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Interdependence of the Two Borders in a Sodium Transporting Epithelium. Possible Regulation by the Transport Pool

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Summary. Specific binding of ^{14}C-amiloride to the mucosal surface of frog skin epithelium (*Rana temporaria*) has been used as a measure of the number of sodium entry sites. All binding measurements were made with the mucosal surface bathed in a solution containing 1.1 mM sodium. When manipulations were used which increased the intracellular concentration of sodium the amount of amiloride bound was reduced. The manipulations included flushing the mucosal surface with solutions containing 111 mM sodium after serosal efflux was inhibited with ouabain or potassium removal. Similar results were obtained when cells were loaded with lithium.

These effects on amiloride binding did not appear to depend on changes in membrane potential or upon changes in affinity of amiloride for its binding site. It appears that inhibition of serosal sodium efflux from the epithelium causes a reduction of mucosal sodium influx by making entry sites unavailable. This latter may be a result, directly or indirectly, of the sodium concentration in the sodium transport pool.

The inhibition of sodium transport in frog skin by ouabain, first described by Koefoed-Johnsen (1957), formed part of the evidence upon which the two-series barrier model of epithelial sodium transport (Koefoed-Johnsen & Ussing, 1958) was based. The Ussing model, as it has become known, has dominated thinking on epithelial transport processes for two decades. During this time there have been numerous studies designed to look at the properties of the two-series barriers individually, that is, the entry step at the mucosal surface and the exit step at the serosal side.

Arguments which favor an action of ouabain at the serosal surface of sodium transporting epithelia are extensive. For example, the glycoside is effective only when applied to the serosal surface (Bonting & Canady, 1964; Asano *et al.*, 1970) and Na. K ATPase has been demonstrated histochemically at the basolateral borders (Keller, 1963; Farquhar & Palade, 1966; Mills & Ernst, 1975).

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Recently evidence suggesting that inhibition of the serosal sodium pump modifies the properties of the mucosal face has been obtained. It was found in frog skin that preincubation with ouabain reduced sodium uptake at the mucosal surface (Biber, 1971), but only if the preincubation was carried out in sodium-containing solutions (Erlij & Smith, 1973). Conductance measurements in toad skin indicate that the sodium conductance of the apical membrane is decreased by inhibition of the serosal pumping mechanism (Larsen, 1973), as is the uptake of sodium through the apical surface of toad bladder (Finn, 1975). Changes in intracellular sodium concentration or of potential across the apical membrane have been suggested as the cause of altered apical membrane permeability.

We have attempted to measure the properties of the apical membrane more directly under both control conditions and after transport had been inhibited with ouabain. To do this we have used frog skin (*Rana temporaria*) and have made binding measurements with ^{14}C-amiloride, an agent known to inhibit sodium entry through the mucosal face by combination with the translocation mechanism (Bentley, 1968; Ehrlich & Crabbé, 1968; Dörge & Nagel, 1970; Nagel & Dörge, 1970; Salako & Smith, 1970*a–b*). It has been found that procedures designed to increase the intracellular concentration of sodium reduce the number of sites which can be labeled by a given amiloride concentration. Other experiments indicate that it is unlikely that increased intracellular sodium leads to substantial changes in affinity for amiloride. Thus, the findings might indicate that sites for sodium entry become increasingly unavailable as the intracellular sodium concentration is raised.

Materials and Methods

All experiments were performed on the abdominal skins taken from frogs, *Rana temporaria*, stored in tanks at room temperature. Binding of ^{14}C-amiloride was measured as described elsewhere in short-circuited skins (Cuthbert, 1973; Cuthbert & Shum, 1974*a–b*; 1967*a–b*). Binding was measured with the mucosal surface bathed in low sodium Ringer's (1.1 mM), while the serosal solution was bathed in normal Ringer's solution (111 mM Na^+). This procedure increases the apparent affinity of amiloride (Cuthbert, 1973; Cuthbert & Shum, 1974*b*) and is an essential requirement with this ligand for the detection of specific binding. Throughout the specific binding has been taken as the difference between the amount of radiolabel retained in the presence of amiloride (10^{-8} M and 54 C/mole) and that retained in the presence of amiloride (10^{-6} M and 0.54 C/mole). Skins (9.6 cm^2) were short-circuited throughout with a Schema Versetae, 360-c voltage clamp. Previously we have stated binding results as binding sites/μm^2 of mucosal surface. To derive these values we have used the product of the amount bound and the reciprocal of the fractional occupancy. The fractional occupancy

was taken as the fractional reduction in short-circuit current (SCC) caused by 10^{-8} M amiloride. On this occasion we are unable to do this because ouabain has been used to inhibit sodium transport. With low sodium, solutions bathing the mucosal surface and, after ouabain, currents are reduced to a fraction of a microampere per cm^2. Inasmuch as such low currents cannot be measured with accuracy, binding in this study is given as pmole/cm^2. The amount of specific amiloride binding was calculated as follows:

$$\text{pmole cm}^{-2} = \frac{\text{DPM} \times 10^{12}}{2.2 \times 10^{12} \times \text{DPM} \cdot \text{C}^{-1} \times 54\ \text{C mole}^{-1} \times 9.6\ \text{cm}^2}.$$

Inhibition curves for amiloride in a variety of conditions were measured in short-circuited skins in conventional Ussing-type cells. The Ringer's solution used had the following composition (mM): NaCl, 111; KCl, 2; $CaCl_2$, 1; glucose, 11.1, and Tris buffer (pH 7.6), 5. Low sodium Ringer's had the same composition except that NaCl was reduced to 1.1 mM. In depolarizing Ringer's all the NaCl was replaced by an equivalent amount of KCl.

Results

Binding Measurements with ^{14}C-Amiloride

In a preliminary set of experiments displaceable ^{14}C-amiloride binding to the mucosal surface was measured first under control conditions and then approximately 1 hr after treatment of the serosal surface with ouabain, 10^{-3} M. Fifteen separate determinations were made in three separate skins, and the results are shown in Table 1.

The upper part of the table shows the displaceable binding measured at an amiloride concentration of 10 nM for 3 separate skins. Displaceable binding accounts for approximately 15% of the total binding, as was found previously (Cuthbert & Shum, 1974*a*, 1976*a–b*). Details are given of the total binding (T) and the nondisplaceable binding (N). It is seen that ouabain has no significant effect on specific amiloride binding (Δ), although there is an increase in total binding. The result fits well with those of Erlij and Smith (1973) who found ouabain had no effect on sodium influx at low sodium concentrations, if the assumption is made that the specific binding sites for amiloride are those through which sodium influx occurs. The results of Table 1 also can be considered as a control to show that high concentrations of ouabain applied to the serosal surface do not *per se* affect amiloride binding at the mucosal surface, assuming that the affinity of amiloride remains unchanged. This latter point will be considered in more detail later on in *Results.* Although binding measurements were made at low sodium (1.1 mM), the SCC was monitored in normal Ringer's during the onset of ouabain

Table 1. ^{14}C-amiloride (10 nM) binding (pmole cm^{-2}) in low sodium (1.1 mM)[a]

	Control		Ouabain	
1	0.026 ± 0.002	(5)	0.026 ± 0.006	(5)
2	0.030 ± 0.004	(5)	0.023 ± 0.006	(5)
3	0.017 ± 0.004	(5)	0.015 ± 0.009	(5)
T	0.151 ± 0.008	(15)	0.208 ± 0.005	(15)
N	0.126 ± 0.008	(15)	0.186 ± 0.005	(15)
Δ	0.025 ± 0.003	(15)	0.021 ± 0.004	(15)
P	<0.001	NS	<0.001	

[a] Effects of ouabain (1 mM).

The results for five determinations on three separate skins are given at the top of the table. Below is given the total binding (*T*) for all measurements using ^{14}C-amiloride (10 nM) and the nondisplaceable binding (*N*) using ^{14}C-amiloride (10 nM) with unlabeled amiloride (1 μM). *Δ* is the difference between *T* and *N* and represents displaceable binding. The significance of this difference was obtained by applying a paired *t*-test using the paired differences obtained in the separate determinations. The significance of the difference between specific binding under test and control conditions was also estimated with a *t*-test. Throughout mean values and standard errors are shown. The numbers of measurements are given in parentheses.

inhibition. After 1 hr at a ouabain concentration of 1 mM the SCC was inhibited by at least 98%; so, it is clear that in these experiments the pump had been inhibited.

The intention in these experiments was to examine the effects of increased intracellular sodium on amiloride binding. Inasmuch as the measurements were made with low sodium Ringer's bathing the mucosal surface, it is unlikely that intracellular sodium was much increased after ouabain. Studies with the electronprobe (Dörge, Rick & Thurau, 1976) have shown that after pump inhibition sodium can enter and leave the transporting cells only through the mucosal face, i.e., the basolateral membranes are tight to sodium after pump inhibition. The electronprobe results suggest a way to increase the intracellular sodium concentration while maintaining the mucosal surface under low sodium conditions. If a tissue bathed on the mucosal face with low sodium Ringer's is flushed with normal Ringer's solution, sodium influx and SCC will increase, as presumably will the concentration of intracellular sodium (Erlij & Smith, 1973). On returning to low sodium conditions, the transport pool will be rapidly emptied by pumping through the serosal face and by efflux through the mucosal face. After ouabain, however, the former of these

two routes will be unavailable, and presumably an increased intracellular sodium concentration will be maintained longer, particularly if serosal pumping is the main exit route in unpoisoned skins, as seems likely. The feasibility of this approach is supported by the experiments reported in Table 2.

Five determinations of amiloride binding were made in each of three skins initially with the mucosal solution containing sodium-free Ringer's solution. Ouabain (1 mM) was applied to the serosal surface for approximately 1 hr, and SCC was monitored with Ringer's solution bathing both sides of the skin. The mean inhibition of SCC was 87% for 3 skins. Afterwards binding was remeasured in zero sodium solution but immediately (within 1 min) following exposure to normal Ringer's solution. In these experiments ouabain significantly ($P<0.02$) reduced the amount of amiloride bound specifically to the skin surface. Before ouabain the amount of specific binding was similar to that found in the experiments reported in Table 1, but afterwards binding was reduced to 50% of the control value. As noted before, ouabain increased the amount of nonspecific binding. This effect is considered unimportant and may not be due to ouabain at all, for we have remarked previously that the amount of nonspecific binding increases gradually during the course of an experiment (Cuthbert & Shum, 1974*a*).

Whereas amiloride and sodium show competition (Cuthbert & Shum, 1974*b*), it might be argued that reduced binding after ouabain is a result

Table 2. ^{14}C-amiloride (10 nM) binding (pmole cm^{-2}) in zero sodium solution[a]

	Control	Ouabain
1	0.030 ± 0.005 (5)	0.011 ± 0.007 (5)
2	0.033 ± 0.008 (5)	0.019 ± 0.011 (5)
3	0.021 ± 0.006 (5)	0.012 ± 0.006 (5)
T	0.176 ± 0.009 (15)	0.211 ± 0.006 (15)
N	0.148 ± 0.009 (15)	0.197 ± 0.007 (15)
A	0.028 ± 0.004 (15)	0.014 ± 0.004 (15)
P	<0.001	<0.02 <0.005

[a] Effects of temporary exposure to high sodium (111 mM) after ouabain (1 mM).

The symbols and arrangement of this table are as for Table 1. Binding was measured initially with the mucosal solution exposed to Ringer's solution without sodium. Following exposure of the serosal surface to ouabain (1 mM) for 1 hr the mucosal surface was exposed to normal Ringer's (111 mM) Na for 5 min, following which it was returned to zero sodium Ringer's (6 changes of solution taking less than 1 min) before binding was remeasured.

of residual sodium (from exposure to Ringer's solution) not being washed away before binding measurements are made. Consequently the experiments were repeated, but with a brief exposure to Ringer's solution preceding all measurements of binding, both before and after exposure to ouabain.

The results of 25 separate measurements made in five separate skins are given in Table 3. In this series the ouabain concentration was reduced to 10 μM. This concentration gave a mean inhibition of SCC of 95% in 1 hr during which the current was monitored with the skins bathed on both sides with normal Ringer's solution. The table shows that a prewash with high sodium Ringer's made no substantial difference to the amount of displaceable (specific) binding which was detected on subsequent exposure to the low sodium Ringer's. However, exposure to ouabain caused, in this series, a 65% reduction in the amount of label specifically bound, so confirming the result given in Table 2.

The effects of ouabain on SCC in frog skin are rather slowly reversible, and the skins are particularly difficult to wash effectively on the serosal side in the type of cell used for making binding measurements (Cuthbert, 1973). We were concerned that some component of the reduction of binding seen with ouabain was a consequence of the duration of the experiment, particularly as control and test parts of the experiments separated by at least one hour, during which time skins were

Table 3. ^{14}C-amiloride (10 nM) binding (pmole cm^{-2}) in low sodium (1.1 mM) following a high sodium (111 mM) flush[a]

	Control	Ouabain
1	0.016 ± 0.003 (5)	0.002 ± 0.009 (5)
2	0.023 ± 0.006 (5)	0.007 ± 0.003 (5)
3	0.020 ± 0.003 (5)	0.002 ± 0.008 (5)
4	0.032 ± 0.007 (5)	0.011 ± 0.004 (5)
5	0.029 ± 0.009 (5)	0.020 ± 0.011 (5)
T	0.168 ± 0.010 (25)	0.202 ± 0.008 (25)
N	0.144 ± 0.009 (25)	0.194 ± 0.007 (25)
Δ	0.024 ± 0.003 (25)	0.008 ± 0.003 (25)
P	< 0.001	< 0.001 < 0.05

[a] Effects of ouabain (10 μM).

The symbols and arrangement of this table are as for Table 2. All binding measurements were made in low sodium (1.1 mM) but always immediately following a 5-min exposure to Ringer's solution (111 mM Na) followed by six washes in low sodium Ringer's taking less than 1 min. Notice that in these experiments the ouabain concentration was reduced to 10 μM.

exposed to ouabain and the SCC allowed to achieve a new steady state. Consequently experiments were performed using potassium-free Ringer's solution to bathe the serosal surface, this strategy producing pump inhibition as an alternative to ouabain. In these experiments binding under potassium-free conditions was measured first, after which normal Ringer's was used to bathe the serosal surface. It will be seen from Table 4 that pump inhibition with potassium-free solution also decreased specific amiloride binding, as judged by the increase which appeared when potassium was restored. Consequently, we can conclude that the results given in Tables 1–3 are not simply a consequence of the duration of the experiments but must be dependent upon changes caused by pump inhibition. In these experiments amiloride binding was measured following a prewash with high sodium solution for the reasons given previously. Table 4 shows once again that the amount of nondisplaceable binding increased in the second part of the experiment (after serosal K^+ was added), providing further evidence that this increase is a function of time, rather than of the various manipulations used or the inhibitors which are applied.

As discussed in the introduction, pump inhibition has been shown to reduce sodium influx through the mucosal face of frog skin, and it was suggested that either changes in intracellular concentration or of membrane potential might be responsible for the decrease in permeability (Erlij & Smith, 1973; Biber, 1971). In order to try to differentiate between these possibilities, we have made use of the potassium depolarized preparation described first by Ussing, Biber and Bricker (1965) and used extensively by Morel and Leblanc (1975*a–b*) and by Fuchs, Hviid Larsen

Table 4. ^{14}C-amiloride (10 nM) binding (pmole/cm^{-2}) in low sodium (1.1 mM) solution following a high sodium (111 mM) flush[a]

	Zero serosal K^+		Plus serosal K^+	
1	0.022 ± 0.002	(5)	0.034 ± 0.005	(5)
2	0.025 ± 0.007	(5)	0.034 ± 0.003	(5)
T	0.136 ± 0.008	(10)	0.196 ± 0.006	(10)
N	0.113 ± 0.008	(10)	0.162 ± 0.006	(10)
Δ	0.023 ± 0.003	(10)	0.034 ± 0.003	(10)
P	<0.001		<0.05	0.001

[a] Effects of omitting serosal K.

The symbols and arrangement of this table are as for Table 1.

and Lindemann (1977). In this preparation the serosal surface is bathed with a solution rich in potassium (all the sodium chloride of Ringer's solution replaced with potassium chloride). In this situation the potential and resistance of the basolateral membranes are reduced to low values. Application of a transepithelial voltage clamp allows the mucosal membrane to be clamped at values close to zero. For a discussion of the clamping errors in this technique, *see* Fuchs *et al.* (1977).

Experiments were performed with 5 skins in which the serosal surface was bathed in potassium chloride Ringer's and the results are given in Table 5. Binding was measured under two conditions, i.e., with the transepithelial potential voltage clamped at either zero or -50 mV. At either potential binding measurements were made with low sodium Ringer's solution bathing the mucosal surface. It proved possible to measure specific amiloride binding in depolarized preparations, and binding was doubled when the transepithelial potential was increased to -50 mV from zero. This finding is in accord with previous findings in which binding was found to be potential dependent (Cuthbert & Shum, 1976*a*). When the transepithelial potential is held at -50 mV, virtually the whole of this potential drop will occur across the mucosal membrane. Recently it has been shown (Nagel, 1976; Helman & Fisher, 1976) that the intracellular potential in short circuited skins is very negative, especially when the mucosal surface is bathed in low sodium. Thus, the mucosal membranes in potassium depolarized skins have more normal potentials when the tissues are voltage clamped at -50 mV than when short circuited.

Table 5. ^{14}C-amiloride (10 nM) binding (pmole cm^{-2}) in low sodium (1.1 mM) in potassium depolarized skins[a]

	zero mV		-50 mV	
1	0.021 ±0.005	(5)	0.031 ±0.005	(5)
2	0.011 ±0.007	(5)	0.023 ±0.006	(6)
3	0.013 ±0.003	(5)	0.028 ±0.003	(5)
4	0.008 ±0.006	(4)	0.021 ±0.005	(5)
5	0.010 ±0.003	(4)	0.017 ±0.003	(5)
T	0.162 ±0.007	(23)	0.163 ±0.008	(26)
N	0.149 ±0.007	(23)	0.139 ±0.007	(26)
Δ	0.013 ±0.003	(23)	0.024 ±0.002	(26)
P	<0.001		<0.005	<0.001

[a] Effects of potential.

The symbols and arrangement of this table are as for Table 1.

In order to avoid any systematic errors in the experiments reported in Table 5, binding measurements in experiments 1, 3, and 5 were made first at zero transepithelial potential and then at -50 mV (serosa negative). In experiments 2 and 4 this order was reversed. Nonspecific binding in the first periods in experiments 1–5 amounted to 0.120 pmole cm^{-2}, whereas in the second periods nonspecific binding increased to 0.167 pmole cm^{-2}. This again confirms that nonspecific binding increases throughout the experiment, a finding not discernable from Table 5 because of the randomization referred to above.

A further six experiments were made with potassium-depolarized skins (Table 6), but in this series the serosal pump was inhibited by ouabain after control measurements of binding had been made. The concentration of ouabain used was high (1 mM) because of the high potassium concentration. However, such high concentrations of ouabain have no effect on amiloride binding *per se* (Table 1). Each binding measurement in this series was made in low sodium Ringer's but following a high sodium flush. As before, when serosal efflux was inhibited with ouabain specific binding with amiloride was reduced. In these experiments the mucosal membrane was clamped at zero both before and after ouabain so it is unlikely that the changes in binding could have been due to changes in mucosal membrane potential.

A final set of binding measurements was made in which we attempted to prevent efflux across the serosal surface without using ouabain to inhibit the pump. To do this, lithium was used as a poorly transported

Table 6. ^{14}C-amiloride (10 nM) binding (pmole cm^{-2}) in low sodium (1.1 mM) following a high sodium (111 mM) flush in potassium depolarized skins[a]

	Control	Ouabain
1	0.030 ± 0.005 (5)	0.014 ± 0.004 (5)
2	0.023 ± 0.005 (5)	0.006 ± 0.002 (5)
3	0.038 ± 0.008 (6)	0.023 ± 0.008 (5)
4	0.019 ± 0.003 (5)	0.011 ± 0.004 (5)
5	0.018 ± 0.003 (5)	0.014 ± 0.004 (5)
6	0.029 ± 0.009 (4)	0.015 ± 0.004 (4)
T	0.141 ± 0.005 (30)	0.188 ± 0.005 (29)
N	0.115 ± 0.005 (30)	0.175 ± 0.005 (29)
Δ	0.026 ± 0.003 (30)	0.014 ± 0.002 (29)
P	<0.001	0.001 <0.001

[a] Effects of ouabain (1 mM).

The symbols and arrangement of this table are as for Table 1.

ion which is less successfully pumped than sodium and so is accumulated in the tissue (Leblanc, 1972). The mucosal surfaces of skins were exposed to Ringer's solution in which NaCl was omitted and substituted with 25 mM LiCl. Apparently the electrical gradient across the mucosal surface allows the intracellular concentration of Li to rise to 60 mM when the outer bathing solution contains 25 mM LiCl (Nagel, 1977).

Binding measurements were made in two separate skins with the mucosal surface bathed in low sodium Ringer's both before and after the mucosal surface had been exposed to Lithium-Ringer's for 5 min. The results are given in Table 7, which shows that prior exposure to lithium decreased specific amiloride binding by approximately 65%. Notice that in one of the skins specific binding of amiloride was high. This skin had a SCC of 3.8 μA cm^{-2} when the mucosal surface was bathed in low sodium Ringer's, whereas the other skin had both a normal amount of binding and a normal SCC (1.6 μA cm^{-2}) in low sodium Ringer's. We have described previously a positive correlation between SCC and channel density (Cuthbert & Shum, 1976*b*; Cuthbert, 1977).

When skins bathed on the mucosal surface with low sodium Ringer's were washed with solutions containing 25 mM LiCl and no sodium, the SCC rose rapidly at first and then declined or showed oscillations. On returning to low sodium Ringer's the SCC was severely depressed and returned only slowly, or incompletely, to the original level (Fig. 1). This behavior is similar to that reported by Nagel (1977). If our interpretation of the binding data presented in this paper is correct, then the slow

Table 7. ^{14}C-amiloride (10 nM) binding (pmole cm^{-2}) in low sodium (1.1 mM)[a]

	Control	Lithium
1	0.028 ± 0.003 (5)	0.012 ± 0.006 (5)
2	0.061 ± 0.004 (5)	0.019 ± 0.004 (5)
T	0.134 ± 0.008 (10)	0.114 ± 0.002 (10)
N	0.089 ± 0.004 (10)	0.098 ± 0.003 (10)
Δ	0.045 ± 0.006 (10)	0.016 ± 0.003 (10)
P	< 0.001	< 0.005 < 0.005

[a] Effects of lithium.

The symbols and arrangement of the table are as for Table 1. Binding was measured initially in low sodium Ringer's (control) and then in low sodium Ringer's following exposure to lithium-Ringer's (25 mM) for 5 min. After exposure to lithium the mucosal surface was washed six times in low sodium Ringer's, this procedure taking less than 1 min.

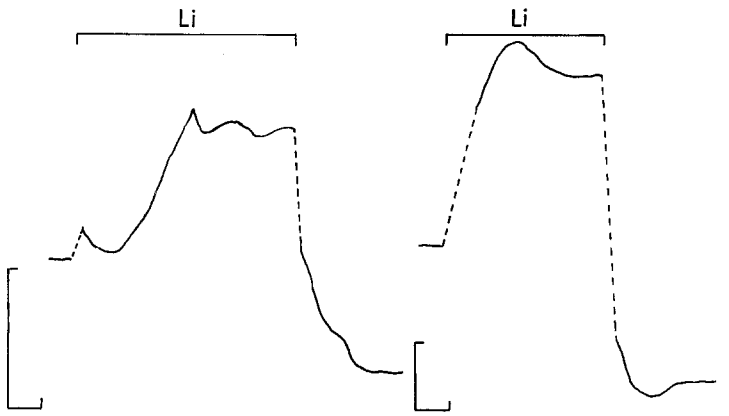

Fig. 1. The effects of Li on SCC. Skins were bathed on the serosal side in normal Ringer's throughout. Initially the mucosal surface was bathed in low sodium Ringer's which was then changed to 25 mM lithium Ringer's. Note that after the lithium was removed the SCC was depressed. Calibrations are 20 μA and 1 min. The time calibration also indicates zero SCC. Skin area 9.6 cm^2

recovery of SCC after Li may represent the reappearance of sodium entry sites in the mucosal face. We hope to be able to study this quantitatively in the future, using improved ligands with higher affinities.

Affinity of Amiloride and Competition of Amiloride with Sodium

Thus far we have described a number of situations in which intracellular sodium concentration is increased and in all instances the amount of specific binding which can be detected with amiloride at a concentration of 10 nM is reduced. At this stage it is important to establish whether inhibition of serosal efflux from the skin is associated with a change in affinity of the entry mechanism for amiloride.

When the mucosal solution contains only 1.1 mM sodium the SCC in *R. temporaria* is about 2 μA cm^{-2}. If transport is further inhibited by addition of ouabain to the serosal solution, currents fall to immeasurable values so it not possible to examine the inhibitory actions of amiloride on current under these conditions. Neither is it possible to determine a binding curve for amiloride in a single tissue. The inhibitory actions of amiloride, therefore, were examined in skins bathed on both sides in Ringer's solution before and after inhibition with ouabain. Affinity is defined as the reciprocal of the concentration of amiloride which inhibits

the amiloride-sensitive SCC by 50%. In most situations the amiloride-sensitive SCC is equal to the total SCC within 1–2%.

Cumulative concentration response curves to amiloride were measured under control conditions with Ringer's bathing both sides of the skin. Thereafter, ouabain at a suitable concentration (2×10^{-7} to 10^{-5} M) was added to the serosal surface to produce a new steady state in which the SCC stabilized at a value of 10–20% of the original SCC. The affinity of amiloride was then redetermined. Figure 2 gives typical concentration response curves for such an experiment, and Table 8 gives the results from 8 similar experiments. The mean value for amiloride affinity was reduced from 4.04×10^6 M^{-1} to 3.5×10^6 M^{-1}. Assuming the binding curve for amiloride is hyperbolic as shown by us previously (Cuthbert & Shum, 1974*a*), this change in affinity would account for only a 12% reduction in binding at an occupancy of 40% (10 nM amiloride causes approximately 40% reduction of SCC when the mucosal solution contains 1.1 mM Na^+), whereas reductions in binding of 33 to 65% have

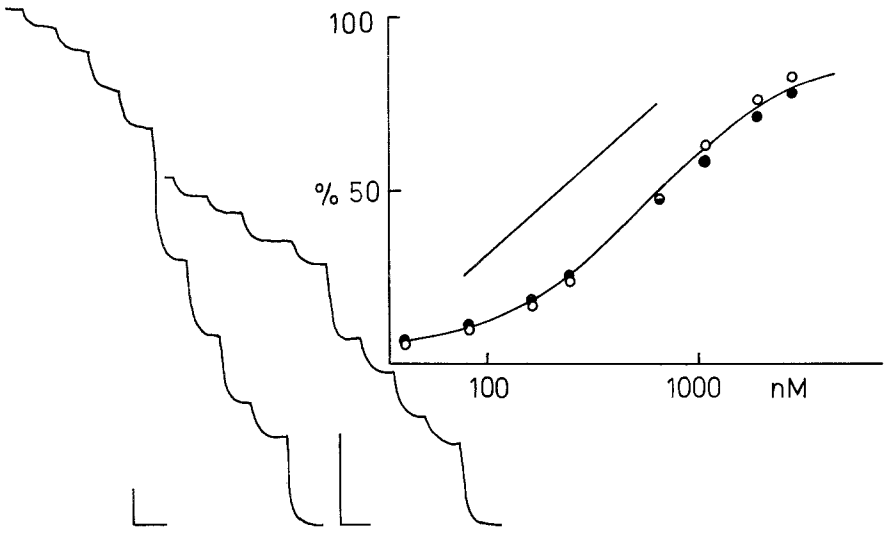

Fig. 2. Lack of effect of ouabain on affinity of amiloride. Responses to cumulative additions of amiloride to the solution bathing the mucosal surface of frog skin. The left hand tracing shows responses for a tissue bathed on both sides with normal Ringer's. Initially the resting SCC was 296 μA. Afterwards amiloride was washed away and ouabain, 2×10^{-7} M was added to the serosal solution. The SCC declined to a new steady-state value (75 μA) in 60 min when the sensitivity to amiloride was retested (right hand curve). Skin area was 7.1 cm^2, and the cumulative amiloride concentrations were 0.042, 0.083, 0.17, 0.26, 0.66, 1.1, 1.9, 2.7 and 100 μM. Calibrations are 20 μA and 2 min for each curve. Inset shows the relation between percentage inhibition and amiloride concentration for the control (○) and ouabain treated (●) condition. The unmarked line shows the theoretical slope, assuming simple mass action kinetics

Table 8. Effect of ouabain on the affinity of amiloride

	SCC (μA/7.1 cm^2)			K ($\times 10^6$ M^{-1})			% Binding (40%)
	Control	Ouabain	% Inhibition	Control	Ouabain	*Kc/Ko*	
1	129	10.8	92	6.67	4.55	1.47	78
1	84	16.8	80	5.55	7.14	0.78	118
3	296	75.0	75	1.43	1.32	1.09	100
4	124	53.0	57	1.96	3.03	0.65	130
5	170	70.5	59	3.44	2.86	1.21	93
6	235	46.5	80	4.76	2.44	1.95	70
7	213	21.5	90	1.67	1.25	1.33	88
8	139	24.8	82	6.90	5.41	1.28	91
	174±25	39.9±8.8	77±5	4.05±0.79	3.50±0.73	1.22±0.14	96±7
P	<0.001			NS			

Table shows affinities expressed as reciprocals of concentrations causing 50% inhibition of SCC. *Kc/Ko* is the ratio of the affinities before and after ouabain. % Binding (40%) indicates the amount of binding expected after ouabain as a percentage of that obtained before inhibition of transport and at an occupancy of 40%. Note there is no significant difference in *K* values before and after ouabain using either an unpaired or paired *t*-test.

been obtained. The last column in Table 8 shows the percentage binding to be expected after ouabain compared to control at an occupancy of 40%, assuming binding and inhibition curves can be superposed (*see* Cuthbert & Shum, 1976*a*).

The overall conclusion from this study is that ouabain has no substantial effect on the affinity of amiloride and the small insignificant change cannot account for the changes in binding reported in the earlier part of this paper. However, it must be remembered that affinity has been measured at high Na concentrations, so we cannot be absolutely sure of the situation at low Na concentrations.

In two experiments not included in Table 8, one of which is illustrated in Fig. 3, the slope of the inhibition curve to amiloride changed after sodium transport had been inhibited by ouabain. The inhibition curves became steeper and no longer had the slope expected from mass action kinetics; furthermore, the apparent affinity was considerably reduced. We feel that the change in slope signifies that in these conditions the entry of sodium though the mucosal face no longer determines the SCC, but rather it is dominated by the availability of pump sites. In such circumstances blockade of sodium entry and efflux though

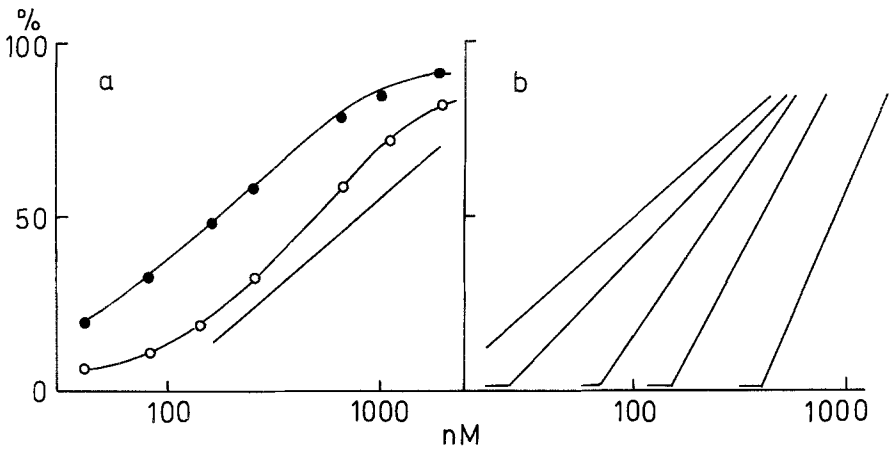

Fig. 3. (*a*): Inhibition curves for amiloride before and after ouabain. Resting SCC for the control (●) condition was 168 μA 7.1 cm^{-2}. Ouabain (2×10^{-7} M) caused the SCC to stabilize at a new steady state of 87 μA 7.1 cm^{-2}, at which time a further inhibition curve for amiloride (○) was determined. Notice the slope of this second curve is steeper than either the control curve or the theoretical slope assuming mass action kinetics. (*b*): Theoretical slopes for inhibition curves, assuming mass action kinetics and an affinity of 10^7 M^{-1}. From left to right the curves represent conditions where the SCC is unaffected until 0%, 20%, 40%, 60% and 80%, respectively, of the entry sites are blocked. In these situations it is assumed that mucosal influx of sodium exceeds serosal efflux, the excess influx being lost by mucosal efflux (*see*, for example, Biber & Sanders, 1973). More complicated situations in which, for example, excess mucosal influx is accompanied by an anion or loss of K^+ through the serosal surface are not considered

the mucosal face will have little effect on the SCC until the influx is reduced below the maximal pumping capacity. When this is achieved there will then be a steep increase in the fractional inhibition associated with a further increase in amiloride concentration. Also shown in Fig. 3 are theoretical inhibition curves for a tissue in which the pumping rate is less than the sodium influx. The general form of the theoretical curves is similar to those obtained experimentally. It is likely that serosal exit will not become rate limiting in low sodium Ringer's as used for measurement of binding.

From the foregoing we can find no substantial evidence that ouabain, and presumably increased intracellular sodium, affects the affinity of amiloride. This is quite different from the situation when the sodium concentration outside the mucosal surface is changed. We have noted before (Cuthbert & Shum, 1974*b*) that when the sodium concentration of the mucosal bathing solutions is reduced, the apparent affinity for amiloride is increased. In fact, the increase in apparent affinity at low

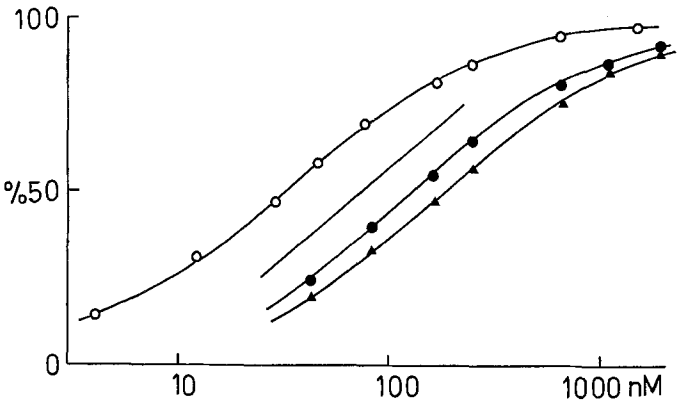

Fig. 4. Inhibition curves for amiloride. Curves show percentage reduction of the amiloride-sensitive SCC *vs.* the amiloride concentration for a single tissue. Initially the skin was bathed on both sides with normal Ringer's (▲) and the resting SCC was 84 μA. When the mucosal solution contained only 5.5 mM Na^+ (○) the SCC fell to 19 μA. After ouabain (10^{-5} M) and when the SCC stabilized at 17 μA with normal Ringer's bathing both sides of the skin, a further inhibition curve was obtained (●). The unmarked line gives the theoretical slope, assuming simple mass action kinetics

Table 9. Comparison of the effect of reduced mucosal sodium (5.5 mM) and of ouabain in the presence of high sodium (111 mM) on the affinity of amiloride

	Current (μA/7.1 cm^2)			K ($\times 10^6$ M^{-1})		
	111 mM Na	5.5 mM Na	111 mM Na plus ouabain	111 mM Na	5.5 mM Na	111 mM Na plus ouabain
1	84	18.9	16.8	5.6	30.3	7.1
2	129	18.5	10.8	6.7	23.3	4.6
3	139	19.8	24.8	6.9	37.7	5.4
mean	117	19.1	17.5	6.4	30.4	5.7

sodium concentration is responsible for the success of the labeling method we have used in this paper.

The increase in affinity at low mucosal sodium concentration is not a consequence of the low SCC, but rather of low sodium concentration. This point is emphasized by the experiment illustrated in Fig. 4. Initially the skin had a SCC of 84 μA, and amiloride had an affinity of 5.6×10^6 M^{-1} when the skin was bathed on both sides with Ringer's. When the sodium concentration of the mucosal bathing solution was reduced to 5.5 from 111 mM the affinity increased to 3.03×10^7 M^{-1} with a current

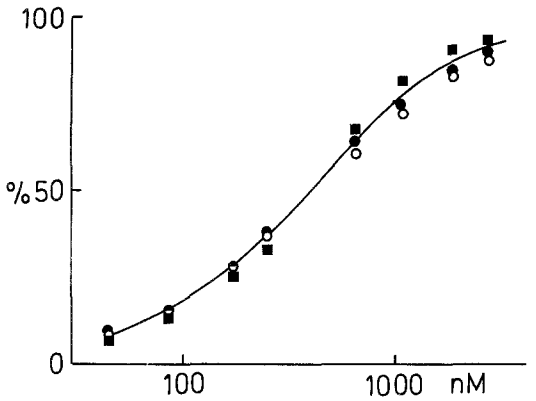

Fig. 5. Inhibition curve for amiloride in depolarized skin. Percentage reduction of the amiloride-sensitive SCC is plotted *vs.* amiloride concentration. Initially the skin was bathed on both sides with normal Ringer's and two control curves were obtained, after which the serosal side was bathed in potassium Ringer's and a further inhibition curve obtained. The amiloride sensitive SCC was 60 μA (●) and 42 μA (○) in the control period and 67 μA (■) in the potassium depolarized condition. Skin area 7.1 cm^2

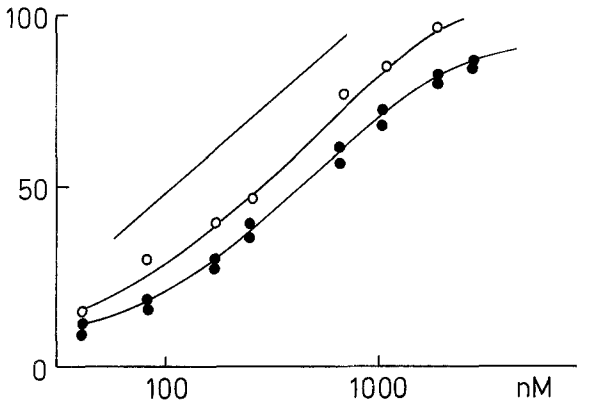

Fig. 6. Effect of ouabain on affinity of amiloride in potassium depolarized frog skin. Inhibition curves to amiloride were obtained with the mucosal surface bathed in Ringer's solution and the serosal surface bathed in potassium Ringer's. Two control curves (●) were obtained with initial amiloride-sensitive currents of 131 and 119 μA for 7.1 cm^2 of skin. Ouabain (10^{-3} M) was applied to the serosal surface for one hr at which time the amiloride-sensitive current was only 9.8 μA. The inhibition curve after ouabain is shown by open symbols (○). The line without symbols gives the theoretical slope, assuming simple mass action kinetics

of 19 μA. However, when the tissue was restored to normal Ringer's and the SCC reduced to 17 μA with ouabain the affinity of amiloride was only 7.14×10^6 M^{-1}. Results from three similar experiments are given in Table 9. It is concluded that when the sodium concentration is reduced

to 5% of normal the current falls to 17% of normal and the apparent affinity of amiloride increases by fivefold. Reduction of the current by the same amount in high sodium by ouabain makes little difference to the affinity.

The apparent affinity of amiloride was also tested in skins depolarized by bathing the serosal surface with potassium Ringer's. There was no change in affinity provided the mucosal sodium concentration remained constant either before or after serosal efflux of sodium had been inhibited by ouabain (Figs. 5, 6).

Sodium Conductance of the Mucosal Face

In the first two sections of *Results* we have found that manipulations which increase the intracellular sodium concentration reduce amiloride binding without apparently affecting affinity. In the discussion we shall consider if the findings can be interpreted in a meaningful way with regard to the mechanism of sodium entry through the mucosal face.

Further information can be derived from measurements of sodium conductance of the mucosal face, which in turn allows some speculation about the turnover number for each entry site.

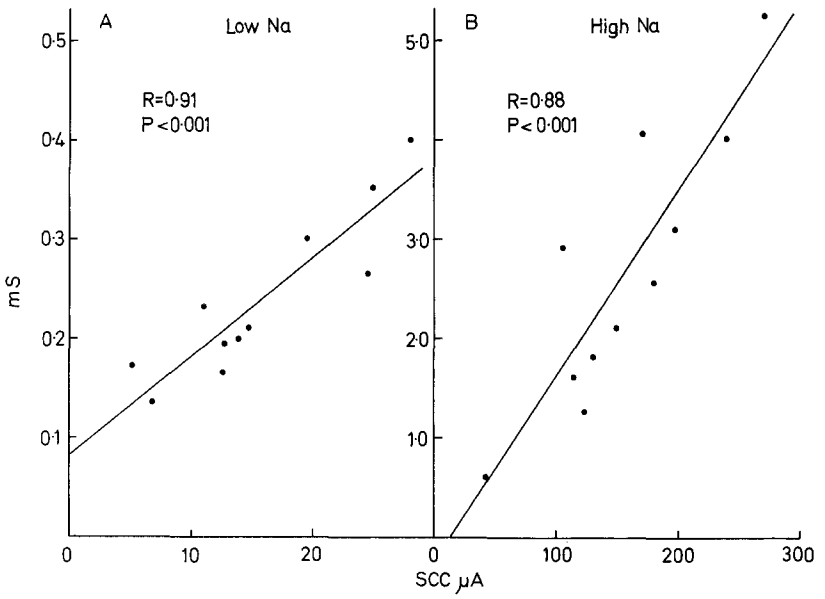

Fig. 7. Short-circuit current of 11 frog skins with mucosal solution containing low Na, 1.1 mM (*A*) and high Na, 111 mM (*B*) *vs.* conductance removed by amiloride, 10^{-4} M. Skin area, 7.1 cm^2. Note: amiloride may have a minor effect on leak conductance at low Na

To measure the conductance in short circuited skins, the potential was clamped for short intervals (2 sec) alternately at voltages 10 mV above and below zero. The resulting current pulses allowed the total skin conductance to be calculated. The sodium conductance of the mucosal face was defined as the conductance removed by the addition of amiloride, 10^{-4} M, to the mucosal bathing solution. Sodium conductance has been measured in eleven skins bathed on both sides with Ringer's solution and in the same eleven skins with low sodium Ringer's substituted for the mucosal solution. In each instance the SCC that could be maintained by the skins was noted and plotted against the sodium conductance for that skin (Fig. 7). A positive correlation was obtained between the SCC and sodium conductance at both high and low sodium concentrations.

Discussion

Binding measurements with ^{14}C-amiloride have revealed that manipulations which increase the intracellular concentration of sodium (or lithium) reduce the extent to which the mucosal face is labeled. Furthermore, this does not appear to be a consequence of a change in affinity, but rather of the availability of binding sites. Alternatively some sites may have zero affinity while the rest are unchanged. Provided specific binding can be taken as binding to sodium entry sites then the implication is that transporting cells can regulate sodium entry. Regulation is by positive feedback, i.e., influx is reduced when the intracellular concentration is increased.

The content of sodium in the compartment identified as the sodium transport pool is apparently unaltered by inhibition of the sodium pump by ouabain (Nagel & Dörge, 1971; Nagel *et al.*, 1974). These conclusions, however, were reached using skins bathed throughout in Ringer's solution. What we have done is to inhibit the pump, expose the mucosal surface temporarily to high sodium, and then return to low sodium to measure binding. After inhibition of the pump the amount of ^{14}C-amiloride binding is reduced only after a high sodium flush, whereas in nonpoisoned skins a high sodium flush makes no difference to the extent of binding. Similarly sodium influx though the mucosal face is inhibited by ouabain only if the incubation is carried in the presence of sodium, but not if it is conducted in sodium-free conditions (Erlij & Smith, 1973). Thus it appears that the high permeability of the mucosal

face which exists under low sodium conditions allows the cells to fill up rapidly when suddenly exposed to high sodium containing solutions, with a consequent reduction in permeability. On restoration of low sodium conditions the compartments into which the sodium has flowed must empty within a minute or so as no reduction in binding is measured at that time. In contrast, if serosal exit is prevented by ouabain or K-free solutions or, alternatively, if the cells are filled with poorly pumped lithium ions, then reduced ^{14}C-amiloride binding is found, indicative of reduced permeability.

The potential across the mucosal face of the transporting cells in short-circuited skins is very negative when the mucosal solution is bathed in low sodium solution (Nagel, 1976). Figure 8 shows diagrammatically proposed potential profiles across the epithelium under a variety of conditions, Fig. 8*A* showing the situation existing in a short-circuited skin under the conditions used for labeling. The mucosal potential may derive, in part, from an *IR* drop across the mucosal face due to extracellular current flow generated at the pump, sodium ions carrying the current across the mucosal face. When the pump is inhibited by ouabain this driving force will be reduced; thus a chemical gradient is necessary to drive sodium into the cells. Presumably potassium also leaves the cells by the serosal face, and it is possible that effects on ^{14}C-amiloride binding may be due to a fall in potassium concentration rather

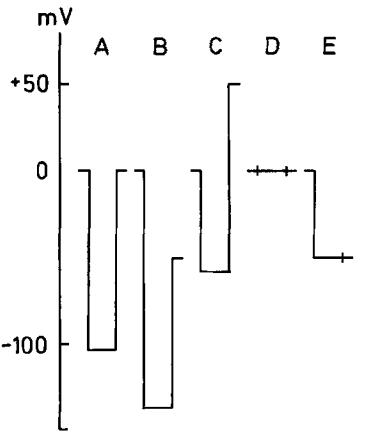

Fig. 8. Potential profiles across *stratum granulosum* of frog skin, moving from outside the mucosal membrane inwards (left to right), low sodium in the mucosal bathing fluid. (*A*): Short circuited. (*B*): Clamped with serosa −50 mV. (*C*): Clamped with serosa +50 mV. (*D*): Short circuited, serosa depolarized with high K. (*E*): Clamped with serosa −50 mV, with serosa depolarized with high K

than a rise in sodium concentration in the cells. Sodium exists in several compartments in frog skin epithelium, and these compartments may be interconnected (Morel & Leblanc, 1975*a*). Also the distribution of sodium within the compartments may be altered by pump inhibition. However, since we loaded epithelia with sodium (or lithium) from the mucosal surface, it is likely that the sodium concentration was increased in the sodium transport pool at the time we detected reduced binding.

We have tried to answer the question as to whether the regulation of amiloride binding sites is governed by the mucosal potential. It has already been demonstrated that the availability of binding sites is dependent on the transepithelial potential (Cuthbert & Shum, 1976*a*). When skins are hyperpolarized (Fig. 8*B*) binding site density increases, while the reverse is true for depolarized tissues (Fig. 8*C*). In the potassium depolarized skin the transmembrane potential of the mucosal face is theoretically zero (Fig. 8*D*). It is of particular interest that Lindemann and Van Dreissche (1977) found only 50 entry sites μm^{-2} in depolarized skins, whereas our values are 200–300 μm^{-2} in skins bathed on the mucosal surface in low sodium. This difference may well be due, in part, to the differences in transmembrane potential obtaining under the different experimental conditions.

When potassium depolarized skins are clamped at -50 mV (serosa) the potential profile to be expected is shown in Fig. 8*E*. Clamping skins at -50 mV would be expected to increase intracellular sodium, yet an increase, not a diminution, in binding was found. This suggests that the effects of potential and of intracellular sodium on binding are separable. Alternatively, when the mucosal membrane is clamped at zero in depolarized skins then an increase in intracellular sodium (following ouabain and a flush with high sodium) leads to a reduction in binding. It seems more reasonable to conclude that the availability of binding sites in this situation is determined by Na_i, since the transmembrane potential is held constant. When skins are bathed on both sides by Ringer's solution, the mucosal transmembrane potential is reduced (Nagel, 1976) and intracellular sodium concentration is increased over that obtaining with low mucosal sodium. Also at high sodium the mucosal surface behaves as if it contained only one entry site μm^{-2} (Lindemann & Van Dreissche, 1977).

It is important to remember at this time that we have measured displaceable binding at a single concentration of amiloride, so that changes in binding could result from either a change in availability of binding sites or a change in affinity. While changes in affinity are seen

with changes in external sodium concentration, we have no evidence of significant changes in affinity when the intracellular concentration of sodium is raised, provided the analysis is confined to those experiments where no change in slope of the inhibitor concentration effect curve from the theoretical slope is found.

The results of this study can be interpreted in, at least, two ways: first, that translocation across the mucosal membrane is by a carrier type mechanism, or, alternatively, that it proceeds by electrodiffusion through channel-like structures. This paradoxical situation implies that insufficient information is available to differentiate between these two, and other, mechanisms.

The instantaneous distribution of carrier molecules between the opposite faces of a membrane is determined by the concentration of ligands at the two faces, and the potential (*see* for example Haydon & Hladky (1972)). We have measured binding under conditions in which the concentration of ligands, sodium and amiloride, at the external face has been kept constant while the sodium concentration at the inner face has been varied. When the intracellular concentration has been (presumably) increased the amount of specific binding has been reduced, even when the membrane potential was held constant.

However, we have no evidence that the binding site can move from one face to the other of the membrane. For example, raised intracellular sodium or, alternatively, lowered intracellular potassium or even altered intracellular calcium might regulate or desensitize the translocation apparatus so that it no longer binds amiloride or sodium. Fuchs *et al.* (1977) have proposed a similar regulatory role for high concentrations of sodium acting from the outer surface. There are precedents for such arguments in other systems of drug receptors where active, inactive, and desensitized receptors can be detected, and where the desensitized receptor has grossly different binding properties for the agonist.

The blockade of sodium efflux from the cell by amiloride (Morel & Leblanc, 1975*b*) does not allow us to differentiate between carriers and channels as efflux would be prevented both if amiloride confined carriers to the outer face, of if channels were blocked by drug.

Arguments which favor a channel mechanism are based upon turnover numbers, but they suffer from the restriction that turnover numbers for known carriers (like valinomycin) artificially impose upper limits for turnover of carrier mechanisms. In various studies reported by us previously we have calculated the current passing each binding site by dividing the total current by the site density. Our values range from 0.7

to 1.3×10^{-16} A per site, this calculation assumes the channels are conducting continuously. The conductance data shown in Fig. 7*A* represents measurements made under conditions identical with those used for binding. From the regression line it is estimated that the sodium conductance of the mucosal face corresponds to 0.0099 mS for each μA of sodium current when the mucosal surface is bathed in low sodium Ringer's. The mean conductance of each site is therefore $7\text{–}13 \times 10^{-16}$ S, and the mean turnover number (400–800 ions sec^{-1}) is low. However, the ratio of mean current to mean conductance may be a true indication of the transmembrane potential. This value is approximately 100 mV which accords with the values reported by Nagel (1976, 1977).

Consider what happens when a skin bathed on the mucosal surface with low sodium Ringer's is changed suddenly to have Ringer's solution on both sides. The SCC increases instantly but then reclines with a half time of a few seconds due to inactivation of some of the entry sites (Zeiske & Lindemann, 1974).

If the total number of entry sites measured with ^{14}C-amiloride in low sodium Ringer's is N then the SCC is given by I_L, where

$$I_L = N \times i_m$$

and where i_m is the mean current per site in low sodium Ringer's. At steady state in Ringer's solution some of these sites will be unavailable to amiloride, and presumably sodium, others may be available but not conducting, so that the new steady-state current, I_H, will be given by

$$I_H = N \times i_i \times P$$

where i_i is the instantaneous current and P is the probability that an entry site is both available and conducting. Thus

$$i_i = i_m \times I_H / I_L \times \frac{1}{P}.$$

From Fig. 7 the ratio I_H/I_L is approximately 10. Lindemann and Van Dreissche (1977) found that instantaneous currents in the skins of *R. esculenta* were 0.3–0.5 pA in normal Ringer's from measurements of current noise. They showed that the SCC was only 1/75 of the current expected if all sites conducted simultaneously. Taking 75 as the value for $1/P$, our values for the instantaneous currents through conducting sites are 0.05–0.1 pA, somewhat less than those obtained from noise analysis. Thus, at high sodium concentrations we estimate turnover numbers of 3–

6×10^5 ions sec^{-1}. This appears to be too fast for a carrier, at least of the types presently known. A similar conclusion was reached by Lindemann and Van Dreissche (1977). From the regression line relating conductance to current at high sodium concentration we estimate that a single conducting site has a conductance of 1–2 pS. This value together with those for instantaneous currents indicate a transmembrane potential of approximately 50 mV, again in accord with the values found with microelectrodes at high sodium concentration (Nagel, 1976). It is also of interest that voltage-dependent sodium channels found in excitable membranes have been found to have conductance of between 1–9 pS from measurements of either channel density or noise (Conti *et al.*, 1976).

From a variety of experimental evidence it seems likely that the sodium translocation mechanism in the apical face of transporting epithelia has more than one conformation (Cuthbert, 1974; Fuchs *et al.*, 1977). An ionized acid grouping seems to be important for both sodium translocation and binding of blocking drugs (Cuthbert, 1976; Lindemann & Voûte, 1976), perhaps not unlike the situation in voltage-sensitive channels in excitable membranes (Hille, 1975). Transport of sodium through channels in which the sodium is "handled" by the channel, for example, facilitating removal of the hydration shell, will depend on the correct conformation. Alternative conformations, such as might be imposed by raised intracellular sodium, will have different binding properties and may impose pseudo-carrier type behavior on the system. Resolution of the mechanism of ion translocation in this system will require much more experimental evidence.

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