Amiloride and Calcium Effect on the Outer Barrier of the Frog Skin

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Summary. Amiloride (0.1 mM) as well as Ca^{++} (10 mM) inhibit Na^+ transport across frog skin by blocking Na^+ entrance across the outer barrier of the epithelium. The inhibition produced by amiloride consists of an "early" and a "late" phase which together account for almost a total inhibition of the short-circuit current (SCC). The analysis of the time course indicates that the two phases are due to the inhibition of superficially and deeply located Na sites, respectively. Ca^{++}, instead, only blocks a fraction of the SCC, and this fraction seems to correspond to the inhibition of the same population of Na sites blocked by the "late" phase of amiloride effect. The location of the two populations of Na sites as well as the possible relationship between them are discussed in terms of maturation of the outermost cell layer.

Amiloride inhibits Na transport across epithelial membranes by reducing Na^+ penetration through the outer barrier (Eigler & Crabbé, 1969; Dörge & Nagel, 1970; Salako & Smith, 1970; Biber, 1971; Moreno *et al.*, 1973). So does calcium (Curran & Gill, 1962). Yet, while amiloride decreases the short circuit current (SCC) to almost zero, calcium reduces only a fraction of this electrical parameter (Curran & Gill, 1962). This reveals a heterogeneity in the population of Na sites of the outer barrier: some sites are sensitive to amiloride and to calcium and some others are only sensitive to amiloride. The aim of the present work is to obtain information on the nature of this difference.

The analysis of the effects of amiloride and calcium is complicated by the existence of a spurious Na pool, which is not located in the main route of Na transport, and that introduces a considerable delay in the decay of the SCC when amiloride is added (Cereijido *et al.*, 1974). Figure 1 illustrates this

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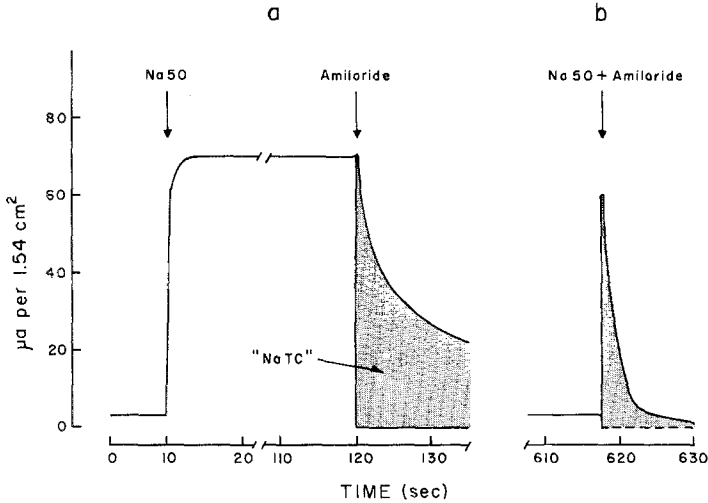
Fig. 1. (a): A frog skin is incubated with chloride Ringer's containing 115 mM Na on the inside, and 1 mM Na plus 114 mM choline on the outside. The outer Ringer's is changed at 10 sec for one containing 50 mM Na and 64 mM choline as chloride salts. The short-circuit current (SCC) rises quickly to a new steady value. Two minutes later the addition of amiloride decreases the short-circuit current. The shaded area under the curve of decay represents a pool of transportable Na^+. The amount of Na in this pool can be calculated by cutting and weighing the piece of paper under the curve. (b): Once amiloride has completely abolished the SCC it can be washed away with fresh Ringer's (not shown) and the skin re-incubated with 1 mM Na-Ringer's on the outside. Then the Na^+ concentration rises again as in a, but this time the new Ringer's also contains 10^{-4} M amiloride. The drug does not prevent the transient increase of SCC

phenomenon and, at the same time, serves to describe the experimental approach used in the present article. When the skin is bathed with 1 mM NaCl on the outside, $(Na)_o$, SCC is low. An instantaneous increase of $(Na)_o$ from 1 to 50 mM elicits a fast increase in SCC which achieves a new steady value within 0.5 sec. This short delay is attributable to an unstirred layer interposed between the bulk solution where one adds the drug, and the outer barrier where it acts, but not to a time required to fill a Na-transporting compartment (NaTC). If, after several minutes (t) of exposure to 50 mM $(Na)_o$ one adds amiloride, the SCC falls exponentially. The shaded area under the curve has the dimensions of (and was often ascribed to) the existence of a "NaTC". The size of this "NaTC" depends on the length of exposure to 50 mM $(Na)_o$; thus, by adding the amiloride at times (t) ranging from 10 sec to 10 min, one can vary "NaTC" over an order of magnitude. The opinion that this "NaTC" is a spurious pool not located along the main route of Na transport, stems from the fact that, while the new value of the

SCC is achieved in a fraction of a second and remains constant thereafter, the size of "NaTC" keeps increasing independently for some 5–10 min (Cereijido *et al.*, 1974). Therefore, the time course of the inhibition by amiloride, as followed by the decrease of SCC, is distorted by the extent of increase of "NaTC" during the exposure to high $(Na)_o$. Guided by the assumption that, because of electroneutrality requirements, Na^+ should accumulate in this "NaTC" with a permeable co-ion, we have previously shown that if the whole experiment is performed using a nonpermeable anion (gluconate), "NaTC" disappears. Under such condition the effect of amiloride is much faster, and the delay can be almost exclusively attributed to the unstirred layer. On the basis of those observations, most experiments described in this paper were performed in the abscence of Cl^- and with gluconate.

The first part of the paper is concerned with the effect of amiloride. It confirms the observations of previous authors (Cuthbert, 1973; Cereijido *et al.*, 1974) that amiloride has an "early" and a "late" effect and affords information indicating that this may be due to the existence of two populations of Na sites: superficial and deep. The second part deals with the effect of Ca^{++} and its interaction with Na^+ and suggests that the delay in Ca^{++} effect can be ascribed to the deep location of the Na sites where it acts. Finally, the third part combines studies on amiloride and on Ca^{++} and concludes that one of the simplest explanations of the experimental results would be given by the existence of Na sites at two different deepnesses and that the inhibition of Ca^{++} as well as the "late" fraction of the effect of amiloride are due to their effect on the sites located in a deeper position. The discussion analyzes the relationship between the two kinds of sites and speculates on whether they represent two stages of the process of maturation of the cell layers from the *stratum germintivum* to the *s. corneum*.

Materials and Methods

The experiments were performed on the abdominal skin of the South American frog *Leptodactylus ocellatus*. Animals of either sex kept in moist sinks with running water were studied at 20–22 °C. The basic Ringer's solution used contained (mM): 115, Na gluconate; 2.4, $KHCO_3$, 1.0, Ca gluconate$_2$; and 2.0, glucose. Other solutions used are specified in *Results*.

In experiments where rapid change of Ringer's was not required, the skin was mounted as a flat sheet between two Lucite chambers of the type designed by Curran, Herrera and Flanigan (1963). The exposed area was 3.14 cm^2 and the volume on each side 5.0 ml. When fast changes of the outer bathing solution were needed, we used the chamber described by Cereijido *et al.* (1974). In this case the skin was also mounted as a flat sheet, but between a set of four individual pairs of chambers. The area of each of these chambers was 1.54 cm^2. The

volume of the outer half was only 0.8 ml and that of the inner, 4.0 ml. The outer bathing solution was changed by injecting 10 ml of the new solution in 0.3 sec through the outer chamber. Electrical potential was measured through agar bridges with 2 M KCl connected to calomel half-cells and these to a voltage clamp apparatus. This apparatus delivered the clamping current through two Cl-coated silver plates. Fast time courses of the short-circuit current (SCC) were followed with a 7B polygraph recorder (Grass) or with a dual beam oscilloscope with two different sweep speeds (Tektronix, Model 565). Changes were made in the outer bathing solution only. This was based both on the necessity of making a change as quickly as possible in the solution in contact with the outer barrier and on the fact that many changes attributed to this barrier are sometimes due to changes in the inner bathing solution (Rabito, Rodríguez Boulan & Cereijido, 1972).

The diffusion coefficient of amiloride was measured with a technique similar to the one described by Kreevoy and Wewerka (1967). Briefly, it compares the escape of amiloride and of a reference substance of known diffusion coefficient through a filter paper diaphragm. In the present case the reference substance was ^{22}Na. Amiloride concentration was determined in an Aminco-Bowman spectrofuorometer. The excitation wavelength was 368 nm. The emitted light was filtered (450 nm) and received in a second monocromator. ^{22}Na was counted in a Nuclear Chicago Autogamma Spectrometer. Na$^+$ concentration was measured in an EEL flame photometer. Ca^{++} activity was measured with a Ca-sensitive electrode (Orion Research; Cambridge, Mass.).

Sources of Material

Amiloride (3,5-diamino-6-chloropyrazinoylguanidine) was from Merck, Sharp & Dohme (West Point, Pa.); choline chloride was from Eastman Organic Chemicals (Rochester, N.Y.); ^{22}Na was from New England Nuclear (Boston, Mass.).

Results are expressed as mean ±SE (number of observations).

Results

As mentioned above, the kinetic of amiloride inhibition at the outer barrier, as followed by the change of SCC, is obscured by the presence of a spurious Na pool. Since the aim is to study in detail the effect of amiloride, we want to get rid of this pool, if possible. To this purpose we planned to use gluconate instead of Cl$^-$. In gluconate, Na does not accumulate in the spurious pool. In this condition, the transient of SCC after addition of amiloride is thought to represent the transport Na$^+$, which is already present near the Na sites of the outer barrier. This Na$^+$ keeps penetrating while the amiloride, just added to the outer solution, diffuses through the unstirred layer and reaches the Na sites. In order to check this assumption, the experiment in Fig. 2 was performed. The abscissa represents the amount of Na$^+$ in the "NaTC" as recorded in gluconate Ringer's. The ordinate represents Na$^+$ present in the unstirred layer and the Na sites, which penetrates between the moment amiloride is added to the outer solution
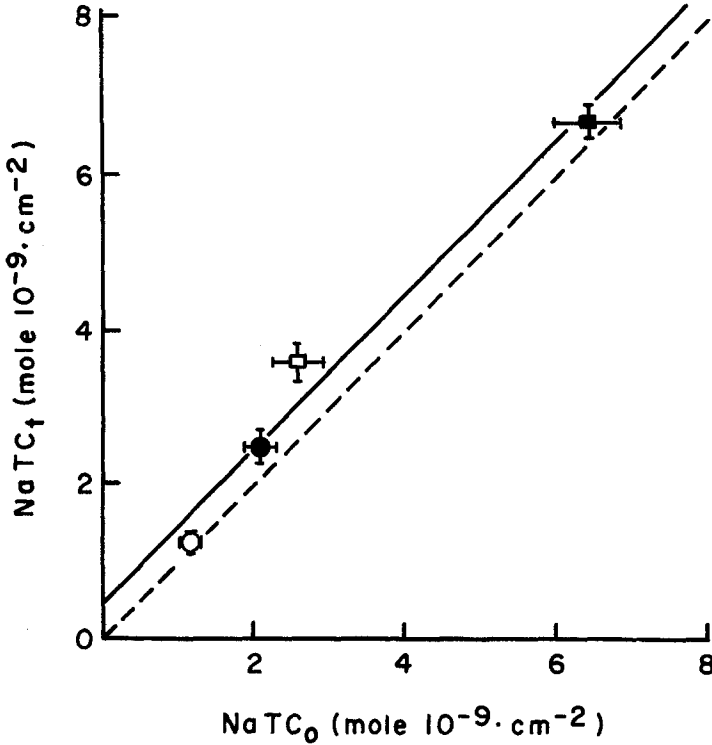
Fig. 2. Relationship between the amounts of Na represented by the area under the curve in Fig. 1a ("NaTC"), and in Fig. 1b (Na due to faster diffusion) in skins bathed with Ringer's solutions where Cl^- was replaced by gluconate. "NaTC" was obtained by adding amiloride 5 min after increase of the concentration of 1 mM to a higher one. The experimental points correspond to concentrations increased to 10 mM (o); 30 mM (●); 50 mM (□); and 115 mM (■). Bars indicate SE; the straight line was obtained by the least squares method: "NaTC" = 0.37 $\times 10^{-9}$ mole $+ 0.99 \times$ (Na due to diffusion). The value of r is 0.98. The slope is not significantly different from 1 $(P > 0.4)$

and the moment it blocks the Na sites. This amount of Na^+ was evaluated as follows (Fig. 1b). When the outer solution contains Ringer's with only 1 mM Na^+, the value of the SCC is low. When this solution is changed to one with 50 mM Na^+ plus 10^{-4} amiloride, the SSC rises to a level comparable to the one it reaches without amiloride and then decays. This effect, first described by Biber (1971), was studied by Cereijido et al. (1974) and attributed to the fact that Na, having a greater diffusion coefficient than amiloride, crosses the unstirred layer faster and achieves a considerable concentration at the level of the Na sites of the outer barrier before amiloride arrives and blocks them. This peak is not observed if amiloride is added before Na so that the sites are already blocked when the new (higher) $(Na)_o$ reaches the outer barrier. Figure 2 compares the amount of Na

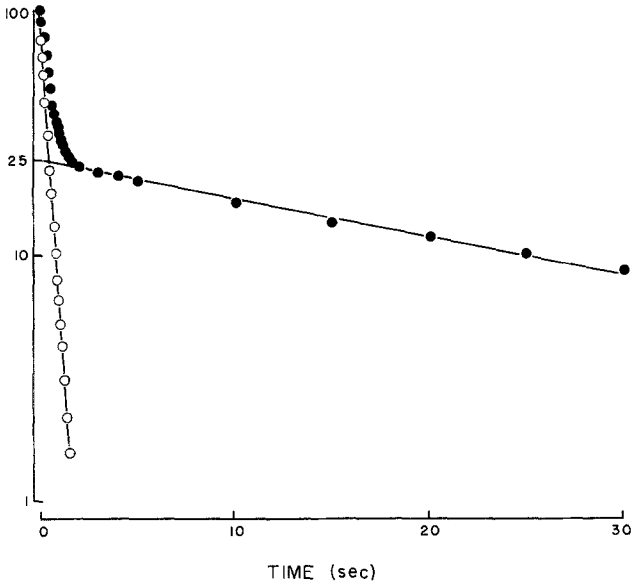C.A. Rabito, C.A. Rotunno, and M. Cereijido

TIME (sec)

Fig. 3. Graphical analysis of the decay of the SCC following the addition of 10^{-4} M amiloride to the outer bathing solution. Amiloride was added 5 min after exposure to 50 mM Na Ringer's. The SCC before amiloride is taken as 100 %. Filled circles are experimental points. Open circles correspond to the difference between the experimental points and the asymptote. 75 % of the Na sites are inhibited by amiloride with a half time of 0.35 sec. The rest are inhibited with a half time of 25 sec

represented by the area under the curve of Fig. 1 a (abscissa) and of Fig. 1 b (ordinate). Both groups of determinations were made in the same set of frog skins, thanks to the reversibility of the effect of amiloride. The slope of the straight line in Fig. 2 is 0.99, which does not differ from 1.0 ($P > 0.4$). The intercept is 0.37×10^{-9} mole of Na per cm^{-2} ($r = 0.982$). The small amount of Na represented by the intercept might be due to the fact that, while Na$^+$ is achieving the same value at the level of the outer barrier that it has in the bulk (50 mM), the small amount of amiloride that has already arrived starts to block sites. This is suggested by the fact that the maximum value reached by SCC in Fig. 1 b is always somewhat lower than the one in Fig. 1 a. On the basis of this set of experiments, gluconate is used as the main anion instead of Cl$^-$.

Cuthbert (1971) has pointed out that the transient following the addition of amiloride is either a single or a double exponential. Figure 3 shows that this is also the case in the presence of gluconate. The SCC inhibited by amiloride may thus be divided into two fractions: An early one constituting some 75 % of the control current registered before amiloride, and a late one
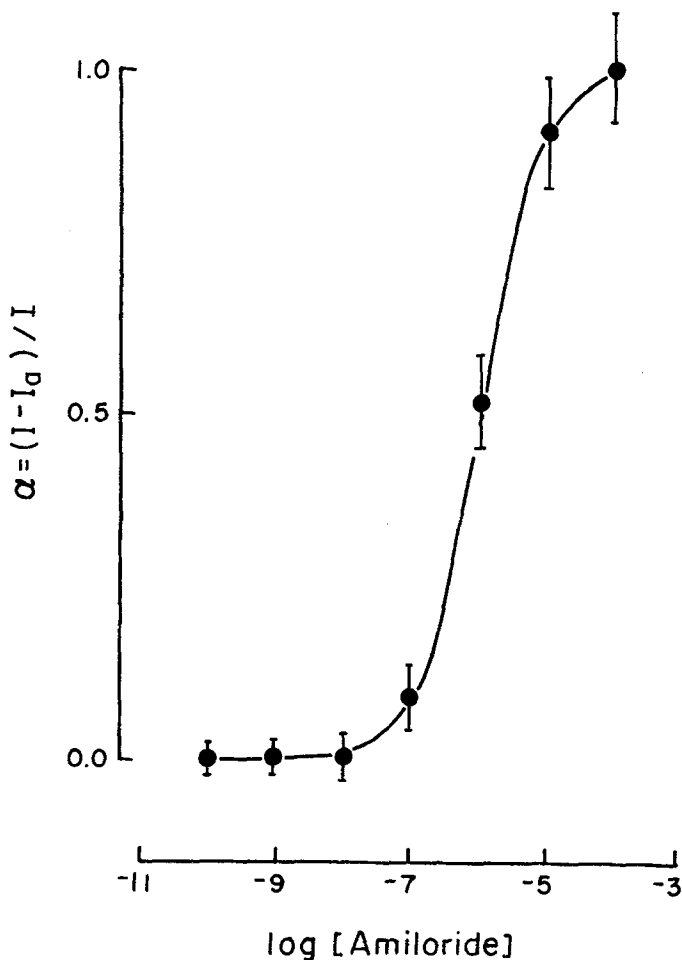
Fig. 4. Relationship between the logarithm of the *dosis* and the response to amiloride. *Ordinate:* total fractional inhibition as defined by Eq. (2). *Abscissa:* logarithm of the molar concentration of amiloride. The curve was drawn using a single affinity constant for amiloride ($k_m = 10^{-6}$ M)

accounting for the remaining 25 %. Their half times are 0.35 and 25 sec, respectively. This effect might reflect a difference in the interaction of amiloride with the sites. However, this is unlikely, because Salako *et al.* (1970), in *Rana temporaria*, and Benos *et al.* (1976), in *Rana pipiens*, have found that in Cl$^-$ Ringer's amiloride interaction can be explained with a single affinity constant. In order to check whether this holds also when gluconate is used instead of Cl$^-$ and in the species used in the present work (*L. ocellatus*), we performed the experiment described in Fig. 4. It shows the relationship between the dose of amiloride and the fractional inhibition α

defined as follows:

$$\alpha = \frac{I - I'}{I - I'_{max}} \tag{1}$$

where I and I' are the values of SCC before and after a given dose of amiloride, and I'_{max} is the inhibition observed at the maximal *dosis* used. It was observed that in gluconate I'_{max} was always close to zero. Therefore, Eq. (1) becomes:

$$\alpha = \frac{I - I'}{I}. \tag{2}$$

The value of the affinity constant used to fit the curve was $10^{-6} \, M^{-1}$, which compares with $0.2 \times 10^{-6} \, M^{-1}$ found by Salako *et al.*, and 0.55 $\times 10^{-6} \, M^{-1}$ found by Benos *et al.* This lends further support to the view that that component of the decay curve (Fig. 1a), which disappears when Cl^- is replaced by gluconate, is related to the elimination of a spurious Na pool and not to a change in the effect of amiloride at the level of the Na sites on the outer barrier.

Another alternative explanation for the two time courses in Fig. 3 is that the late components were given by Na sites located in a deeper position. In order to evaluate the depth of the Na site blocked by amiloride, one may use the following solution of the diffusion equation (Crank, 1956).

$$[A]_o = [A]_\infty - \frac{4}{\pi} [A]_\infty \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} \exp - \left(\frac{(2n+1)^2 \pi^2 D'_A t}{4\delta^2} \right) \tag{3}$$

where $[A]_o$ is the concentration of amiloride in a barrier located at a depth δ from the bulk solution as a function of time (t), $[A]_\infty$ is concentration in the bulk, and D'_A is the apparent diffusion coefficient of amiloride.

The value of D'_A was measured with the method of Krevoy and Wewerka, (1967), as described in *Methods*: $(1.69 \pm 0.23) \times 10^{-6} \, cm^2 \, sec^{-1}$. The length δ corresponds to the position of the Na sites where amiloride acts, i.e., the depth of the "outer barrier". Therefore, δ is common for both Na^+ and amiloride. This parameter can be evaluated with an equation analogous to Eq. (3) assessing $(Na)_o$, the concentration at the level of the outer barrier, in terms of its effect on the SCC. The necessary modification of Eq. (3) is described in the *Appendix*. The experimental approach consists of measuring the SCC in Ringer's with 1 mM $(Na)_o$, then changing instantaneously to 50 mM $(Na)_o$, using the value of the diffusion coefficient for Na $(D'_{Na} = 5 \times 10^{-6} \, cm^2 \, sec^{-1}$; Kidder, Cereijido & Curran, 1964), and
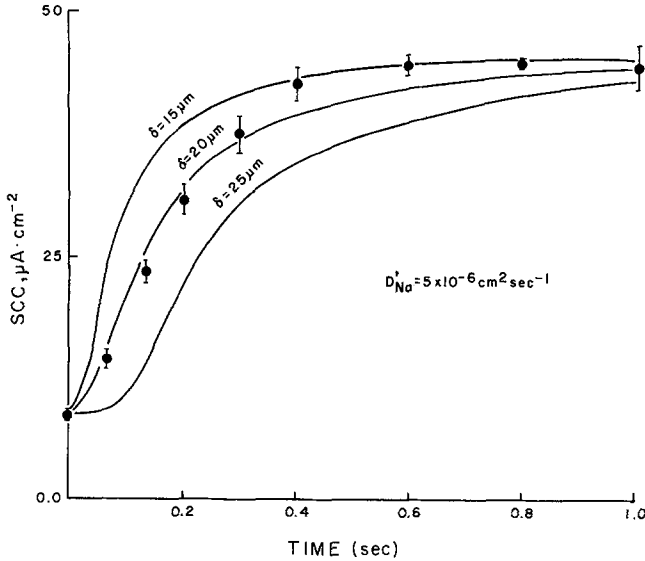
Fig. 5. Time course of the increase of SCC following a change in the concentration of Na in the outer solution from 1 to 50 mM. The theoretical curves were obtained with Eq. (A1), using an apparent Na diffusion coefficient of 5×10^{-6} cm² sec⁻¹ and the δ specified on each curve

adjusting the curve to the experimental points by varying δ as depicted in Fig. 5. In this way δ is found to be 15–25 μm.

Figure 6 shows the time course of the concentration of amiloride at the level of the Na sites of the outer barrier. Full circles represent the concentration of amiloride (as read on the curve of Fig. 4) necessary to produce the inhibition of the early component of amiloride (Fig. 3). Open circles represent the concentrations corresponding to the late component. The lines were drawn with Eq. (3) using the value of D'_A, measured as described above, and several values of δ which gives the best fit is one which agrees with the position of the outer barrier. The late component, instead, is given by Na sites located in a much deeper position.

As mentioned in the introduction, while amiloride blocks all Na sites and drops the SCC to zero, calcium only inhibits a fraction of the total current (Curran & Gill, 1962). The following experiment was designed to study whether the fraction of Na sites inhibited by Ca^{++} corresponds to one of the two groups blocked by amiloride. Figure 7 shows the inhibition of the SCC produced by amiloride 10^{-4} M in skins bathed with Ringer's with different concentration of Ca^{++}. In order to simplify comparisons, in this figure the SCC was taken as 100 % just before the addition of amiloride. At 10 mM, calcium produces its maximal effect. Fifty percent of this inhibition
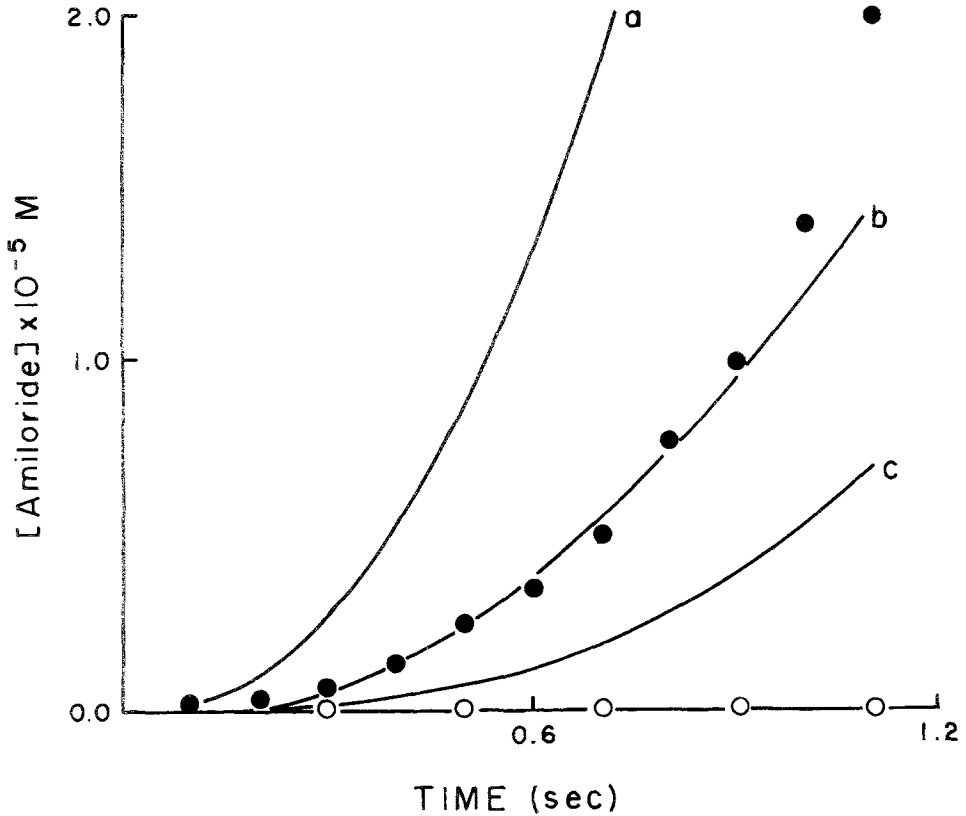
Fig. 6. Time course of the increase in concentration of amiloride at the level of the Na-sensitive barrier, as reconstructed from the relationship between its concentration/effect curve (Fig. 4) and the time course of its "early" (filled circles) and "late" (open circles) effects. The theoretical curves were drawn with Eq. (3). The value of D'_A is 1.69×10^{-6} cm^2 sec^{-1}. The values of δ are 20 µm (curve a), 25 µm (curve b), and 30 µm (curve c)

is elicited by 1 mM Ca^{++}. At 0, 1 and 10 mM Ca^{++}, the half times of the early component of amiloride inhibition are 300, 250 and 250 msec, respectively; half times of the late component are 32, 32 and 19 sec. Therefore, Ca^{++} seems to have little effect on the time course, but it does reduce the size of the late component. Thus, while skins bathed in Ringer's without Ca^{++} (upper curve) have a fraction of 26 % of sites reachable by amiloride with a slow time course, in skins treated with 1 mM Ca^{++} these (i.e., the concentration which corresponds to the k_m) sites are reduced to one half (13 %), and with 10 mM Ca^{++} they are reduced to less than 9 % in 5 min of exposure to Ca^{++}. The view that Ca^{++} acts on the deeply located Na sites agrees with the observation of Curran & Gill (1962) that, in spite of the fact that Ca^{++} acts on the outer barrier, it takes some 10 min to develop its maximal effect.
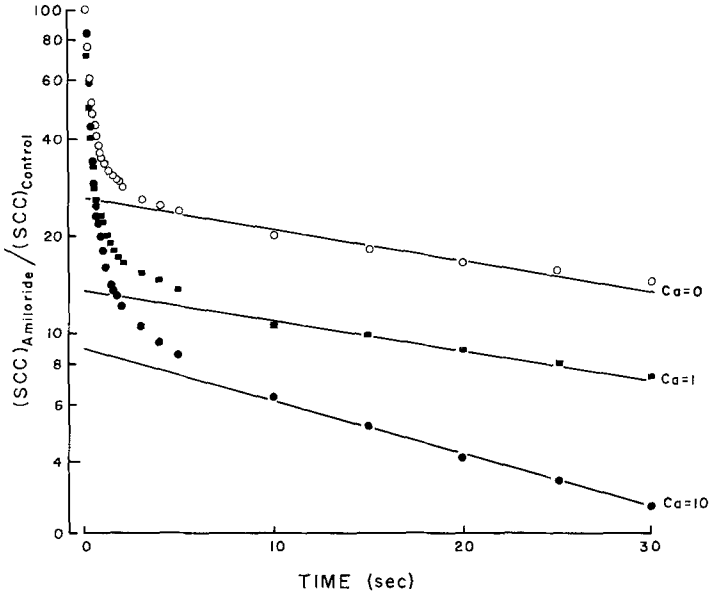
Fig. 7. Effect of 10^{-4} M amiloride on skins incubated with 50 mM Na$^+$ and different concentrations of Ca^{++}. *Ordinate:* fractional inhibition of the SCC produced by amiloride

    Another characteristic of the inhibition produced by Ca^{++} is that it depends on the concentration of sodium. Thus, at a constant concentration of Na$^+$, the inhibition of transport increases with increasing calcium concentration and approaches a limiting value, but this maximum degree of inhibition depends on the concentration of Na$^+$ (Curran & Gill, 1962). This is illustrated in Fig. 8 where 10 mM Ca inhibits the SCC by only 10 % at 15 mM (Na)$_o$, but by 50 % at 90 mM (Na)$_o$. The analysis of this phenomenon might afford more information on the group of deeply located Na sites. In order to perform this analysis, it may be convenient to divide the phenomenon into two steps (i) the modification of the Na sites by Ca^{++} and (ii) the penetration of Na$^+$ through these sites. Thus one does not know whether the high (Na)$_o$ is needed to allow Ca^{++} to modify the sites or whether Ca^{++} can act by itself, and the high (Na)$_o$ is only needed to put in evidence the modification. This was studied in the experiment described in Fig. 9. On the left hand side of the figure there is a group of skins (open circles) bathed in 1 mM (Na)$_o$ without Ca^{++}. At a given moment (Na)$_o$ is suddenly increased to 50 mM and the SCC raises to more than 100 µA. When 10 mM Ca^{++} is added to this group, the SCC begins to fall with a slow time course (filled circles). If $(Na)_o$ is increased but at the same time 10 mM Ca^{++} is also added (filled squares), the initial part of the increase of SCC is
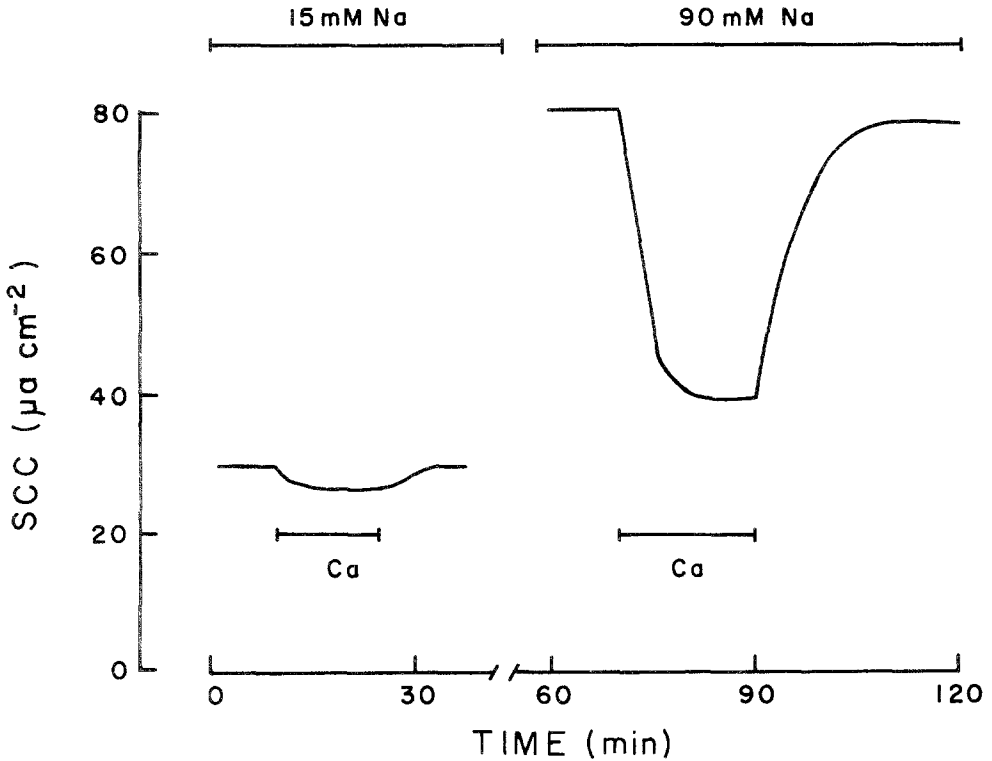
Fig. 8. Effect of 10 mM Ca^{++} on the SCC of a frog skin incubated at 15 (left) and 90 (right) mM Na^+. Notice the slow time course of the effect (as compared to that of amiloride, Fig. 3) and higher fractional inhibition elicited at the higher concentration of Na^+

essentially equal to the one in the previous group, indicating that the state of the Na sites is similar to the state they have in the absence of Ca^{++}. In a couple of minutes though, the effect of Ca^{++} begins to show: the SCC decreases. A third group of skins was also incubated in 1 mM $(Na)_o$, but in the presence of 10 mM Ca^{++} (triangles). As expected in this low $(Na)_o$, the inhibition produced by Ca^{++} is minimal and not significant. When $(Na)_o$ is increased the SCC does not increase to the level it reached in the two previous groups. Na^+ finds the sites already modified by calcium, i.e., the effect of Ca^{++} has no delay. This suggests that Ca^{++} can act on the Na sites by itself, and that the enhancement of α', the fractional inhibition of the SCC, produced by the high $(Na)_o$ is achieved in a later step. Figure 10 shows again the same phenomenon, except that in this series of experiments, the attention was focused in the change from a low to a high α' as the concentration of $(Na)_o$ increased at the level of the outer barrier. The right hand side of Fig. 10 is a control showing that the addition of 10 mM
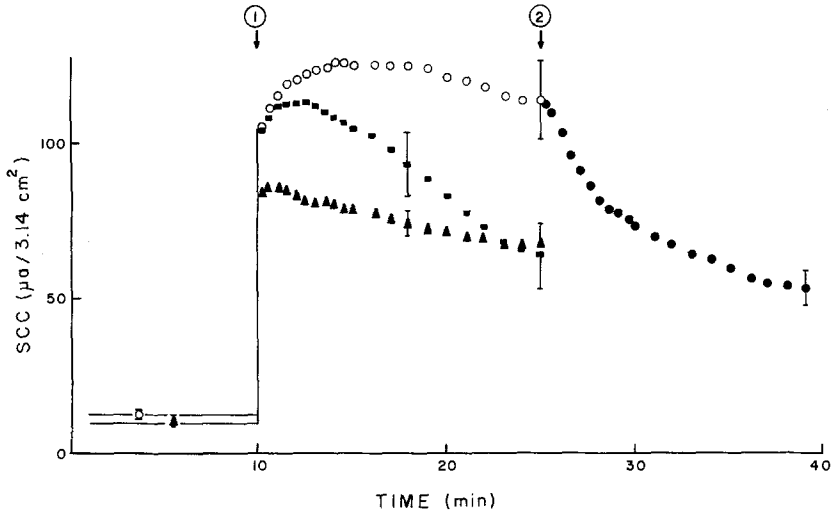
Fig. 9. Time course of the effect of Ca^{++} and Na$^+$ on the SCC. Skins were initially incubated with 1 mM Na$^+$ on the outside, both without Ca^{++} (open circles) and with 10 mM Ca^{++} (filled circles). At arrow, 1 mM Na concentration is suddenly increased to 50 mM. The upper curve (open circles) corresponds to an increase of Na$^+$ in the group incubated at 1 mM Na without Ca^{++} which was switched to 50 mM Na$^+$ without Ca^{++}. Squares correspond to skins incubated at 1 mM without Ca^{++}, which was switched to 50 mM Na with 10 mM Ca^{++}. Triangles correspond to skins incubated with 1 mM Na$^+$ and 10 mM Ca^{++}, which received 50 mM Na$^+$ with 10 mM Ca^{++}. At arrow 2 the group of skins that have so far been without Ca^{++} are suddenly treated with 50 mM Na with 10 mM Ca^{++}
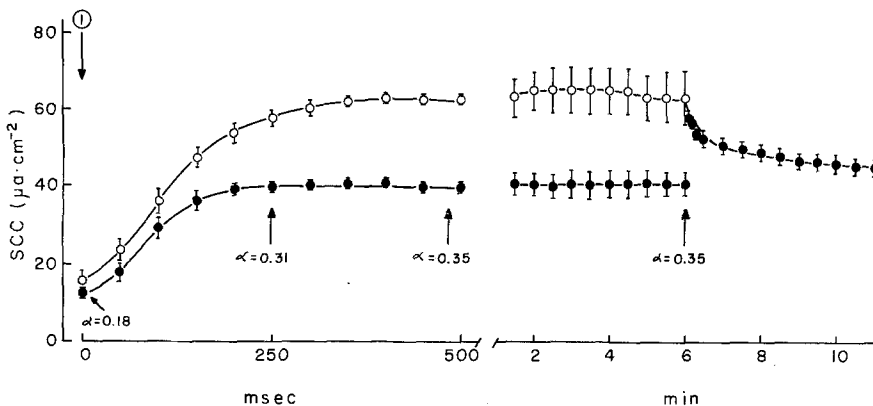

Fig. 10. Analysis of the early effect of Na$^+$ and Ca^{++} on the SCC; skins were pre-incubated in Ringer's with 1 mM Na$^+$ with 10 mM Ca^{++} (filled circles) and without Ca^{++} (open circles). At arrow 1 the Na$^+$ concentration of the outer bathing solution is increased without modifying the concentration of Ca^{++}. The fractional inhibition obtained by comparing the simultaneous values of SCC in the two curves increases as the Na$^+$ concentration at the level of the outer barrier increase. Six minutes later 10 mM Ca^{++} is added to the outer Ringer's (arrow 2). This reduces the SCC with a slow time course (filled circles) which approaches the value of the skins that had calcium added from the beginning

Ca^{++} to the group, which was so far without this ion, produces a decay of SCC in the course of several minutes, and not in milliseconds as in the left hand side.

Discussion

The two-barrier hypothesis of Koefoed-Johnsen and Ussing (1958) of Na^+ transport across frog skin postulates that the outer one behaves as a Na-sensitive K-impermeable boundary. It seems now well established that this barrier is located at the outer anatomical border of the *stratum granulossum:* (i) All the K in the epithelium exchanges with ^{42}K in the inner solution, indicating that there is no K-impermeable barrier interposed (Curran & Cereijido, 1965); (ii) the time course of changes in potential difference across frog skin following rapid changes in composition of the bathing solutions indicates that most of the delay (0.2–0.4 sec) is accounted for solely by unstirred layers and the *stratum corneum* (Kidder *et al.*, 1964; Gebhardt, Fuchs & Lindemann, 1972; Cereijido *et al.*, 1974; Morel & Leblanc, 1975); (iii) tight junctions, which confer to the epithelium the property of an effective permeability barrier, are located at the external border of the *stratum granulossum* (Farquhar & Palade, 1963); (iv) interaction of amiloride, Li^+, and K^+ with Na^+ penetration across the outer barrier takes place at the outer anatomical border of the epithelium (Biber & Curran, 1970; Rotunno *et al.*, 1970; Cereijido *et al.*, 1972; Moreno *et al.*, 1973); (v) the effect of ADH on the Na-sensitive barrier is elicited at the outer solution/epithelium boundary (Cereijido & Rotunno, 1971); (vi) studies performed with microelectrodes by different workers show a considerable discrepancy with respect to the electrical profile found, to the value of the well of negative electrical potential recorded under short-circuit conditions and on the position of the tip. Yet, all workers found that most of the potential change elicited by varying the Na^+ concentration in the outer solution is always sensed between the microelectrode tip and the outer solution, regardless of the exact location of this tip in the epithelium (Cereijido & Curran, 1965; Whittembury, 1964; Nagel, 1976); (vii) morphological changes associated with Na^+ penetration across the Na-sensitive barrier and into the epithelium are located at the outermost cell layer which is not yet cornified (Vôute & Ussing, 1970).

However, the epithelium is not a static structure. Since it matures from the innermost (*stratum germinativum*) to the outermost cell layer (*stratum granulossum*), but only this last one has on one of its sides the properties of

the "outer facing barrier", it follows that in a given moment and position the cells develop the "outer barrier". Also, when the outermost cell layer degenerates to form the *s. corneum*, the replacement cells coming from the *s. spinosum* should, in turn, develop an "outer barrier".

Under experimental treatment with aldosterone this process is synchronized, and the skin goes through alternating periods of low (right after moulting) and high SCC (Nielsen & Tomilson, 1970). Therefore, when the cells of the *stratum granulossum* undergo a process of cornification, the "outer facing barrier" is likely to switch positions (Fig. 11). Although moulting is a macroscopic phenomenon in which a whole layer detaches, at the microscopic level the cells might be in different stages of cornification or might have lost their "outer facing barrier" in different degrees until the whole cell layer is ripe to be shed.

Figure 11 depicts a situation where two cells (light shaded) in the outermost cell layer (dark shaded) are in an advanced state of transformation to *corneum* and those behind are already prepared to take over the role of "outer barrier". In order to reach the portion of outer barrier (bold line) on these replacement cells, amiloride might have to circumvent the cells which have lost their own outer barrier. This may explain the existence of superficially and deeply located Na sites, but not on those in a superficial position; it will mean that the difference between the two populations of sites would not be only a difference in position, but that it reflects a certain process of maturation of the sites. During this process the affinity for Ca^{++}, as compared to that for Na^+, may be significantly reduced.

The effect of Ca^{++} cannot be explained by a direct competition between Na^+ and Ca^{++} since, in such case, more sites should be available to calcium at low Na concentrations (Curran & Gill, 1962). The possibility exists that, in order to reach the deep portion of the outer barrier, Ca^{++} may need to diffuse through a tortuous path lined with negative charges. If because of the spacing of these charges Ca^{++} binds with only one of its valences, it may reverse the charge of the path blocking the diffusion of a significant amount of Ca^{++}. A high $(Na)_o$ may displace Ca^{++} from these sites, neutralize the charge of the path, and allow Ca^{++} to reach and modify the deep portion of the outer barrier.

There is no information with respect to why amiloride acts on both populations of Na sites but Ca^{++} only acts on one of them. According to Eisenman (1960), the main factor which allows ion exchangers to discriminate between mono and divalent cations is the spacing between the fixed sites. This suggests that the decrease of the ability of calcium to inhibit Na sites may be due simply to a change in the spacial distribution of the sites on
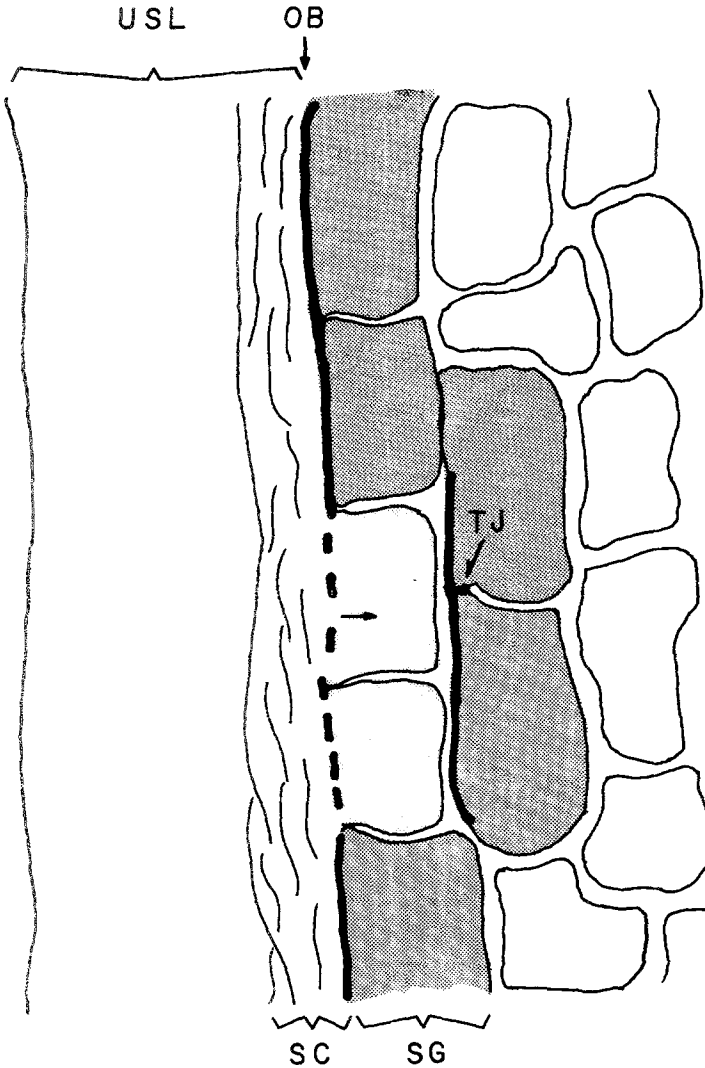
Fig. 11. Schematic representation of the "outer barrier" (bold line). *USL:* unstirred layer formed by the outer bathing solution and the *stratum corneum* (*SC*). Lightly shadowed cells of the *stratum granulossum* (*SG*) represent two cells in advanced state of cornification (*see* text)

the outer barrier. However, in both states the sites would instead prefer amiloride to Na^+, regardless of their spacing.

The information obtained in the last years indicates that the picture of a uniform outer barrier having a single kind of Na site is an over-simplification. Thus Cuthbert (1973) could not explain the interaction between amiloride and Na^+ in terms of a single homogeneous population

of receptors; Benos *et al.* (1976) have shown that the effect of the ionic composition of the outer bathing solution on the SCC and on the inhibitory effect of amiloride are exerted at separate and distinct regions on the transport sites; and Nielsen *et al.* (1970) studying Na^+ transport in skins before and just after moulting have obtained some evidence that amiloride-sensitive sites are already present in the cell layer which becomes exposed when the outermost layer is shed. In conclusion, these observations, together with the data of the present paper, complicate our view of the outer barrier, but this complication may result in a better understanding of the genesis of the outer barrier, which is one of the most important steps in Na^+ transport.

Appendix

When the concentration of sodium in the outer bathing solution is instantaneously changed from 1 to 50 mM, the concentration at the level of the outer barrier $((Na)_{OB})$ changes according to the following equation (Crank, 1956):

$$(Na)_o = (Na)_{o\infty} + \frac{4}{\pi}((Na)_{oi} - (Na)_{o\infty}) \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} \exp - \left(\frac{(2n+1)^2 \pi^2 D'_{Na} t}{4\delta^2} \right)$$

(A1)

where D'_{Na} is the apparent diffusion coefficient of Na and δ is the thickness of the unstirred layer. This change of the concentration of Na^+ at the level of the outer barrier increases the influx of Na^+ and the short-circuit current (I). The values of I and $(Na)_{OB}$ are related by the following equation (*see* Moreno *et al.*, 1973; Cereijido *et al.*, 1974):

$$I = \frac{I_{max}(Na)_{OB}}{(Na)_{OB} + K_m}$$

(A2)

where I_{max} is the value of I at infinite $(Na)_{OB}$ (in gluconate this value is 92.2 µA cm^{-2}; Rabito *et al.*, *unpublished results*) and K_m is the value of $(Na)_{OB}$, that gives $0.5 I_{max}$ (in gluconate it is 16.7 mM). Therefore, when the value of the concentration of Na^+ in the bulk is raised from 1 to 50 mM, the value of the SCC increases with time from the value it has at 1 mM (5.22 µA

cm^{-2}) to a higher value according to the following equation:

$$I = 5.22 + \frac{92.2\,(\text{Na})_{OB}}{(\text{Na})_{OB} + 16.7} \tag{A3}$$

combining Eqs. (A 1) and (A 3) and assigning values to D'_{Na} and δ, a series of theoretical curves of I_t as a function of time can be drawn. In Fig. 5 these curves are compared to the experimental points.

References

Benos, D.J., Simon, S.A., Mandel, L.J., Cala, P.M. 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

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

Biber, T.U.L., Curran, P.F. 1970. Direct measurement of uptake of sodium at the outer surface of the frog skin. *J. Gen. Physiol.* **56**:83

Cereijido, M., Curran, P.F. 1965. Intracellular electrical potentials in frog skin. *J. Gen. Physiol.* **48**:543

Cereijido, M., Moreno, J.H., Rodríguez Boulan, E., Rotunno, C.A. 1972. On the evaluation of fluxes across the outer border of the epithelium. *In:* Role of Membranes in Secretory Processes. L. Bolis, editor. p. 279. North Holland, Amsterdam

Cereijido, M., Rabito, C.A., Rodríguez Boulan, E., Rotunno, C.A. 1974. The sodium-transporting compartment of the epithelium of frog skin. *J. Physiol. (London)* **237**:555

Cereijido, M., Rotunno, C.A. 1971. The effect of antidiuretic hormone on Na movement across frog skin. *J. Physiol. (London)* **212**:119

Crank, J. 1956. In the Mathematics of Diffusion. p. 45. Oxford University Press, London

Curran, P.F., Cereijido, M. 1965. K fluxes in frog skin. *J. Gen. Physiol.* **48**:1011

Curran, P.F., Gill, J.R. 1962. The effect of calcium on sodium transport by frog skin. *J. Gen. Physiol.* **45**:625

Curran, P.F., Herrera, F.C., Flanigan, W.J. 1963. The effect of Ca and antidiuretic hormone on Na transport across frog skin. II. Sites and mechanisms of action. *J. Gen. Physiol.* **46**:1011

Cuthbert, A.W. 1971. Neurohypophyseal hormones and sodium transport. *Phil. Trans. R. Soc. B.* **262**:103

Cuthbert, A.W. 1973. Evidence for multiple forms of receptors for amiloride in transporting epithelia. *Eur. J. Pharmacol.* **33**:187

Dörge, A., Nagel, W. 1970. Effect of amiloride on sodium transport in frog skin. II. Sodium transport pool and unidirectional fluxes. *Pfluegers. Arch. Gesamte Physiol.* **321**:91

Eigler, J., Crabbé, J. 1969. Effects of diuretics on active sodium transport in amphibian membranes. *In:* Renal Transport and Diuretics. K. Thurau and H. Jahmarker, editors. pp. 195–208. Springer-Verlag, Berlin

Eisenman, G. 1960. On the elementary atomic origin of equilibrium ionic specificity. *In:* Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editors. p. 172. Academic Press, London-New York

Farquhar, M.G., Palade, G.E. 1965. Cell junction in amphibian skin. *J. Cell Biol.* **26**:263

Kidder, G.W., Cereijido, M., Curran, P.F. 1964. Transient changes in electrical potential differences across frog skin. *Am. J. Physiol.* **207**:935

Koefoed-Johnsen, V., Ussing, H.H. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298

Kreevoy, M.M., Wewerka, E.M. 1967. A filter paper diaphragm technique for diffusion coefficients. *J. Phys. Chem.* **71**:4150

Gebhardt, U., Fuchs, W., Lindemann, B. 1972. Resistance response of frog skin to brief and long lasting changes of $(Na)_o$ and $(K)_o$. *In:* Role of Membranes Secretory Processes. L. Bolis, editor. p. 284. North Holland, Amsterdam

Morel, F., Leblanc, G. 1975. Transient current changes and Na compartmentalization in frog skin epithelium. *Pfluegers Arch.* **388**:135

Moreno, J.H., Reisin, I.L., Rodríguez Boulan, E., Rotunno, C.A., Cereijido, M. 1973. Barriers to sodium movement across frog skin. *J. Membrane Biol.* **11**:99

Nagel, W. 1976. The intracellular electrical potential profile of the frog skin epithelium. *Pfluegers Arch.* **365**:135

Nielsen, R., Tomilson, R.W.S. 1970. The effect of amiloride on sodium transport in normal and moulting frog skin. *Acta Physiol. Scand.* **79**:238

Rabito, C.A., Rodríguez Boulan, E., Cereijido, M. 1973. Effect of the composition of the inner bathing solution on transport properties of the frog skin. *Biochim. Biophys. Acta* **311**:630

Rotunno, C.A., Vilallonga, F., Fernández, M., Cereijido, M. 1970. The penetration of sodium into the epithelium of frog skin. *J. Gen. Physiol.* **55**:716

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

Vôute, C.L., Ussing, H.H. 1970. Quantitative relation between hydrostatic pressure gradient, extracellular volume and active sodium transport in the epithelium of the frog skin (*R. temporaria*). *Exp. Cell Res.* **62**:375

Whittembury, G. 1964. Electrical potential profile of the toad skin epithelium. *J. Gen. Physiol.* **47**:795