulation (Segel, 1975). In the present paper, the use of Michaelis-Menten kinetics is based on three major assumptions which are evaluated and justified below. These assumptions are: (a) the net transport of sodium across the skin of these amphibia can be accurately described by classical Michaelis-Menten type kinetics; (b) the entry of sodium across the outer epithelial surface is rate-limiting for net transport, and therefore changes in $I_{sc}$ produced by alterations in sodium or calcium ion concentrations and (or) additions of amiloride reflect events produced at this border only; and (c) the interaction between amiloride and its receptor site occurs on
the external aspect of the outer surface of the epithelium, and this interaction is completely reversible.

(a) The observations that the curves describing the relationship between \( I_{sc} \) and sodium concentration are rectangular hyperbolas, and that linearization of these plots (Figs. 2, 3, and 4) conform with a high degree of correlation to reciprocal rearrangements of the Michaelis-Menten equation (Kirschner, 1955; Cereijido et al., 1964, Biber, 1971) substantiate the first assumption. There are other mathematical relationships (namely, \( I_{sc} = a \log [Na] \)) which can adequately describe the experimental \( I_{sc} \) vs. [Na] curves. However, these formulations cannot account for the saturation of these curves and, most importantly, fail to describe competition effects with other cations. The reader is referred to Mandel (1978) for further discussion of this matter. The basic Michaelis-Menten equation can be rewritten as:

\[
I_{sc} = \frac{(I_{\text{max}}) [Na]}{K_t + [Na]}
\]

where \( I_{\text{max}} \) represents the maximal (saturating) level of \( I_{sc} \) that can be achieved under a given set of conditions, and \( K_t \) is operationally defined as the value of [Na] required to maintain \( I_{sc} \) at one-half its saturating level.

(b) The clearest experimental evidence demonstrating that the outer entry barrier is rate-limiting for sodium transport across amphibian skin comes from experiments in which active sodium transport, as measured either by \( I_{sc} \) or net Na influx, as well as rapid \( ^{22}\text{Na} \) entry into the skin were measured simultaneously. Both the transepithelial Na transport and the rapid entry were found to be saturable functions of external [Na] (Biber, 1971; Moreno et al., 1973) and identical in magnitude.

(c) The third major assumption made is that the inhibitory effects of amiloride are completely reversible and that amiloride exerts its actions on the external surface of the skin. Results have been obtained in this (Benos and Mandel, 1978) and other laboratories (Cuthbert, 1973) demonstrating that the inhibition of \( I_{sc} \) by amiloride in frog skin can be rapidly reversed (and complete recovery obtained) by washing with drug-free Ringer; this is true even after exposure periods of 30 min. Furthermore, a given concentration of amiloride produces a given level of inhibition; no progressive inhibition with time is observed. Amiloride is presumed to interact with externally located sites because of its rapid inhibitory action when applied to the external solution in frog skin (Biber and Sanders, 1973). In addition, the drug is ineffective from the serosal side in frog skin (Salako and Smith, 1970) and does not inhibit the enzymatic activity of the Na, K-ATPase (Baer et al., 1967).

Two types of experiments were performed to test whether the inhibitory action of amiloride on active Na transport was competitive or non-competitive. The first type consisted of determining amiloride dose-response curves at different Na concentrations of the bathing media. A competitive interaction would be indicated by parallel dose-response curves shifting to lower drug concentrations as the Na concentration decreased; that is, less amiloride would be required to elicit the same inhibitory level at lower Na concentrations. Conversely, a noncompetitive inhibitor would be equally effective at all Na concentrations and, consequently, the dose-response curves at various Na concentrations would be superimposed.

The second type of experiment was the reciprocal of the previous one, measuring \( I_{sc} \) as a function of Na concentration at two constant external amiloride concentrations. These results produced hyperbolic plots of \( I_{sc} \) vs. [Na]. However, these plots are not useful to determine \( I_{\text{max}} \) and \( K_t \) because such a procedure involves the determination of an asymptotic value as [Na] approaches infinity. A more appropriate and useful way to
determine these constants is to recast Eq. 1 into a single reciprocal, linear form (Eadie, 1942; Dowd and Riggs, 1965):

$$I_{sc} = I_{\text{max}} - K_t \left( I_{sc}/[Na] \right).$$

(2)

A plot of $I_{sc}$ vs. $I_{sc}/[Na]$ yields a straight line if the system obeys Michaelis-Menten kinetics; the y intercept equals $I_{\text{max}}$ and the slope is equal to $-K_t$.

A plot of $I_{sc}$ vs. $I_{sc}/Na$ in the presence of a constant inhibitor concentration is a useful way of graphically determining the type of inhibition produced (e.g., competitive or noncompetitive with substrate), and thus provides insight into the molecular architecture of the receptor site (Dixon and Webb, 1964). With fully competitive inhibitors, $I_{\text{max}}$ is left unchanged but the apparent $K_t$ for the substrate reaction is increased. In the noncompetitive case, $K_t$ is unaltered, but the maximal transport velocity is decreased.

RESULTS

Effects of Amiloride on $I_{sc}$ at Different Sodium Concentrations

The effect of variations in the sodium concentration of the bathing media on the inhibition of short-circuit current ($I_{sc}$) by amiloride was examined in isolated abdominal skin preparations obtained from bullfrog, grassfrog, toad, and R. temporaria. These results are presented in terms of the apparent inhibitory dissociation constant ($K_t$) of amiloride (defined as the concentration of amiloride necessary to produce 50% inhibition of $I_{sc}$) and the maximal level of inhibition obtained at high amiloride concentrations (Table I). There was a slight leftward shift in the inhibition curves obtained at sodium concentrations of 55 and 20 mM (with respect to 110 mM Na) in the bullfrog (Fig. 1). The $K_t$ of amiloride averaged 8.0 (± 1.4) × 10^{-7} M at 110 mM Na in the bullfrog. This apparent $K_t$ was significantly different from that observed at 55 or 20 mM Na, namely, 3.1 (± 0.6) × 10^{-7} M ($P < 0.01$) and 2.7 (± 0.4) × 10^{-7} M ($P < 0.005$), respectively. The $K_t$ of amiloride in grassfrog skin averaged 6.2 (± 1.0) × 10^{-7} M at 110 mM Na, and was not significantly different in magnitude at sodium concentrations of 55, 20, and 6 mM. Similar results were obtained in the toad. However, decreasing the sodium concentration to 6 or 3 mM in bullfrog and 3 mM in

| Na  | Bullfrog (n=8) | Grassfrog (n=3) | Toad (n=4) | R. Temporaria (n=3) |
|-----|----------------|-----------------|------------|---------------------|
|     | $K_t$ $\times 10^{-7}$ M | $I_{\text{max}}$ inhibition | $K_t$ $\times 10^{-7}$ M | $I_{\text{max}}$ inhibition | $K_t$ $\times 10^{-7}$ M | $I_{\text{max}}$ inhibition |
| 110 | 8.0±1.4        | 91.3±0.9        | 97.6±1.2   | 90.8±3.1           | 91.6±3.7        |
| 55  | 3.1±0.6        | 93.4±1.2        | 97.6±2.7   | 94.0±3.3           | 94.0±2.8        |
| 20  | 2.7±0.4        | 89.3±2.9        | 94.7±4.1   | 97.8±1.0           | 92.4±2.2        |
| 6   | 10.6±5.9       | 67.7±3.4        | 95.0±4.6   | 96.6±2.6           | 92.0±4.7        |
| 3   | 35.0±5.8       | 64.3±3.4        | 99.5±1.4   | 94.0±5.3           |

The maximal percentage inhibition of $I_{sc}$ was taken as that produced by 10^{-7} M amiloride in all preparations except R. temporaria where 10^{-8} M amiloride was the highest concentration used.
grassfrog resulted in a rightward shift of the dose-response curves. In addition, sodium concentrations of 6 mM (bullfrog) and 3 mM (bullfrog and grassfrog) depressed the maximal level of inhibition obtained at high amiloride concentrations. We do not attach much weight to these latter observations, inasmuch as these curves were always determined late in the experiment and in a region (low sodium, high amiloride) where sensitive measurements of current changes cannot be made. Furthermore, these results (i.e., rightward shifts of the inhibition curves and depression of the maximal inhibition level at low sodium) were not always obtained. This was especially true if the dose-response curves at low [Na] were determined at the beginning of the experiment (see Table II below).

The $K_t$ of amiloride in *R. temporaria* skin averaged $3.0 (\pm 3.6) \times 10^{-7} \text{M}$ at 110 mM Na (Table I). Reducing the Na concentration resulted in a concomitant leftward shift in the dose-response curves. At 3 mM Na, the $K_t$ of amiloride equalled $2.7 (\pm 2.8) \times 10^{-8} \text{M}$, a decrease of one order of magnitude as compared to the value measured at 110 mM Na. No depression in the maximal level of inhibition at high amiloride concentrations was observed in these experiments.

Another series of experiments was performed to determine in more detail to what extent the inhibitory effect of amiloride is dependent upon external sodium. These experiments, performed on bullfrog skin, are summarized in Table II. In Table II A and B, the inhibitory effects of amiloride on $I_{sc}$ were assessed at only two concentrations of sodium. Both $10^{-7} \text{M}$ and $10^{-6} \text{M}$ amiloride inhibited $I_{sc}$ to a greater extent at 6 and 3 mM Na than at 110 mM Na. However, as can be seen in Table II C, the percent inhibition produced by $10^{-7} \text{M}$ amiloride became independent of [Na] at concentrations below 75 mM. There is, however, a statistically significant difference between the percent inhibition of $I_{sc}$ observed at 110 mM Na and that observed at 75 mM Na ($P <$
0.001) or 6 mM Na (P < 0.005). These results are consistent with those observed in Table II A and B and in Fig. 1.

Figs. 2, 3, and 4 present a summary of experiments in which the $I_{sc}$ was measured as a function of [Na] both in the absence and presence of two constant amiloride concentrations. The results have been normalized to the value of $I_{sc}$ obtained at [Na] = 110 mM (141.7 ± 16.5 μA in bullfrog, 123.8 ± 11.2 μA in grassfrog, and 150.1 ± 15.8 μA in *R. temporaria*). The data from these

| TABLE II |
|-----------|
| PERCENT INHIBITION OF SHORT-CIRCUIT CURRENT IN BULLFROG SKIN PRODUCED BY AMILORIDE AT DIFFERENT SODIUM CONCENTRATIONS |
| Inhibition of $I_{sc}$ |
| Concentration | $10^{-7}$ M Amiloride | $10^{-8}$ M Amiloride |
| A. [Na], mM | % | % |
| 110 | 39.8±3.1 | 71.5±2.8 |
| 6 | 57.4±3.4 | 83.1±2.5 |
| B. [Na], mM | % | % |
| 110 | 43.2±2.0 | 79.6±0.8 |
| 3 | 56.3±2.8 | 86.1±2.1 |
| C. [Na], mM | % |
| 110 | 35.1±2.1 |
| 90 | 46.1±3.3 |
| 75 | 51.2±1.3 |
| 55 | 54.8±0.9 |
| 45 | 54.4±2.7 |
| 30 | 54.9±2.4 |
| 20 | 51.6±3.0 |
| 6 | 56.1±3.5 |
| 3 | 53.8±3.7 |

In each group of experiments, the sodium concentration was varied on both sides of the preparation and the osmolality maintained with choline chloride. The results are expressed as the mean ± 1 SEM (n = 8).

In the absence of drug, the $K_t$ for Na was 17.2 ± 3.0 mM for bullfrog, 8.6 ± 0.8 mM for grassfrog, and 12.4 ± 1.3 mM for *R. temporaria*. In the bullfrog and grassfrog, $10^{-7}$ M amiloride resulted in no significant change in $K_t$, but $I_{max}$ was drastically reduced from 115.1 ± 4.1 to 85.9 ± 5.6 μA and from 108.2 ± 2.4 to 87.5 ± 4.6 μA, respectively. These data clearly show that amiloride, at least at $10^{-7}$ M, is a noncompetitive inhibitor of
Na transport in these frog skin preparations. Addition of $10^{-6}$ M amiloride also resulted in no significant change in $K_t$ with respect to the controls (19.2 ± 3.7 vs. 17.2 ± 3.0 mM in bullfrog and 7.8 ± 2.7 vs. 8.6 ± 0.8 mM in grassfrog), but elicited a substantial decrease in $I_{\text{max}}$ (41.0 ± 6.0 as compared to 115.1 ± 4.1 μA, and 37.4 ± 6.0 vs. 108.2 ± μA in bullfrog and grassfrog, respectively). These results again are consistent with noncompetitive inhibition. However, there are

![Figure 2](Image)

**Figure 2.** Single reciprocal (Eadie) plot ($I_{sc}$ vs. $I_{se}/[Na]$) of $I_{se}$ vs. [Na] data obtained from isolated bullfrog skin in the absence and presence of $10^{-7}$ and $10^{-6}$M amiloride. The ionic strength was maintained constant with choline chloride. Each point represents the mean value of 12 experiments; the bars indicate 1 SEM. The linear correlation coefficients are 0.98 for the control curve, and 0.96 and 0.86 for the data acquired at $10^{-7}$ and $10^{-6}$M amiloride, respectively. The y intercepts ($I_{\text{max}}$) and slopes ($K_t$) of the lines are: 115.1 ± 4.1 μA and −17.2 ± 3.0 mM (control); 85.9 ± 5.6 μA and −19.2 ± 5.3 mM ($10^{-7}$M amiloride); and 41.0 ± 6.0 μA and −19.2 ± 3.7 mM ($10^{-6}$M amiloride).

![Figure 3](Image)

**Figure 3.** Eadie plot ($I_{sc}$ vs. $I_{se}/[Na]$) of $I_{se}$ vs. [Na] data obtained from isolated grassfrog skin in the absence and presence of $10^{-7}$ and $10^{-6}$M amiloride. Each point represents the mean of 12 experiments. The linear regression correlation coefficient, y intercept, and slope of the lines are: 0.98, 108.2 ± 2.4 μA, and −8.6 ± 0.8 mM (control); 0.94, 87.5 ± 4.6 μA, and −9.9 ± 1.5 mM ($10^{-7}$M amiloride); and 0.67, 37.4 ± 6.0 μA, and −7.8 ± 2.7 mM ($10^{-6}$M amiloride).
several problems with this interpretation at the higher amiloride concentration. First, the linear correlation coefficient becomes poorer at 10^{-6} M amiloride ($r^2 = 0.86$ in bullfrog and 0.67 in grassfrog), i.e., the experimental points deviate from predicted linear behavior. Secondly, the maximal level of amiloride inhibition is reduced at low external sodium concentrations (Fig. 1). These two observations may be attributable to either experimental error due to decreased sensitivity of $I_{se}$ measurements at lower currents (as is the case at low [Na] and high amiloride), or a change in the mechanism of inhibition under conditions of low sodium. This latter possibility will be considered in detail in the Discussion.

![Eadie plot](image)

**Figure 4.** Eadie plot ($I_{se}$ vs. $I_{se}$/[Na]) of $I_{se}$ vs. [Na] data obtained from isolated *R. temporaria* skin in the absence and presence of 10^{-7}M amiloride. Each point represents the mean of seven experiments. The linear regression correlation coefficient, y intercept, and slope of the lines are: 0.99, 113.2 ± 2.4 μA, and -12.4 ± 1.3 mM (control); and 0.99, 96.0 ± 3.6 μA, and -33.8 ± 3.9 mM (10^{-7}M amiloride).

Addition of 10^{-7} M amiloride to the solution bathing the outside surface of *R. temporaria* skin (Fig. 4) resulted in a 2.7-fold increase in $K_I$ (to 33.8 ± 3.9 mM) with little change in $I_{max}$ (96.0 ± 3.6 as compared with 113.2 ± 2.4 μA). These results, taken together with the leftward displacements of the dose-response curves with decreasing sodium are indicative of competitive or mixed inhibition of $I_{se}$ by amiloride in this system.

This same type of experiment was also performed in eight isolated toad skin preparations. Addition of 10^{-6} M and 10^{-5} M amiloride increased $K_I$ (51.7 ± 8.4
and 80.1 ± 5.0 mM, respectively, as compared to 29.1 ± 10.7 mM in the absence of the drug, and decreased $I_{\text{max}}$ (105.5 ± 8.4 μA (10^{-6}M) and 53.2 ± 4.8 μA (10^{-5}M) compared to the control value of 116.9 ± 8.0 μA). These results indicate that the inhibition produced by amiloride in this system is mixed.

The validity of this type of analysis in determining the mechanism of $I_{sc}$ inhibition was tested with a known competitive inhibitor of $I_{sc}$ from the external solution, namely, potassium (Mandel and Curran, 1973). Potassium does not penetrate the epithelium from the outside. Sodium was varied between 55 and 3 mM in the absence and presence of 25 mM external potassium in the bullfrog skin. Fig. 5 shows that the apparent $K_i$ of Na is increased from 5.3 mM to 10.5 mM by 25 mM external potassium, with no significant change in $I_{\text{max}}$. These results are in accord with those of a pure competitive inhibitor.

![Figure 5. Eadie plot of $I_{sc}$ vs. $I_{sc}/[\text{Na}]$ in isolated bullfrog skins at external potassium concentrations of 0 and 25 mM. The potassium concentration of the inside solution was always 2.5 mM. The sodium concentrations utilized were 55, 20, 6, and 3 mM. The ionic strength was adjusted with choline chloride to equal that of the regular Ringer solution. Each point is the average of seven individual experiments.](image)

**Stoichiometry of the Amiloride-Sodium Entry Site Interaction**

In many enzyme systems the binding of substrate and (or) inhibitor molecules to one site can affect the binding at another site so as to exhibit cooperative interactions. A useful method to determine whether such cooperativity exists as well as the stoichiometry of substrate-enzyme and (or) inhibitor-enzyme binding is to employ the Hill equation (Segel, 1975). If an enzyme is composed of multiple catalytic subunits, each subunit being identical and totally independent, the binding of substrate at one subunit will in no way influence the binding of substrate at another (i.e., zero cooperativity). Under these conditions, $n$ molecules of a single-site enzyme behave identically to a single molecule of an $n$-site enzyme. This situation would result in a hyperbolic velocity vs. substrate concentration graph (as is observed in amphibian skin). Analysis of $n$ by the Hill equation (see Appendix for derivation):

$$\log \left( \frac{I}{I_{\text{max}} - I} \right) = n (\log [\text{Na}]) - \log K',$$

(4)
where \( I = I_{sc} \) at a given \([Na]\), and \( K' \) is equal to a constant which includes \( K_t \), would yield \( n = 1. \) \( I_{sc} \) vs. \([Na]\) data obtained from these epithelia were subjected to this analysis, the results being presented in Table III. The value of \( n \) was determined from the slope of the graph of \( \log \left( \frac{I}{I_{max} - I} \right) \) vs. \( \log [Na] \). The points were fitted by linear regression, and all plots had correlation coefficients better than 0.97. The Hill coefficient \( (n) \) for the interaction of Na with its 'receptor' was 1.16 (bullfrog), 1.03 (grassfrog), 0.99 (\( R. \) temporaria), and 1.27 (toad), numbers not very different from 1. These values are not significantly different from one another (\( P > 0.25 \)).

Likewise, a Hill-type equation for multisite noncompetitive inhibition analysis can be derived from rapid-equilibrium assumptions (Segel, 1975; see Appendix):

\[
\log \left( \frac{I_{am}}{I - I_{am}} \right) = -n \log \text{[amiloride]} + \log C',
\]

where \( I_{am} \) is the \( I_{sc} \) observed at a given amiloride concentration, \( I \) is the \( I_{sc} \) measured in the absence of amiloride, and \( C' \) is a constant whose interpretation depends upon the type (i.e., competitive, noncompetitive, etc.) of inhibition produced, as well as the substrate concentration used. This equation was applied to the data summarized in Table I and \( n \) computed (Table IV). In all skin preparations and at all substrate concentrations, \( n \) was found to be less than 1. Fig. 6 shows a Hill inhibitory plot of the amiloride inhibition of \( I_{sc} \) of isolated bullfrog at \([Na] = 110 \text{ mM}\). The slope of the regression line fitted to the

### Table III

| Skin epithelium     | Hill coefficient |
|---------------------|------------------|
| Bullfrog (\( n = 12 \)) | 1.16 ± 0.04      |
| Grassfrog (\( n = 11 \)) | 1.03 ± 0.04      |
| \( R. \) temporaria (\( n = 7 \)) | 0.99 ± 0.08      |
| Toad (\( n = 8 \))     | 1.27 ± 0.08      |

Mean ± SEM.

### Table IV

| Na  | Bullfrog (\( n = 8 \)) | Grassfrog (\( n = 5 \)) | \( R. \) temporaria (\( n = 5 \)) | Toad (\( n = 4 \)) |
|-----|------------------------|-------------------------|----------------------------------|-------------------|
| mM  |                        |                         |                                  |                   |
| 110 | 0.54 ± 0.02            | 0.77 ± 0.05             | 0.75 ± 0.07                       | 0.42 ± 0.05       |
| 55  | 0.57 ± 0.03            | 0.68 ± 0.08             | 0.74 ± 0.06                       | 0.70 ± 0.08       |
| 20  | 0.51 ± 0.02            | 0.70 ± 0.09             | 0.68 ± 0.03                       | 0.67 ± 0.05       |
| 6   | 0.45 ± 0.04            | 0.67 ± 0.09             | 0.67 ± 0.09                       | 0.68 ± 0.06       |
| 3   | 0.34 ± 0.03            | 0.43 ± 0.02             | 0.67 ± 0.07                       | –                 |
Experimental points was 0.54 ± 0.04. Additional experiments were performed at a Na concentration of 110 mM to compute the value of \( n \) for the interaction between amiloride and the Na entry site. The values of \( n \) calculated from these experiments were 0.54 ± 0.02 for bullfrog (\( n = 12 \)) and 0.53 ± 0.05 for grassfrog (\( n = 10 \)). These results indicate that the inhibition of the Na entry site elicited by amiloride increases with the square root of the amiloride concentration. Thus, the amiloride concentration needed to achieve complete inhibition is higher than would be required if the inhibitory action was related to the first power of the amiloride concentration. Possible interpretations of this finding are treated in the Discussion.

**Figure 6.** Hill inhibitory plot of the amiloride-induced reduction of \( I_{sc} \) in bullfrog skin (see Eq. 5 in text). The data points were taken from Fig. 1, [Na] = 110 mM (bullfrog). The line was computed by linear regression. The correlation coefficient is 0.98 and the line has a slope of 0.54 ± 0.02.

**Effect of External Calcium on Amiloride-Induced Inhibition of \( I_{sc} \)**

The effect of external Ca on the amiloride-induced inhibition of \( I_{sc} \) has proven difficult to understand. It is well known that external Ca inhibits \( I_{sc} \) in *R. pipiens* (Curran and Gill, 1962) and *R. catesbeiana;* in addition, the presence of external Ca is required to obtain the complete inhibition of \( I_{sc} \) by amiloride in *R. temporaria* (Cuthbert and Wong, 1972) and *R. pipiens* (Benos et al., 1976). The latter investigators found evidence that these two actions of Ca could be separated from each other by the use of other multivalent cations, particularly uranyl. Cuthbert and Wong (1972) interpreted the Ca requirement for amiloride's action in terms of the formation of an amiloride-Ca-Na entry site ternary complex which prevented Na translocation. In view of these findings, it became important to investigate further the role of Ca to obtain a clearer understanding of the molecular nature of the interaction between amiloride and the Na entry site.

Through a kinetic analysis similar to the one presented herein, Mandel (1978) demonstrated that the inhibitory action of Ca on the \( I_{sc} \) was noncompetitive in *R. pipiens*. The same experiments, performed in *R. catesbeiana* and *B. marinus*, elicited identical results.¹ An important difference between these two species of

¹ Benos, D. J., L. J. Mandel, and R. S. Balaban. Unpublished observations.
frogs and the toad is manifested in their Ca requirement for the inhibitory action of amiloride, as shown below.

The inhibition of the $I_{sc}$ as a function of amiloride concentration in the presence and absence of 1 mM external Ca in toad skin was determined and is summarized in Table V. This table shows the effect of external Ca in promoting amiloride inhibition of $I_{sc}$. Removal of Ca from the external solution not only decreased the maximal percent inhibition obtained by amiloride but also increased the amiloride concentration necessary for 50% inhibition of $I_{sc}$. Similar log dose-response curves of amiloride inhibition of $I_{sc}$ plus and minus external Ca were performed using bullfrog skin (Table V). Amiloride, at all concentrations, is equally effective in inhibiting $I_{sc}$ independently from the presence or absence of external calcium. This finding demonstrates that the external divalent cation requirement for the action of amiloride is not absolute and that, at least in bullfrog, the inhibitory actions of amiloride and calcium appear to be independent of each other.

### Table V

The effect of external calcium on $K_I$ and on the maximal level of $I_{sc}$ inhibition produced by amiloride in isolated toad and bullfrog skin preparations

| External calcium | Toad $(n=10)$ | Bullfrog $(n=10)$ |
|------------------|-------------|------------------|
| mM               | $K_I \times 10^{-7}$ | % Maximal inhibition | $K_I \times 10^{-7}$ | % Maximal inhibition |
| 1                | 4.4 (±1.3) | 93.0 (±1.7) | 2.4 (±0.3) | 91.9 (±2.3) |
| 0                | 38.8 (±10.6) | 77.2(±5.7) | 2.2 (±0.7) | 94.2 (±1.3) |

Mean ± (SEM).

**Discussion**

Benos et al. (1976) have shown that a complex interaction exists between amiloride, multivalent cations, and the Na-entry site in frog skin epithelium. In the present studies, these interactions were examined in more detail utilizing the methods of enzyme kinetics. The justification for the use of enzyme kinetics in this system rests on the observation that the interaction between external Na and its entry site may be described by the Michaelis-Menten formulation. The experiments reported in this communication support this idea because amiloride was found to interact with external Na in ways predictable by this kinetic formalism. Large species variability was found in this interaction, amiloride being noncompetitive in the bullfrog and grassfrog, and mixed in *R. temporaria* and the toad. A unifying hypothesis, discussed in detail below, which can account for such multiplicity of behavior is one in which amiloride blocks Na entry by reacting at an external receptor site spatially distinct from the actual sodium translocation site. In addition, two other conclusions concerning the nature of the amiloride-sensitive transport site can be drawn from this study. First, the inhibition of $I_{sc}$ by amiloride may involve the interaction of more than one molecule of amiloride per Na entry site, i.e., amiloride binding displays
negative cooperativity. Second, the inhibitory actions of amiloride do not absolutely require the presence of divalent cations in the external bathing medium.

The type of inhibition produced by a substance has a direct bearing on its molecular mechanism of action. In noncompetitive inhibition the binding of either substrate or inhibitor in no way influences the binding of the other. In order for this to be true, the binding sites of inhibitor and substrate have to be separate. Conversely, the binding of a competitive inhibitor prohibits the simultaneous binding of the substrate, i.e., the enzyme can be bound to one or the other, not both. This, however, does not necessarily imply that the substrate and inhibitor bind at an identical locus. One can envisage a situation in which the binding of inhibitor at one site prevents the binding of substrate at another either sterically or by inducing a conformational rearrangement of the enzyme.

In frog skin and toad bladder, various interpretations have been made regarding the interaction between amiloride and the Na-entry site. Salako and Smith (1970) and Cuthbert and Shum (1974; 1976) reported that the effect of amiloride in R. temporaria was potentiated when the sodium concentration of the mucosal bathing solution was lowered, as evidenced by leftward displacements of the dose-response curves with decreasing [Na]. The former investigators, upon replotting their data in the double-reciprocal form, demonstrated mixed inhibition. On this basis, Salako and Smith (1970) suggested that the interaction between amiloride and Na may be noncompetitive or mixed. In addition, Bentley (1968) concluded that amiloride is a noncompetitive antagonist of Na entry in toad bladder. Biber (1971), in R. pipiens, found that 10^{-5}M amiloride inhibited I_{se} to the same extent when the sodium concentration was either 115 mM (87.6% inhibition) or 6 mM (86.1% inhibition). Superficially, this behavior is consistent with noncompetitive inhibition. More recently, Cuthbert (1976 a, b), from double-reciprocal kinetic plots of data from his laboratory, proposed that amiloride is competitive with sodium and further, that it acts in a manner analogous to that of the axonal Na channel blocker, tetrodotoxin (Kao and Nishiyama, 1965; Hille, 1975 a, b), namely, by binding to an ion selectivity filter, thereby plugging the Na channel.

The data presented in Table I and Figs. 1, 2, and 3 of this paper support the notion that amiloride and Na are noncompetitive in the skins of R. pipiens and R. catesbeiana, i.e., these molecules interact with a different region of the Na entry protein. These results are clearly different from those shown in Table I and Fig. 4 for R. temporaria in which competitive inhibition is suggested. The implication is that a species difference does exist across the Atlantic regarding the amiloride-Na entry site interaction in frog skin. A possibility that cannot be ignored is that sufficient diversity exists among species to allow amiloride to block Na transport in R. temporaria as Cuthbert (1976 a, b) describes, i.e., by plugging the "Na channel", whereas in R. pipiens and R. catesbeiana amiloride interacts at a different region of the Na entry site. Alternatively, amiloride may bind in all these anuran skin epithelia not at the mouth of the "Na channel" but at a separate locus on this Na translocation moiety, which has been termed the 'modifier-site' (Lindemann, 1975). The kinetic differences observed between the various epithelial preparations may then be attributed to variations in the
chemical properties of this modifier site. Competitive inhibition would be observed if amiloride and Na interacted at the modifier site to exclude each other, whereas noncompetitive inhibition would be obtained if Na did not affect amiloride binding at this site. The toad skin, by virtue of its displaying mixed-type inhibition kinetics, would occupy an intermediate position in this scheme. In point of fact, mixed-type kinetics are a common occurrence because even the results obtained in bullfrog show that the interaction between amiloride and Na contains elements of competitive-type behavior, namely, the increased effectiveness of amiloride as an inhibitor of $I_{sc}$ at sodium concentrations below 75 mM when compared to that observed with $[\text{Na}] = 110$ mM (Table I). It is important to note that this enhancement of inhibitory ability with decreasing sodium was evident only to 75 mM Na (Table I C), a concentration still on the saturating portion of the $I_{sc}$ vs. $[\text{Na}]$ plot. The degree of competition at this modifier site may well vary from preparation to preparation: differences in the structural components (e.g., $-\text{SH}$, $-\text{COOH}$, or $-\text{NH}_3$ groups) of the amiloride interaction region may mediate this property. Recently, Gottlieb et al. (1978) reported that $p$-chloromercuribenzene sulfonate (PCMBS), a sulfhydryl-specific mercaptide bond reagent, increases $I_{sc}$ of isolated rabbit colon and decreases the effectiveness of amiloride. Similar experiments performed in our laboratory on bullfrog skin show that amiloride is equally effective in inhibiting $I_{sc}$ in the presence or absence of PCMBS despite the profound alterations of $I_{sc}$ produced by PCMBS. We have also shown that the skins of grassfrog, bullfrog, and toad respond quite differently when exposed to other chemical site specific modifying reagents (Mandel et al., 1978).

In order to acquire knowledge concerning the stoichiometry of the amiloride-receptor site interaction from kinetic data, it is convenient to rearrange the Michaelis-Menten equation for noncompetitive inhibition into a Hill-type equation. From this type of analysis, we found that, at all sodium concentrations and for all epithelial skin preparations, the value of $n$ for the amiloride-receptor site interaction was significantly less than 1 (Table IV), while that for the Na, Na-site interaction was 1 (Table III). In the bullfrog, $n$ for amiloride was about 0.5, and for grassfrog, $R. \text{temporaria}$, and toad, $n$ was 0.7. A value of $n$ below 1 is a sign of negative cooperativity among the receptor sites, that is, the binding of amiloride at one entry site appears to decrease the affinity of adjacent sites. One implication of this result is that amiloride binding sites are in sufficient proximity to interact with each other, suggesting at least two, not mutually exclusive, possibilities: (a) more than one amiloride molecule may bind with each receptor site and/or (b) receptor sites may be in close proximity to each other by either clustering of proteins or by having more than one Na-entry site per translocation protein. It should be noted that a value of $n$ less than 1 provides no indication about the actual amiloride-receptor stoichiometry. These values for $n$ obtained with amiloride should be contrasted with the value of $n = 1$ calculated for the inhibition of $I_{sc}$ elicited by triaminopyrimidine (TAP), a less potent inhibitor of $I_{sc}$ than amiloride. The present results with amiloride, again, indicate that there appear to be differences between species. Cuthbert

---

2 Balaban, R. S., L. J. Mandel, and D. J. Benos. Manuscript in preparation.
(1974) reported that the slopes of the Hill inhibition plots for toad bladder and *R. temporaria* skin were 0.96 and 1.22, respectively. This observation has been used to substantiate the assumption of a 1:1 stoichiometry employed in the calculation of the number of Na entry sites (Cuthbert, 1973) and, subsequently, turnover numbers (Cuthbert, 1976 b), from [14C]amiloride binding studies. Our results indicate that the stoichiometry of binding is not necessarily 1:1, even in *R. temporaria* skin. The determination of the actual number of amiloride binding sites per Na-translocation unit must await future equilibrium binding studies on the isolated protein.

We favor the hypothesis that amiloride and Na interact at separate loci on the apical entry mechanism in all of these skin preparations for the following reasons: (a) The implications of a Hill coefficient less than 1 are such that it is easier to conceptualize negative cooperativity and (or) multiple binding sites for amiloride at modifier sites rather than at the mouth of the "channel". Of course, one possibility that cannot be excluded is that the mouth of the channel may be one of the amiloride binding sites. (b) This is the more general model and it can account for variabilities in behavior among species where Cuthbert's (1976 a, b) simple plugging model cannot. (c) Preliminary experiments with chemical site specific reagents performed in this laboratory (Mandel et al., 1978) indicate that the action of each reagent on I$_{sc}$ appears to be independent from its action on the effectiveness of amiloride as an inhibitor of I$_{sc}$.

Deviations from simple kinetics were observed in Figs. 1, 2, and 3, especially at high amiloride and low Na concentrations. These results indicate that the actual interaction between amiloride and the Na entry site is rather complex and the noncompetitive model is only a first approximation. Numerous kinetic models with varying degrees of complexity could be devised to explain these results. Qualitatively, it is possible to explain this behavior as complexities added to the simple noncompetitive model whose basic postulate is that amiloride inhibits at a site separate from the "mouth of the Na channel". Possible complexities are: (a) the negative cooperativity noted earlier could cause nonlinear behavior, especially at high amiloride concentrations because it could give the appearance of amiloride receptor sites with varying affinities. A characteristic of receptors with different affinities is curvature of single or double reciprocal kinetic plots which becomes more apparent at higher inhibitor concentrations (Segel, 1975). (b) The lack of complete I$_{sc}$ inhibition by high amiloride concentrations at low external [Na] is similar to the amiloride-insensitive fraction of I$_{sc}$ observed in the absence of external Ca (Table V, and Benos et al., 1976). External Ca has been shown to be a noncompetitive inhibitor of Na entry in frog skin (Mandel, 1978). Therefore, it is possible that both external Ca and Na affect the conformation of the Na entry site in such a way as to influence allosterically its properties. For example, the Na entry sites could exist in two populations, one amiloride-sensitive and the other one not, with a constant interconversion between the two dependent on the external Na and (or) Ca concentration. At high external Na (or Ca) all the sites could be in the amiloride-sensitive configuration, whereas at low Na a fraction of the sites could be in the amiloride-insensitive form.
Another aim of the present paper was to study the interaction between amiloride and external Ca. This study was prompted by the observation of Cuthbert and Wong (1972) that the concentration of amiloride required to produce 50% inhibition of $I_{sc}$ in *R. temporaria* was increased about 400 times subsequent to the removal of external calcium (from 0.24 μM to 0.1 mM). The authors suggested that amiloride and Ca may form a ternary complex with the membrane receptor controlling Na entry in the frog skin. Species differences appear, again, to become manifest in these experiments because *R. pipiens* and the toad show much less sensitivity of the amiloride response to the presence of external Ca (Benos et al., 1976; Table V), and the present results in *R. catesbeiana* show that external Ca does not affect the inhibitory action of amiloride at all. Thus, it may be concluded that external Ca need not complex with amiloride to enhance the latter's ability in the inhibition of Na transport. Because Ca itself can influence $I_{sc}$ (Curran and Gill, 1962), and because this effect is separate from any it might have on amiloride binding (Benos et al., 1976), any change that calcium can mediate in promoting the effectiveness of amiloride may in some way be dependent upon the structural 'flexibility' of the receptor moiety. Furthermore, because this action of Ca is independent of its effects of $I_{sc}$, these effects must be occurring at separate regions of the membrane, one perhaps being absent in bullfrog skin. Insight into the molecular architecture of the Na entry mechanism in these amiloride-sensitive epithelia will be achieved once the chemical structures of the calcium and amiloride binding regions are learned.

**APPENDIX**

Derivation of Hill Equations for Multi-Substrate and Multi-Inhibitor Analyses

*Enzyme with Multiple Catalytic Sites*

In this analysis we will assume, as before, that the Na entry unit is analogous to an enzyme with sodium as its substrate. The velocity equation for a unit containing $n$ identical sites can be derived from rapid equilibrium assumptions and is given by (see Segel, 1975) for derivation):

$$I_{sc} = \frac{I_{max}[Na](1 + [Na]/K_d)^{n-1}}{K_t(1 + [Na]/K_t)^n}, \quad (1a)$$

where $n$ is the number of identical sites and the other terms have the meanings defined earlier. This equation reduces to Eq. 1 in the text, if $n = 1$.

The main assumption made in deriving the Hill equation is that a strong cooperative interaction occurs upon the binding of substrate to the enzyme. Under these conditions, most of the enzyme-substrate complexes will be in the form containing $n$ molecules of substrate per enzyme and, thus, the velocity equation will be dominated by the $[Na]^n$ terms. Eq. (1a) can then be simplified to

$$I_{sc} = \frac{I_{max}[Na]^n}{[Na]^n + K_t^n}, \quad (2a)$$
Multiplying both sides by \((\lbrack \text{Na} \rbrack_n + \lbrack \text{K} _t \rbrack_n)\) and rearranging, we obtain

\[ I_{sc} \cdot K_t^n = (I_{\text{max}} - I_{sc}) \lbrack \text{Na} \rbrack^n. \]  

(3 a)

Dividing both sides by \((I_{\text{max}} - I_{sc})\) and \(K_t^n\):

\[ \frac{I_{sc}}{I_{\text{max}} - I_{sc}} = \frac{\lbrack \text{Na} \rbrack^n}{K_t^n} \]  

(4 a)

where \(K' = K_t^n\).

Taking logarithms, we have

\[ \log \left( \frac{I_{sc}}{I_{\text{max}} - I_{sc}} \right) = n \log \lbrack \text{Na} \rbrack - \log K'. \]  

(5 a)

which is Eq. 4 of the text.

Thus, a plot of \(\log (I_{sc}/I_{\text{max}} - I_{sc})\) vs. \(\log \lbrack \text{Na} \rbrack\) should yield a straight line in slope \(n\).

In practice, when experimental velocity data are analyzed in terms of a Hill equation, deviations from linearity can occur and \(n\) can take on non-integral values. The major reason for this is that if the cooperativity of substrate binding is not very marked (i.e., the sites are indeed independent), then the velocity equation will not reduce to the Hill equation, and thus \(n\) will not equal the actual number of substrate binding sites. The Hill equation ignores the contributions towards the observed velocity of complexes containing less than \(n\) molecules of \(\lbrack \text{Na} \rbrack\). This contribution may become significant if the binding interactions are weak. The true number of binding sites per molecule of enzyme can only be determined from equilibrium binding studies.

**Multisite, Nonexclusive, Noncompetitive Inhibition**

If an inhibitor molecule is noncompetitive with substrate (i.e., no effect on \(K_t\), as shown for amiloride), if only a single substrate molecule is involved, and if all terms containing less than \(n\) molecules of amiloride can be ignored, the following rate equation can be derived from rapid-equilibrium assumptions (Segel, 1975):

\[ I_{am} = \frac{I_{\text{max}} \lbrack \text{Na} \rbrack}{(\lbrack \text{Na} \rbrack + K_t)(1 + \lbrack \text{amiloride} \rbrack^n/K_t^n)}, \]  

(6 a)

where \(K_t\) is the intrinsic dissociation constant of amiloride binding, and \(I_{am}\) is the short-circuit current observed at a particular amiloride concentration.

Inasmuch as \(I_{sc} = I_{\text{max}}\lbrack \text{Na} \rbrack/(K_t + \lbrack \text{Na} \rbrack)\), dividing both sides of Eq. 6 a by \(I_{sc}\) (the current observed in the absence of amiloride), we obtain

\[ \frac{I_{am}}{I_{sc}} = \frac{1}{1 + \lbrack \text{amiloride} \rbrack^n/K_t^n}. \]  

(7 a)

Rearranging this equation and taking logarithms of both sides, we obtain

\[ \log \left( \frac{I_{am}}{I_{sc} - I_{am}} \right) = -n \log \lbrack \text{amiloride} \rbrack + \log C'. \]  

(8 a)

where \(C' = K_t^n\).

This equation has the same basic assumptions as that made in the first section of this Appendix. With reference to the data presented in Fig. 6, the \(y\) intercept is equal to \(-3.18\). If we assume that \(K_t = 8 \times 10^{-7}\text{M}\) (Fig. 1, \([\text{Na}] = 110\ \text{mM}\)), we can predict from Eq. 8 a that the \(y\) intercept should be \(\log ((8 \times 10^{-7})^{8.3}) = -3.05\). We thus conclude that Eq. 8 a adequately describes the velocity data of amiloride-induced inhibition of \(I_{sc}\) in frog skin as well as supporting the contention that amiloride is a noncompetitive inhibitor.
We would like to thank Ms. Teresa Rummans and Ms. Kathie Collatos for their valuable technical assistance, and Ms. Joyce Cannon for typing the manuscript. We also thank Dr. Edward J. Cragoe, Jr., of Merck Sharp & Dohme, West Point, Pa., for the generous gift of amiloride. This work was supported by grants AM-05624 and AM-16024 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

Received for publication 12 May 1978.

REFERENCES

BAER, J. C., C. B. JONES, S. A. SPITZER, and H. E. Russo. 1967. The potassium sparing and natriuretic activity of N-amidino-3,5-diamino-6-chloropyrazine carboxamide hydrochloride dihydrate (amiloride hydrochloride). J. Pharmacol. Exp. Ther. 157:472–485.

BENOS, D. J., and L. J. MANDEL. 1978. Irreversible inhibition of sodium entry sites in frog skin by a photosensitive amiloride analogue. Science (Wash. D.C.). 199:1205–1206.

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.

BENTLEY, P. J. 1968. Amiloride: a potent inhibitor of sodium transport across the toad bladder. J. Physiol. (Lond.). 195:317–330.

BIBER, T. U. L. 1971. Effect of changes in transepithelial transport on the uptake of sodium across the outer surface of the frog skin. J. Gen. Physiol. 58:131–144.

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: 83–99.

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.

CANDIA, O. A. and P. S. REINACH. 1977. Sodium washout kinetics across inner and outer barrier of the isolated frog skin epithelium Biochem. Biophys. Acta. 468:341–352.

CEREIJIDO, M., F. HERRERA, W. J. FLANIGAN, and P. F. CURRAN. 1964. The influence of Na concentration of Na transport across frog skin. J. Gen. Physiol. 47:879–893.

CEREIJIDO, M., and C. A. ROTUNNO. 1968. Fluxes and distribution of sodium in frog skin: a new model. J. Gen. Physiol. 51:280s–289s.

CURRAN, P. F., and J. R. GILL. 1962. The effect of calcium on sodium transport by frog skin. J. Gen. Physiol. 45:625–649.

CUTHBERT, A. W. 1973. An upper limit to the number of sodium channels in frog skin epithelium. J. Physiol. (Lond.). 228:681–692.

CUTHBERT, A. W. 1974. Interactions of sodium channels in transporting epithelial: a two-state model. Mol. Pharmacol. 10:892–903.

CUTHBERT, A. W., 1976 a. Importance of guanidinium groups for blocking sodium channels in epithelia. Mol. Pharmacol. 12:945–957.

CUTHBERT, A. W. 1976 b. Receptors controlling sodium ion translocation in biological membranes. J. Pharm. Pharmacol. 28:383–388.

CUTHBERT, A. W., and W. K. SHUM. 1974. Amiloride and the sodium channel. Naunyn-Schmiedeberg's Arch. Pharmacol. 281:261–269.

CUTHBERT, A. W., and W. K. SHUM. 1976. Characteristics of the entry process for sodium in transporting epithelia as revealed with amiloride. J. Physiol. (Lond.). 255: 587–604.

CUTHBERT, A. W., and P. Y. D. WONG. 1972. The role of calcium ions in the interaction of amiloride with membrane receptors. Mol. Pharmacol. 8:222–229.
Dixon, M., and E. C. Webb. 1964. Enzymes. Academic Press, Inc., New York. 2nd edition. 950 pp.

Dowd, J. E., and D. S. Riggs. 1965. A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. J. Biol. Chem. 240: 863-869.

Eadie, G. S. 1942. The inhibition of cholinesterase by phystostigmine and prostigmine. J. Biol. Chem. 146:85-93.

Gottlieb, G. P., K. Turnheim, R. A. Frizzell, and S. G. Schultz. 1978. p-Chloromercuribenzenene sulfonate blocks and reverses the effect of amiloride on sodium transport across rabbit colon in vitro. Biophys. J. 22:125-129.

Hille, B. 1975 a. An essential ionized acid group in sodium channels. Fed. Proc. 34:3118-3121.

Hille, B. 1975 b. The receptor for tetrodotoxin and saxitoxin. A structural hypothesis. Biophys. J. 15:615-618.

Kao, C. Y., and A. Nishiyama. 1965. Actions of saxitoxin on peripheral neuromuscular systems. J. Physiol. (Lond.). 180:50-66.

Kirschner, L. B. 1955. On the mechanism of active sodium transport across the frog skin. J. Cell. Comp. Physiol. 45:61-87.

Lindemann, B. 1975. A modifier-site model for passive Na+ transport into frog skin epithelium. In Intestinal Permeation, Vol. 4. M. Kramer and F. Lauterbach, editors. Excerpta Medica, Amsterdam. 217-228.

Lindemann, B., and W. Van Driessche. 1977. Sodium-specific membrane channels of frog skin are pores: current fluctuations reveal high turnover. Science (Wash. D.C.). 195:292-294.

Mandel, L. J. 1978. Effects of pH, Ca, ADH, and theophylline on the kinetics of Na entry in frog skin. Am. J. Physiol. 235:C35–C48.

Mandel, L. J., D. J. Benos, and S. A. Simon. 1978. Chemical probing of the Na-entry mechanism in frog skin epithelium with site-specific reagents. Sixth International Biophysics Congress, Kyoto, Japan.

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.

Moreno, J. H., I. L. Reisin, E. Rodriguez Boulan, C. A. Rotunno, and M. Cerrejido. 1973. Barriers to sodium movement across frog skin. J. Membr. Biol. 11:99–115.

Rawlings, F., L. Mateu, F. Fragachan, and G. Whittenbury. 1970. Isolated toad skin epithelium: transport characteristics. Pfluegers Arch. Eur. J. Physiol. 316:64–80.

Rotunno, C. A., F. A. Vilallonga, M. Fernandez, and M. Cerrejido. 1970. The penetration of sodium into the epithelium of the frog skin. J. Gen. Physiol. 55:716-735.

Salako, L. A., and A. J. Smith. 1970. Changes in sodium pool and kinetics of sodium transport in frog skin produced by amiloride. Br. J. Pharmacol. 39:99–109.

Segel, I. H. 1975. Enzyme Kinetics. John Wiley & Sons, Inc., New York. 957 pp.

Schultz, S. G., and R. Zalusky. 1964. Ion transport in isolated rabbit ileum. I. Short-circuit current and Na fluxes. J. Gen. Physiol. 47:567–584.

Ussing, H. H., and K. Zerahs. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol. Scand. 23:110-127.