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# Effect of External Cation and Anion Substitutions on Sodium Transport in Isolated Frog Skin

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**Summary.** Effects of changes in external ionic strength, external cation and/or anion substitution on transepithelial influx and efflux of sodium, short-circuit current and on transepithelial potential difference and resistance were studied in isolated frog skin. Active transport of Na was found to be highly dependent on both anionic and cationic composition of external medium. Relative abilities of external monovalent cations to inhibit active Na transport were H > Li > K > Rb > Cs > choline. Relative abilities of external monovalent anions to stimulate active Na transport were I > Br > Cl. Sequences of anion interaction and of resistance changes suggest that anionic stimulation of Na transport is not due to electrical coupling across outer cell membrane. The ability of different anions and cations to alter Na transport suggests that externally located charged groups act as important barriers or filters to ion movement. In addition, the experiments suggest that an increase in ionic strength of external medium has an effect on active transport of Na, a finding that indicates interference of surface charges with Na entry. Directional changes in efflux of Na due to changes in ionic composition of external medium usually paralleled changes in active Na transport. It is possible that the observed relationship between influx and efflux of Na is the result of common pathways and of interaction of the active transport system with Na efflux.

According to the model put forward by Koefoed-Johnsen and Ussing (1958) for active sodium transport across isolated frog skin, Na enters the cell across the outwards facing cell boundary by simple diffusion along an electrochemical gradient and is expelled into the serosal medium across the serosal border via an active transport process. However, direct measurements of the influx across the outer border reveal saturation kinetics and competition (Biber & Curran, 1970). Both phenomena are incompatible with the concept of free diffusion and indicate a lack of independence of ion movement. There are a large number of factors which must be considered as possible sites of interaction or barriers, such as internal binding sites, flux coupling, external charged groups, pore constrictions, or carriers. A number of studies concerning the effects of external anions (Ferreira, 1968; Huf, 1972) and cations (Biber & Curran, 1970; Rotunno et al., 1970) indicate that charged sites may act as barriers or filters to ion movement.

We explored this possibility in greater detail by undertaking a systematic study of effects of substitutions of monovalent cations and anions and of changes in ionic strength in the external medium on active Na transport across the frog skin. The strategy was the following: effects on Na transport were studied when the ionic strength was increased by addition of various combinations of monovalent cations and anions to the external medium and also when the corresponding combinations of ions were used in substitutions which did not involve a change in ionic strength. It was anticipated that such an approach would provide new information not only about the relative ability of different ions to stimulate or inhibit Na transport but also about effects exerted by ionic strength on Na entry across the external (apical) surface of the epithelial cells.

Throughout the experiments, the external Na concentration was maintained at 15 mM. At this concentration the Na transport proceeds at a considerable rate. Yet at this concentration Na is low enough to permit a sizeable increase in ionic strength by addition of various anions and cations without exceeding the ionic strength and osmolality which is normally used in amphibian Ringer's solution. Instead of following the usual assumption that the

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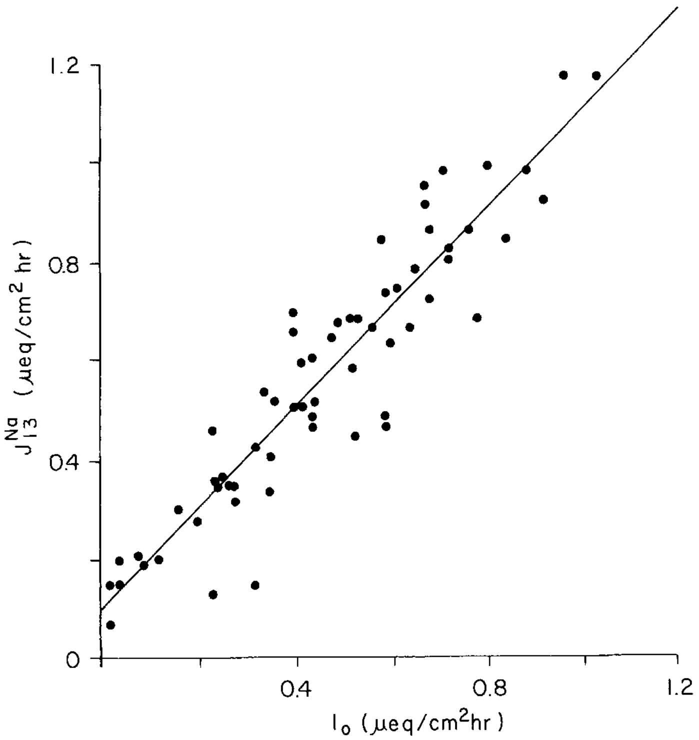


**Fig. 1.** A plot of the transepithelial influx of Na ($J_{13}^{Na}$) *vs.* the short-circuit current ($I_o$) in skins exposed to a low ionic strength external solution (15 mM NaCl, 200 mM mannitol, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$). $N=62$. For details *see* text

**Table 1.** Fluxes and electrical phenomena at low and high external ionic strength

| | Low ionic strength | High ionic strength |
|---|---|---|
| Transepithlial potential (mV) | 49 ± 2 | 33 ± 2 |
| Resistance ($\Omega$ cm²) | 3813 ±249 | 1617 ±122 |
| Short-circuit current (μeq/cm² hr) | 0.489 ± 0.023 | 0.785 ± 0.039 |
| Na influx ($J_{13}^{Na}$) | 0.572 ± 0.035 | 1.004 ± 0.048 |
| Na efflux ($J_{31}^{Na}$) | 0.021 ± 0.002 | 0.023 ± 0.002 |
| Choline efflux ($J_{31}^{Ch}$) | 0.045 ± 0.004 | 0.046 ± 0.008 |
| $J_{13}^{Na}$ – short-circuit current | 0.105 ± 0.013 | 0.078 ± 0.017 |

Fluxes are in μeq/cm² hr. Number of preparations: 192 for electrical measurements, 62 and 64 for flux determinations at low and high ionic strength, respectively. External medium contained 15 mM NaCl, 200 mM mannitol, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$ for "low ionic strength" condition and 15 mM NaCl, 100 mM choline Cl, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$ for "high ionic strength" condition. For details *see* text

short-circuit current is an accurate indicator for the rate of active Na transport, we felt that it was necessary to measure the actual ion fluxes, not only of Na but also of choline. There are at least three reasons for not relying solely on current measurements for information about Na transport. First, even in absence of electrochemical gradients, the short-circuit current may not be identical with net Na transport. For example, it is well know that Li which is actively transported contributes to the current (Zerahn, 1954). Furthermore, after addition of $FeCl_3$ to the external medium, active Na transport in inward direction is replaced by Cl transport in outward direction so that the current observed represents exclusively net Cl movement (Biber & Mullen, 1979). Second, other ions besides Na may contribute to the current in the presence of an electrochemical gradient for these ions across the skin. Third, the assumption is usually made that the Na flux in inward direction (Na influx) is for practical purposes equal to net transfer of Na and current across the skin. However, this assumption may not be valid in the presence of large electrochemical gradients, especially when the Na permeability is increased. For example, in voltage-clamp experiments we found evidence for very large discrepancies between current and Na influx across the entire epithelium as well as across the external (apical) cell membrane (Biber & Sanders, 1973).

The following study provides information about the relative abilities of different anions and cations to stimulate or inhibit Na transport and illustrates that under short-circuit conditions active Na transport is strongly dependent on both the cationic and anionic composition of the external solution.

Effects connected with changes in ionic strength suggest that surface charges exert a screening effect on the entry of Na into the epithelial cells.

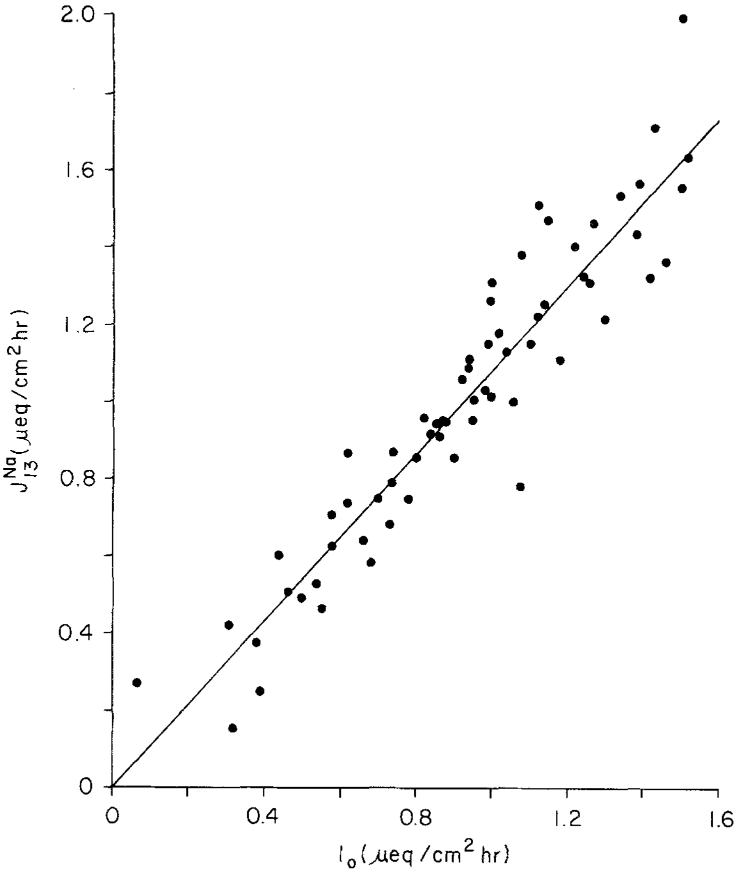


**Fig. 2.** A plot of the transepithelial influx of Na ($J_{13}^{Na}$) *vs.* the short-circuit current ($I_0$) in skins exposed to a high ionic-strength external solution (15 mM NaCl, 100 mM ChCl, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$). $N=64$. For details *see* text

## Materials and Methods

Frogs (southern variety of *Rana pipiens*) were purchased from a commercial source in Connecticut (Connecticut Valley) throughout the year. They were kept, unfed, at room temperature (21–23 °C) in bins with free access to water. They were used within 2 weeks of arrival.

After each frog had been double pithed, the abdominal skin was carefully removed and circular pieces were cut out with a punch. These circular pieces were mounted between two halves of a new chamber which had been specially designed to reduce edge-damage (Biber & Mullen, 1977). The mounted frog skin (exposed area of 0.98 cm$^2$) divided the chamber into serosal and external compartments, each containing 0.5 ml of bathing solution. The solutions in both compartments were stirred vigorously by a stream of small air bubbles.

The transepithelial potential difference across the skin (TEP) was measured by calomel half cells which were connected to the solution by agar bridges. A second pair of bridges led to Ag-AgCl electrodes for applying the short-circuit current ($I_0$). TEP and $I_0$ were determined by using an automatic clamping device which could be adjusted for changes in fluid resistance between the bridges for measuring the TEP. The $I_0$ was monitored continuously by a recorder (Gould Brush Model 220). The resistance across the isolated frog skin was obtained either by computations from the transepithelial potential under open-circuit conditions and the short-circuit current or by calculations from the difference between the short-circuit current and the current determined when the tissue was clamped for a few seconds at 5 or 10 mV. There was no significant difference between the results obtained with these two methods.

The serosal solution was a modified Ringer's solution consisting of 5 or 15 mM NaCl, in combination with 110 or 100 mM choline Cl (ChCl), respectively, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$. The "low ionic strength" control external solution consisted of 5 or 15 mM NaCl, in combination with 220 or 200 mM mannitol, respectively, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$. The "high ionic strength" control external solution consisted of 5 or 15 mM NaCl, in combination with 110 or 100 mM ChCl, respectively, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$. Experimental cationic substitution solutions were identical to "high ionic strength" control solution except for the replacement of ChCl by another monovalent chloride salt. Experimental anionic substitution solutions were identical to "high ionic strength" control solution except for the replacement of ChCl by another monovalent choline salt.

Fluxes of ions were measured by adding the appropriate isotope to one side of the skin and measuring its rate of appearance on the "cold" side. Experiments were arranged so that specific activity on the "hot" side did not change appreciably over the experimental period and the backflux of isotope was negligible. The "cold" bathing medium was entirely emptied and replaced at 10-min intervals, and during the procedure the current flow through the chamber and the skin had to be interrupted briefly (< 5 sec). Radioactivity was determined by liquid scintillation spectrometry (Intertechnique Model SL 30) with appropriate corrections for quenching and background.

Concentration of sodium and chloride was measured by flame photometry (Instrumentation Laboratory Model IL343) and chloridometry (Buchler Model 4-2008), respectively. Osmolality and pH were determined with an osmometer (Advanced Instruments Model 3D) and pH meter (Radiometer Model PHM 64), respectively.

Flux measurements were made only under short-circuit conditions and are reported only for intervals where a steady state had been achieved unless a time sequence is specified. Fluxes are

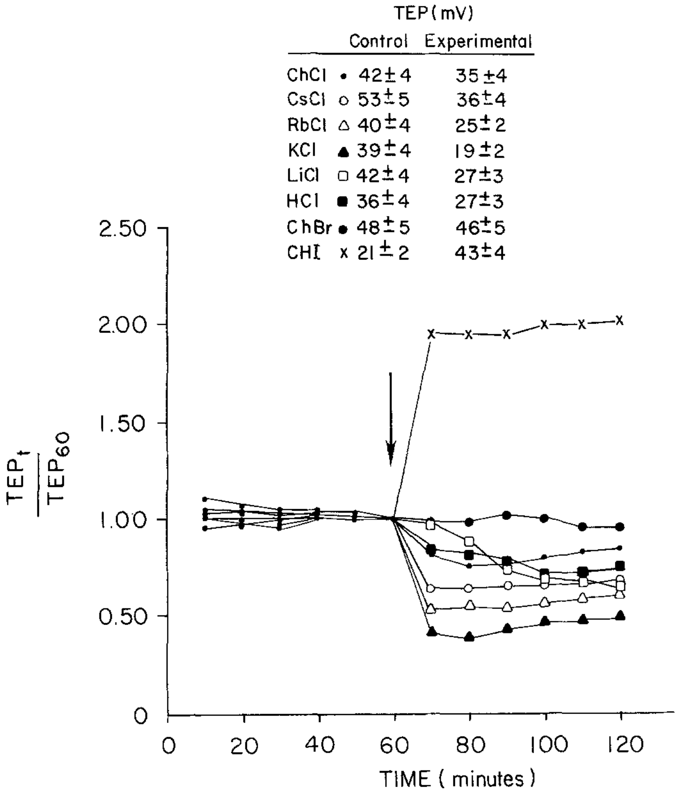


**Fig. 3.** The effect of a change in the external ionic strength by substitution of 100 mM of test salt for 200 mM mannitol and the effect of decreasing the pH of the low ionic-strength external medium from 8.0 to 4.0 on the transepithelial potential difference (TEP) across isolated frog skin. All values have been normalized in relation to the mean TEP measured at the last interval of the control period ($TEP_{60}$). Mean absolute values with SE are given for the last intervals of the control and experimental periods. $N=24$ for each point

expressed in terms of a 3-compartment model in which compartments 1, 2 and 3 are the external bathing solution, cell compartment, and serosal bathing solution, respectively. $J_{ij}^{s}$ represents the solute flux from compartment *i* to compartment *j*. Throughout the paper, the errors are given as standard errors of the mean (SEM) and the number of observations is given in parenthesis.

## Results

In initial experiments the electrical characteristics and the unidirectional Na fluxes across the isolated frog skin were compared in the standard "low ionic strength" external solution and the standard "high ionic strength" external solution. Data from all skins tested have been averaged and are listed in Table 1. Clearly, at high ionic strength transepithelial potential (TEP) and resistance (*R*) were substantially lower than at low ionic strength. On the other hand, short-circuit current ($I_0$) and Na influx($J_{13}^{Na}$) were larger by some 60 and 75%, respectively. There was no significant difference between Na effluxes ($J_{31}^{Na}$) or between choline effluxes ($J_{31}^{Ch}$) obtained under these two conditions.

At low as well as at high ionic strength $J_{13}^{Na}$ was greater than the simultaneously measured $I_0$. At low

**Table 2.** Percentage changes in Na influx ($J_{13}^{Na}$), short-cirucuit current ($I_0$) and transepithelial potential (TEP)

| Salt | Ionic strength increased | | | Ionic strength not changed | | |
|---|---|---|---|---|---|---|
| | $J_{13}^{Na}$ | $I_0$ | TEP | $J_{13}^{Na}$ | $I_0$ | TEP |
| LiCl | −85±13 | −30±12 | −36± 9 | −86±14 | +20± 7 | +38± 9 |
| KCl | −23± 7 | −41± 9 | −62±11 | −45± 9 | −54±10 | −24± 3 |
| RbCl | NS | +11± 3 | −48± 9 | −28± 5 | −32± 6 | −15± 4 |
| CsCl | +25± 4 | +33± 4 | −36± 5 | −18± 5 | −26± 4 | −21± 4 |
| ChBr | +51±10 | +62±11 | NS | +12± 6 | +14± 6 | +18± 7 |
| ChI | +86±14 | +150±20 | +102±15 | +28± 8 | +23± 7 | +74±12 |

Values calculated from changes observed in individual skins. 8 skins were used for $J_{13}^{Na}$ determinations. Measurements of $I_0$ and TEP were made in 24 skins. Because of initial transient changes only the last experimental period was used for calculations of LiCl experiments. For details *see* text.

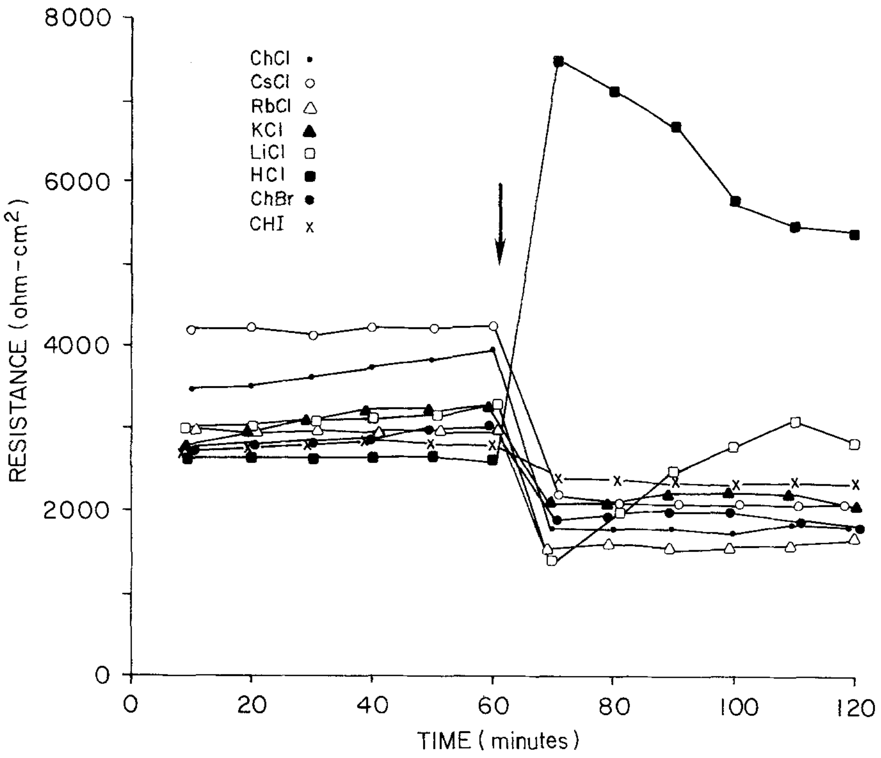


**Fig. 4.** The effect of an alteration in the external ionic strength by substitution of 100 mM of test salt for 200 mM mannitol and the effect of decreasing the pH of the low ionic-strength external medium from 8.0 to 4.0 on resistance across isolated frog skin. Points represent averages obtained from observations on 24 skins

**Table 3.** Effect of external ionic strength on the efflux of Na($J_{31}^{Na}$)

| Experimental | $J_{31}^{Na}$ control (low ionic strength) | $J_{31}^{Na}$ experimental (high ionic strength) | $J_{31}^{Na}$ experimental / $J_{31}^{Na}$ control |
|---|---|---|---|
| Choline Cl | 19±2 | 27±3 | 1.44±0.13 |
| CsCl | 20±2 | 27±3 | 1.35±0.14 |
| RbCl | 22±2 | 23±2 | 1.05±0.15 |
| KCl | 19±2 | 16±2 | 0.84±0.04 |
| LiCl | 23±2 | 14±2 | 0.61±0.10 |
| Choline Br | 10±2 | 29±3 | 1.45±0.15 |
| Choline I | 20±2 | 30±3 | 1.43±0.13 |

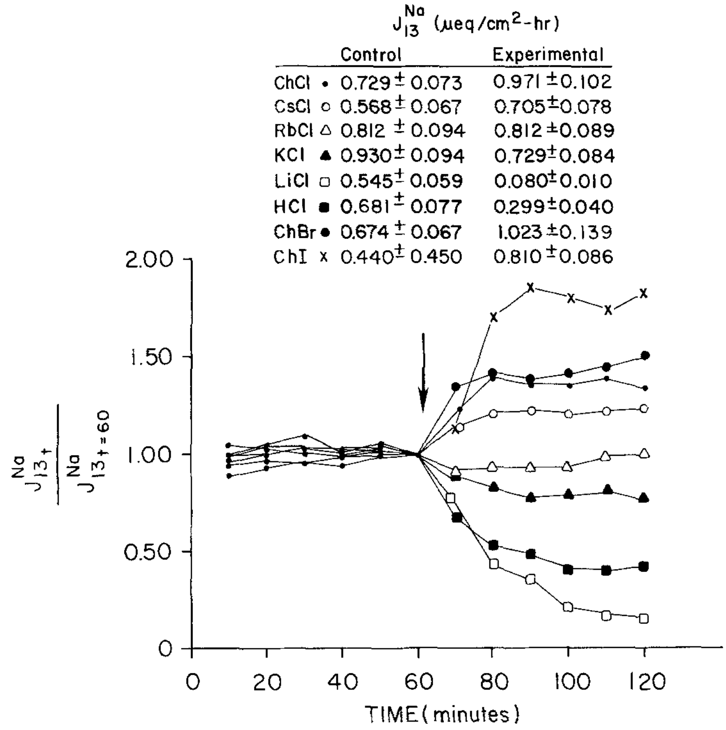
Efflux values are in neq/cm$^2$ hr. $n=16$ for each value. The external solution initially consisted of "low ionic strength" solution containing 15 mM NaCl, 200 mM mannitol, 2.5 mM KHCO$_3$ and 1 mM CaCl$_2$ (control). During the experimental manipulation the ionic strength of the external solution was increased by replacing mannitol with either 100 mM monatomic chloride salt or a 100 mM monatomic choline salt. Each line represents experiments on 8 skins. Lowering of the pH from 8 to 4 in the external "low ionic strength" solution decreased $J_{31}^{Na}$ from 21±2 to 10±1 neq/cm$^2$ hr ($n=16$ for each average value).

**Table 4.** Effect of changes in external cations and anions on the efflux of Na ($J_{31}^{Na}$)

| Experimental condition | $J_{31}^{Na}$ control | $J_{31}^{Na}$ experimental | $J_{31}^{Na}$ experimental / $J_{31}^{Na}$ control |
|---|---|---|---|
| CsCl | 26±3 | 24±2 | 0.92±0.11 |
| RbCl | 22±2 | 22±2 | 1.01±0.03 |
| KCl | 23±2 | 13±1 | 0.57±0.10 |
| LiCl | 25±2 | 10±1 | 0.39±0.12 |
| Low pH | 24±2 | 10±1 | 0.42±0.12 |
| Choline Br | 20±2 | 34±3 | 1.69±0.22 |
| Choline I | 21±2 | 26±3 | 1.24±0.10 |

Efflux values are in neq/cm$^2$ hr. $n=16$ for each value. The external solution initially consisted of 15 mM NaCl, 100 mM ChCl, 2.5 mM KHCO$_3$ and 1 mM CaCl$_2$ (control). The data shown on each line were obtained from experimental procedures on 8 skins in which either the ChCl in the external solution was replaced with another monatomic chloride salt or another monatomic choline salt, or in which the pH of the external solution was lowered from 8 to 4.

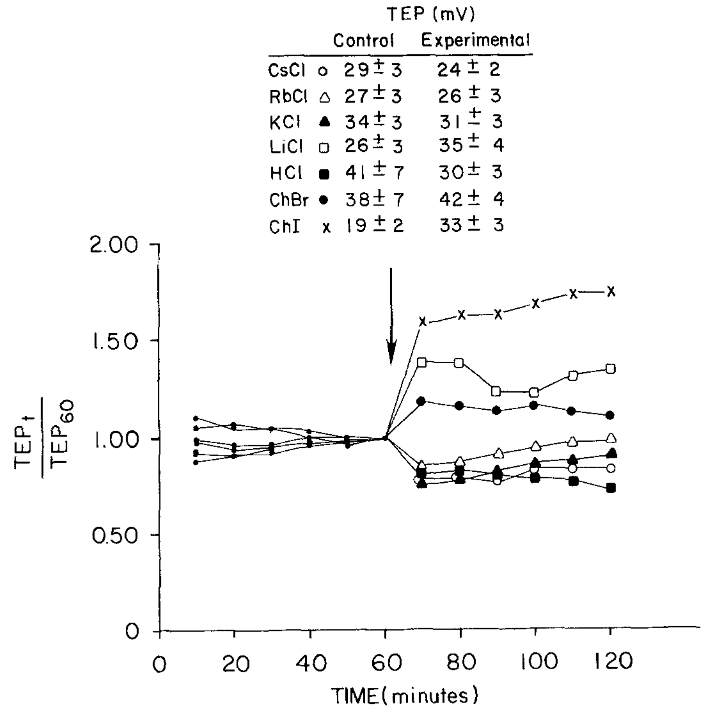
ionic strength the difference between the two amounted to 0.105 μeq/cm$^2$ hr ($n=62$; *see* last line on Table 1; $I_0$ in this group was 0.467±0.031 μeq/cm$^2$ hr). The individual values for $I_0$ and $J_{13}^{Na}$ are plotted in Fig. 1. Calculations from such a plot give a correlation coefficient and slope of 0.93 and 1.021±0.052, respectively, and an intercept of 0.095±0.028 μeq/cm$^2$ hr. At high external ionic strength the difference between simultaneously measured $J_{13}^{Na}$ and $I_0$ amounted to 0.078 μeq/cm$^2$ hr ($n=$



$J^{Na}_{13}$ (μeq/cm²-hr)

| | | Control | Experimental |
|---|---|---|---|
| ChCl | • | 0.729±0.073 | 0.971±0.102 |
| CsCl | ○ | 0.568±0.067 | 0.705±0.078 |
| RbCl | △ | 0.812±0.094 | 0.812±0.089 |
| KCl | ▲ | 0.930±0.094 | 0.729±0.084 |
| LiCl | □ | 0.545±0.059 | 0.080±0.010 |
| HCl | ■ | 0.681±0.077 | 0.299±0.040 |
| ChBr | ● | 0.674±0.067 | 1.023±0.139 |
| ChI | x | 0.440±0.450 | 0.810±0.086 |

**Fig. 5.** The effect of an alteration in the external ionic strength by substitution of 100 mM of test salt for 200 mM mannitol and the effect of decreasing the pH of the low ionic-strength external medium from 8.0 to 4.0 on the transepithelial influx of Na ($J^{Na}_{13}$) across isolated frog skin. All values have been normalized in relation to the mean influx measured at the last interval of the control period ($J^{Na}_{13t=60}$). Mean absolute values with SE are given for the last intervals of the control and experimental periods. $N=8$ for each point



TEP (mV)

| | | Control | Experimental |
|---|---|---|---|
| CsCl | ○ | 29±3 | 24±2 |
| RbCl | △ | 27±3 | 26±3 |
| KCl | ▲ | 34±3 | 31±3 |
| LiCl | □ | 26±3 | 35±4 |
| HCl | ■ | 41±7 | 30±3 |
| ChBr | ● | 38±7 | 42±4 |
| ChI | x | 19±2 | 33±3 |

**Fig. 6.** The effect of the substitution of 100 mM of test salt for 100 mM ChCl and the effect of decreasing the pH of the high ionic-strength external media from 8.0 to 4.0 on the transepithelial potential difference (TEP) across isolated frog skin. All values have been normalized in relation to the mean TEP measured at the last interval of the control period ($TEP_{60}$). Mean absolute values with SE are given for the last intervals of the control and experimental periods. $N=24$ for each point

64; Table 1; $I_0$ in this group was $0.926 \pm 0.017$ μeq/cm² hr). Figure 2 represents a plot of $J^{Na}_{13}$ *vs.* $I_0$ for the individual values obtained at high ionic strength. The data yield a correlation coefficient and slope of 0.94 and $1.073 \pm 0.049$, respectively, and an intercept of $0.011 \pm 0.048$ μeq/cm² hr.

These results suggest that the ionic strength of the external solution has a large influence on the rate of active Na transport in the isolated short-circuited frog skin. However, the data were collected from different skins. In order to test the effect of external ionic strength on transport parameters more

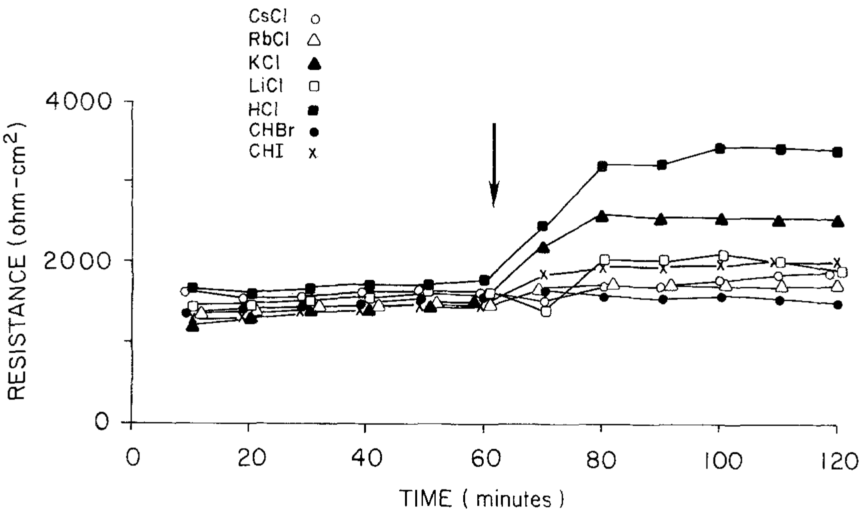
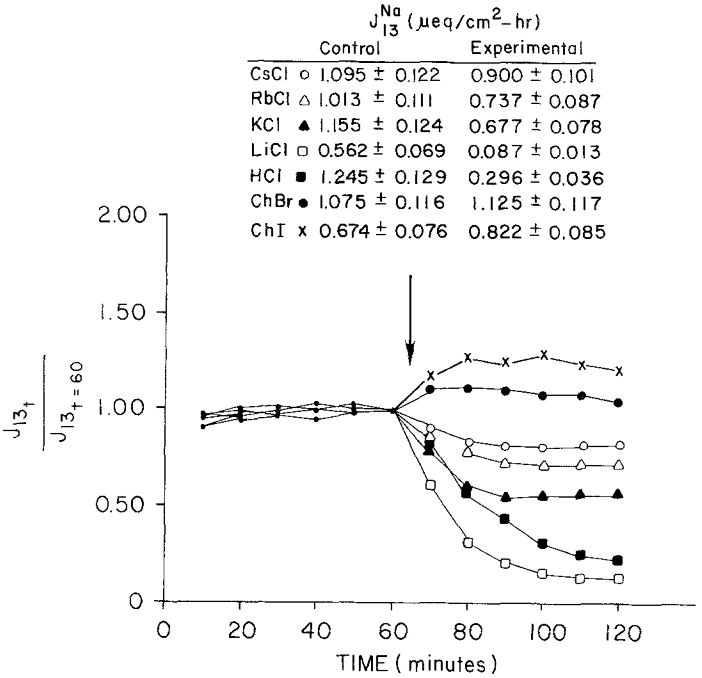


Fig. 7. The effect of the substitution of test salt for 100 mM ChCl and the effect of decreasing the pH of the high ionic-strength external media from 8.0 to 4.0 on the resistance across isolated frog skin. Points represent averages obtained from observations on 24 skins



| $J_{13}^{Na}$ (μeq/cm²-hr) | | Control | Experimental |
|---|---|---|---|
| CsCl | ○ | 1.095 ± 0.122 | 0.900 ± 0.101 |
| RbCl | △ | 1.013 ± 0.111 | 0.737 ± 0.087 |
| KCl | ▲ | 1.155 ± 0.124 | 0.677 ± 0.078 |
| LiCl | □ | 0.562 ± 0.069 | 0.087 ± 0.013 |
| HCl | ■ | 1.245 ± 0.129 | 0.296 ± 0.036 |
| ChBr | ● | 1.075 ± 0.116 | 1.125 ± 0.117 |
| ChI | x | 0.674 ± 0.076 | 0.822 ± 0.085 |

Fig. 8. The effect of the substitution of 100 mM of test salt for 100 mM ChCl and the effect of decreasing the pH of the high ionic-strength external medium from 8.0 to 4.0 on the transepithelial influx of Na ($J_{13}^{Na}$ or $J_{13}$) across isolated frog skin. All values have been normalized in relation to the mean $J_{13}^{Na}$ measured at the last interval of the control period ($J_{13_{t=60}}$). Mean absolute values with SE are given for the last intervals of the control and experimental periods. $N=8$

directly $J_{13}^{Na}$, $J_{31}^{Na}$, TEP, $I_0$ and resistance were followed with time after an abrupt increase in the ionic strength of the external solution by switching rapidly from the low to the high ionic strength medium in the same skin. Under these conditions the results were quantitatively similar to those obtained in a steady state (vide supra). There was 27±4% decrease in TEP (Fig. 3) and 78±9% increase in $I_0$. The simultaneous decrease in TEP and increase in $I_0$ resulted in a 56±7% fall in skin resistance (Fig. 4). The values of $J_{13}^{Na}$ and $J_{31}^{Na}$ increased by 40±6% (Fig. 5) and 44±13% (Table 3), respectively. These changes were even more marked if the NaCl concentration of both the low and high ionic strength bathing solutions was reduced from 15 to 5 mM (220 mM mannitol replaced with 110 mM ChCl in the change from low to high ionic strength medium). In these circumstances $I_0$ increased by 95±10% (0.164 to 0.319 μeq/cm² hr) and $J_{13}^{Na}$ by 46±6% (0.275 to 0.402 μeq/cm² hr, $n=8$).

These data show that the substitution of 100 mM ChCl for 200 mM mannitol results in a significant increase in the active transport of Na. In order to determine whether this stimulation is due to an increase in ionic strength or to a change in the external concentrations of Ch or Cl, the effects of a series of monatomic chloride and choline salts were examined. In one group of experiments the frog skin was first bathed in the standard low ionic strength external solution (200 mM mannitol) and then with a high ionic strength external test solution in which 100 mM of the salt of interest replaced 200 mM mannitol (Figs. 3–5). In a second set of experiments the skins were bathed initially in the standard high ionic strength external solution (100 mM ChCl) and this was then replaced with the high ionic strength test solution containing 100 mM of the appropriate salt in place of ChCl (Figs. 6–8). The results of these two series of experiments will be considered separately.

Figures 3 through 8 each present separate data for changes in TEP, $R$ or $J_{13}^{\mathrm{Na}}$. The data are plotted as a function of time and are expressed as fractional changes with respect to the last control period. To avoid crowding, the SE of the fractional changes are not given in the figures but SE of percentage changes are included in Table 2 which summarizes the changes in TEP, $J_{13}^{\mathrm{Na}}$ and $I_0$. The percentage changes in Table 2 were calculated from measurements done on individual skins each of which served as its own control. Such percentage changes seem to be most meaningful in these experiments since there were large variations in transport rates between different preparations. However, to provide an impression of the variability of transport rates in different groups of experiments we have added the averages plus SE for the absolute values on top of the figures dealing with changes in TEP and $J_{13}^{\mathrm{Na}}$ (Figs. 3, 5, 6, 8).

### *Increase in Ionic Strength*

As Fig. 3 indicates, TEP decreased after replacement of mannitol by KCl, RbCl, CsCl and LiCl but remained unchanged when exposed to ChBr. TEP increased sharply with exposure to ChI. $R$ decreased with every substitution except LiCl (Fig. 4). Initially LiCl caused a substantial decrease in $R$, but $R$ returned gradually towards the control level. The effects of changes in pH will be reported at the end of the Results section. Changes in $J_{13}^{\mathrm{Na}}$ observed after these various substitutions are shown on Fig. 5. They indicate an ever stronger stimulation with CsCl, ChCl, ChBr and ChI, a moderate inhibition with KCl, and a strong inhibition after introduction of LiCl to the external medium.

Table 2 provides information about changes in $I_0$ and about differences in sequences for inhibitory or stimulatory effects on $I_0$, TEP and $J_{13}^{\mathrm{Na}}$. As mentioned above, the table represents percentage changes obtained from individual skins. $I_0$ seems to be following the same trend as $J_{13}^{\mathrm{Na}}$, although there are some differences in the magnitude of the response to LiCl and RbCl. After change-over to LiCl there was first an increase in $I_0$ above control levels. However, in subsequent periods $I_0$ decreased to reach stable values at 30% below control levels. Since after LiCl replacement part of the current is carried by Li ions (*see* Introduction), it was not surprising to find that $J_{13}^{\mathrm{Na}}$ was much lower than $I_0$ and that the percentage decrease in $J_{13}^{\mathrm{Na}}$ was much greater than the one observed for $I_0$. As a result of this, LiCl occupies a different position in the stimulation-inhibition sequence depending on whether $J_{13}^{\mathrm{Na}}$ or $I_0$ was measured. The position of LiCl is also different in the TEP measurements in that the percent decrease due to LiCl is less pronounced than the decrease observed in presence of either KCl or RbCl.

Determinations of Na efflux ($J_{31}^{\mathrm{Na}}$) are listed in Table 3. $J_{31}^{\mathrm{Na}}$ is substantially increased after addition of ChCl, CsCl, ChBr and ChI, and significantly reduced during exposure to KCl and LiCl. These changes proceed in the same direction as those observed for $J_{13}^{\mathrm{Na}}$ under corresponding conditions.

### *No Change in Ionic Strength*

In the second set of experiments the external surface of the frog skin was exposed to various salts without a change in ionic strength by substitution of ChCl. As shown in Fig. 6, TEP was in general either less depressed or more stimulated when compared to the changes observed in the first series of experiments. Figure 7 indicates further that, with the exception of LiCl, there was, in contrast to the previous experiments, no decrease in $R$ in this set of experiments: An increase in $R$ did occur in presence of KCl, but no substantial changes were measured with the other salt replacements. This lack of a decrease in $R$ goes along with a general trend towards greater inhibition of $J_{13}^{\mathrm{Na}}$ (Fig. 8) when compared to the corresponding changes seen in the first set of experiments. Even the stimulation of $J_{13}^{\mathrm{Na}}$ in the presence of ChI and ChBr is less pronounced than in the previous set.

A comparison of changes in $J_{13}^{\mathrm{Na}}$, $I_0$ and TEP observed under these conditions can be made in Table 2 (right-hand side) and shows that there is again a disparity between the effects on these three measurements. The sequence of effects on $J_{13}^{\mathrm{Na}}$ from greatest inhibition to strongest stimulation is the same as the one observed in the first series of experiments. For $I_0$ measurements the sequence is also the same except for LiCl which stimulates instead of inhibiting $I_0$. The sequence observed with TEP measurements is similar to the one seen under the same conditions for $I_0$, with possible exception of RbCl and CsCl.

Measurements of $J_{31}^{\mathrm{Na}}$ are listed in Table 4. They indicate that $J_{31}^{\mathrm{Na}}$ decreases in presence of LiCl and KCl, the two salts which inhibited $J_{13}^{\mathrm{Na}}$ most. On the other hand, $J_{31}^{\mathrm{Na}}$ increased in the presence of ChBr and ChI, both of which also stimulated $J_{13}^{\mathrm{Na}}$.

### *Changes in pH*

A sudden decrease in the pH of the low ionic strength external solution (pH 8.0 to pH 4.0) resulted in a 28±8% fall in the TEP (Fig. 3) and a 71±12% decrease in the $I_0$ which was accompanied by a 188±4% increase in skin resistance (Fig. 4). In addition, the decrease in external pH gave rise to a 60±11% decrease in $J_{13}^{Na}$ and a 52±10% decrease in $J_{31}^{Na}$ (Table 1). When the pH of the high ionic strength external solution was decreased suddenly from pH 8.0 to 4.0, there was a 27±8% decrease in the TEP (Fig. 6), a 63±11% decrease in the $I_0$ and a 106±16% increase in skin resistance (Fig. 7). Other changes included a 72±12% decrease in $J_{13}^{Na}$ (Fig. 8) and a 58±11% decrease in $J_{31}^{Na}$ (Table 2). A further decrease in the pH of the external solution to 3.0 resulted in a much more complete inhibition of TEP, $J_{13}^{Na}$ and $I_0$, but there was also a large increase in $J_{31}^{Na}$ and a large decrease in skin resistance which probably indicate a breakdown of the skin as a permeability barrier.

All the effects of changes in ionic composition of the external medium reported here are reversible.

## Discussion

### *Relationship between $I_0$ and $J_{13}^{Na}$. Changes in Electrical Properties*

The short-circuit current ($I_0$) was only about 82% of the simultaneously measured transepithelial influx of Na ($J_{13}^{Na}$) in skins exposed to low ionic strength external medium. A discrepancy, however, should be expected because contributions to the current from the transepithelial efflux of Na ($J_{31}^{Na}$) and the assymetrical gradients for Ch and Cl. Mean values for $J_{31}^{Na}$, $J_{31}^{Ch}$, and ($J_{13}^{Na}-I_0$) were on the order of 0.021, 0.045, and 0.105 μeq/cm$^2$ hr, respectively. If these values are taken into consideration, a small component, in the range of 0.040 μeq/cm$^2$ hr, remains unaccounted for. The passive diffusion of Cl down its concentration gradient would only make the discrepancy larger. However, it seems possible that this difference represents net inward movement of Cl as recently demonstrated in isolated frog skin (Alvarado, Dietz & Mullen, 1975; Alvarado, Poole & Mullen, 1975), although it might be due to the variability in the different measurements.

The increase in the ionic strength of the external medium by the substitution of ChCl for mannitol led to a significant increase in the short-circuit current. Koblick (1959) noted a similar phenomenon and suggested that choline might be transported in the same direction as, and possibly in competition with, sodium. Kirschner (1960), however, showed that although the skin of *R. pipiens* was not impermeable to choline, there was no evidence for active choline transport. We have found a mean flux ratio for choline of 1.05±0.12 ($n=24$) with the high ionic strength Ringer's. Although we found no evidence for the active transport of choline, the permeability was significantly higher than that demonstrated for Na in a previous paper (Biber & Mullen, 1976). The high permeability of choline cannot be explained by molecular size but has been observed previously in frog skin (Kirschner, 1960) and the kidney tubule (Boulpaep, 1976). It has been suggested that the high permeability of choline in relation to Na might be due to radiolysis of choline (Kirschner, 1960), and there is some evidence that the low permeability values for Na in frog skin might be due to an interaction of the active transport system with $J_{31}^{Na}$ (Biber & Mullen, 1976; Biber & Mullen, 1977; Huf & Howell, 1974; Kirschner, 1959).

The observation that these choline fluxes are relatively large does not necessarily imply that these fluxes proceed through the cells (i.e., via transcellular path). In fact, the choline fluxes reported here are in the same range as the fluxes measured in identical setups for several nonelectrolytes (Biber & Mullen, 1977). The kinetic properties of these nonelectrolyte fluxes suggested passage through an extracellular shunt (i.e., via paracellular path).

The increase in short-circuit current after an abrupt change in the ionic strength of the external medium was accompanied by a corresponding increase in $J_{13}^{Na}$ and thus the increased current seems to be due to an alteration in active Na transport. Despite corresponding increases in $I_0$ and $J_{13}^{Na}$, and a lack of concentration assymetries, a small component of the $I_0$ (0.060 μeq/cm$^2$ hr) remains unaccounted for. Again, active transport of Cl could be the source for such a difference.

The increase in the ionic strength of the external solution by the replacement of mannitol with ChCl also resulted in a large decrease in the transepithelial potential difference (TEP) and total skin resistance. These alterations are probably due to the shunting effect of increased external Cl. Not only should there be a direct increase in conductance because of high Cl concentration, but it has also been shown that high external Cl concentrations cause large increases in the permeability of the skin to Cl, either because of the opening of paracellular shunts (Alvarado, Dietz & Mullen, 1975; Alvarado, Poole & Mullen, 1975; Mandel & Curren, 1972) or the activation of an exchange diffusion system (Alvarado, Dietz & Mullen, 1975; Alvarado, Poole & Mullen, 1975; Braus, Kristensen & Larsen, 1976).

### *Differential Effects of Cations*

We studied the effects of several monatomic chloride and choline salts in order to determine whether the stimulation of active Na transport seen when 100 mM ChCl is substituted for 200 mM mannitol is due to increased ionic strength, increased external [Ch] or increased external [Cl]. The results presented here demonstrate that reversible changes in TEP, $I_0$, $J_{13}^{\mathrm{Na}}$, $J_{31}^{\mathrm{Na}}$ and skin resistance can be observed when, in the presence of constant external [Na], the cationic composition of the external medium is altered. The differential inhibitory effects of cations suggest that the general ionic strength of the external medium is not the only factor involved. The relative abilities of external cations to inhibit $J_{13}^{\mathrm{Na}}$ are $H > Li > K > Rb > Cs > Ch$. The sequence is consistent with Eisenman's X or XI series for ion selectivity (Eisenman, 1962) and Chandler's and Meves' alkali ion selectivity for squid giant axon Na channels (Chandler & Meves, 1965). It is not surprising that the sequence observed here for Na transport differs in some respects from the sequence obtained by Lindley and Hoshiko (1964) from changes in TEP in the frog skin since changes in TEP across the entire skin can be due to many factors including the development of diffusion potentials across the cell membranes on either side of the epithelial cells or across the extracellular shunt, changes in Na or anion permeability, or alteration of an electrogenic transport process.

In general, the inhibitory effect of cations on Na transport decreases with an increase in crystal radius of the ion. The relative cation sequence is indicative of high anionic field strength. Recent studies on excitable membranes have led to the conclusion that there is an essential negatively charged group within the Na channel (Hille, 1975) and Zeiske and Lindemann (1975) have recently suggested the existence of charged Na pores (carboxyl groups) at the external cell surface of frog skin in order to explain the inhibitory action of low pH on Na transport.

### *Effects of Anions*

Huf (1972) and Ferreira (1968) have shown that the substitution of Cl for $SO_4$ in the external bathing medium gives rise to a substantial increase in the $I_0$ and $J_{13}^{\mathrm{Na}}$, and it has been suggested that at least a component of active Na transport is anion dependent. Under short-circuit conditions, however, only the external and internal solutions are short-circuited and significant potentials exist across the individual cell membranes. One, then, cannot entirely rule out the possibility of electrical coupling across individual cell membranes. Our studies show, however, an increase in both, $J_{13}^{\mathrm{Na}}$ and resistance after replacement of Cl with I in the external solution. This mitigates against electrical coupling across the outer cell membrane. The relative abilities of external anions to stimulate the short-circuit current and $J_{13}^{\mathrm{Na}}$ are $I > Br > Cl$, i.e., the stimulation becomes stronger with an increase in the crystal radius of the ion. This sequence is very similar, but much less extensive, to that presented by Singer and Civan (1971) for the amphibian urinary bladder but is inconsistent with ionic size and the sequence of anion selectivity put forward by Lindley and Hoshiko (1964) for frog skin. Singer and Civan (1971) concluded that the effects of anion substitution could be explained by an interaction of anions with one or more sites with a net positive charge in a complex system.

### *Ion Effects at Outer Barrier*

The experiments show that replacement of anions and cations in the external solution causes changes in $J_{13}^{\mathrm{Na}}$. But alterations in $J_{13}^{\mathrm{Na}}$ are practically identical to changes in active Na transport across the epithelium under the experimental conditions used in these studies (skins short-circuited, Na concentration kept constant at the same level on both sides of the preparation) because the Na efflux values measured under control as well as experimental condition are so small (Tables 3 and 4) that they make up for only a small fraction of $J_{13}^{\mathrm{Na}}$. In addition, it seems reasonable to assume that under the conditions present in this study the observed changes in $J_{13}^{\mathrm{Na}}$ reflect accurately changes in Na entry into the epithelial cells across the outward facing (apical) cell membrane of the outermost living cell layer ($J_{12}^{\mathrm{Na}}$) since Na movement across this border has been shown to be rate-limiting for Na transport across the epithelium under may different conditions (*see,* e.g., Biber & Cruz, 1973; Cruz & Biber, 1976; Mullen & Biber, 1978). This assumption is also supported by studies in which inhibitory effects of external Li and K were observed by direct measurements of Na entry across the outer border (Biber & Curran, 1970; Rotunno et al., 1970).

It is not known how such ion replacements act on Na entry. However, two of the most obvious possibilities for specific ion effects on Na entry deserve to be mentioned here. First, it is possible that these ions interact either with sites in Na channels in the apical cell membrane or with sites on a carrier which is involved in Na transfer acros the apical cell border. The kinetics of Na entry have been explained both on the basis of a carrier model (Biber & Sanders, 1973) and more recently on the basis of channel kinetics (Lindemann & Van Driessche, 1977). The present results do not permit a distinction to be made between the two models. Second, diffusion potentials generated by various ions across the apical cell membrane could cause changes in driving force for Na across this membrane and therefore, changes in Na entry. Hence, permeable cations would change the potential difference across the membrane so that Na entry is inhibited and permeable anions would change the membrane potential towards values which are more favorable for Na entry. Lithium enters the cells via the apical cell membrane. In fact, in direct measurements Li uptake was found to compete with Na uptake (Biber & Curran, 1970) and recent measurements of membrane potentials indicate that changes in Li concentration in the external medium induce changes in the potential across the apical cell membrane (Nagel, 1977). However, Li takes a special position: Its crystal radius is smaller than that of Na and it is actively transported across the frog skin (*see* Introduction). Koefoed-Johnsen and Ussing (1958) observed that the apical side of the skin is impermeable to K, and it seems likely that the apical cell membrane is also relatively impermeable to the other cations tested here.

**Table 5.** Effect of changes in ionic strength on active transport of sodium

| Solution change from control to | A Change in $J_{13}^{Na}$ when ionic strength is increased | B Change in $J_{13}^{Na}$ when ionic strength remains same | A–B |
|---|---|---|---|
| Choline Cl | +242 | 0 | +242 |
| CsCl | +137 | −195 | +332 |
| RbCl | 0 | −276 | +276 |
| KCl | −201 | −488 | +287 |
| Choline Br | +349 | + 50 | +299 |
| Choline I | +370 | +148 | +222 |
| Mean (A–B) +SE ($N$=6) | | | +276 ± 16 |

Changes in $J_{13}^{Na}$ taken from the average values listed in Fig. 5 (increase in ionic strength) and on Fig. 8 (no change in ionic strength). All values in neq/cm$^2$ hr. For details *see* text.

### *Ionic Strength Effect*

A comparison of the changes in $J_{13}^{Na}$ observed with initial "low ionic strength" solution (Fig. 5) with the changes in $J_{13}^{Na}$ determined with initial "high ionic strength" solution (Fig. 8) reveals significant differences in the response of $J_{13}^{Na}$ to changes in ionic composition of the external medium. The average changes in $J_{13}^{Na}$ are summarized in Table 5 in two columns. Column *A* lists the change in $J_{13}^{Na}$ observed when the initial "low ionic strength" solution is replaced by a "high ionic strength" solution. Column *B* lists the stimulation or inhibition of $J_{13}^{Na}$ observed when specific ion replacements are carried out in the absence of a change in ionic strength. When these changes are subtracted from the corresponding changes seen in the presence of an increase in ionic strength (column *A*), one obtains essentially the same amount of change in $J_{13}^{Na}$ (column *A* minus *B*). In other words, regardless of stimulatory or inhibitory effects of specific ion replacements on $J_{13}^{Na}$, $J_{13}^{Na}$ is increased in every instance by essentially the same amount when corresponding ion replacements are combined with a given increase in ionic strength. Given the assumption[1] that, in the presence of 15 mM NaCl, neither the choline nor the chloride ion *per se* cause changes in $J_{13}^{Na}$, this observation suggests that a change in ionic strength by itself causes an increase in Na entry. Since changes in ionic strength are bound to cause changes in the configuration of surface charges, this observation suggests the presence of surface charges and thus the changes in $J_{13}^{Na}$ may be explained by a change in the screening effect of surface charges. Surface charges, on the other hand, should be strongly affected by changes in pH. Indeed, the experiments presented here show that a change in pH has a strong effect on $J_{13}^{Na}$ to the extent that $J_{13}^{Na}$ decreases to essentially the same level (0.299 ± 0.040 μeq/cm$^2$ hr and 0.296 ± 0.036 μeq/cm$^2$ hr on Figs. 5 and 8, respectively) in low ionic strength and high ionic strength solution despite the fact that, under control conditions, $J_{13}^{Na}$ in high ionic strength solutions was significantly higher than the corresponding value in low ionic strength solution (1.245 ± 0.129 and 0.681 ± 0.077 μeq/cm$^2$ hr, respectively.

One might be inclined to think that an increase in ionic strength should increase a screening effect exerted by surface charges with the result that the Na concentration at or near the Na entry site is decreased. Hence one might anticipate that under such conditions the Na permeability of the outside barrier is diminished and that $J_{13}^{Na}$ is inhibited. This expectation would apply to the situation in which the surface charges are nontitrable. However, the observation that a decrease in pH causes a decrease in $J_{13}^{Na}$ indicates that the surface charges are titrable. If this is so, then one would expect an increase in surface pH and consequently in surface charge density when the ionic strength is increased (DeSimone, Heck & DeSimone, 1979). Due to its effect on surface potential, such an increase in surface charge density would tend to cause an increase in Na concentration at or near the Na entry site. Such a mechanism would explain

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[1] This assumption is probably correct since recent experiments carried out in presence of 15 mM NaCl and with minimized or no changes in ionic strength give no indication of changes in Na transport when external choline chloride is replaced either by choline bicarbonate or by Tris salts (T.U.L. Biber, *unpublished observations*).

the observed stimulation of $J_{13}^{Na}$ as the ionic strength is increased. Obviously, the Na concentration at the membrane surface will be the result of a balance between the "diluting" effect through screening of surface charges and the "concentrating" effect through an increase in surface-charge density.

Thus, the present study suggests that Na entry involves the passage of Na across at least two barriers, one which is connected with the ionic strength effect and at least one additional barrier, probably in a deeper location, which is involved in the more specific ion effects, has charged sites which serve as filters or binding sites for Na transfer. These charge interactions at least give a partial explanation for some of the published variation in Na transport kinetics data (Kirschner, 1974; Koblick, 1959), and one might expect difficulties in describing fluxes according to the Goldman-Hodgkin-Katz approach.

### *Relationship Between $J_{31}^{Na}$ and $J_{13}^{Na}$*

Tables 3 and 4 demonstrate that Na effluxes are low under control and experimental conditions. Alterations in ionic strength or substitution usually produced directional changes in the efflux of Na ($J_{31}^{Na}$) similar to those demonstrated for the influx of Na ($J_{13}^{Na}$). It is possible that the observed relationship between $J_{13}^{Na}$ and $J_{31}^{Na}$ might be the result of common pathways and of the interaction of the active transport system with $J_{31}^{Na}$ as suggested by Biber and Mullen (1976, 1977), Kirschner (1959) and Huf and Howell (1974). On the other hand, corresponding changes in the efflux of Na might result indirectly from cellular transport due to alterations in the paracellular shunt via cellular swelling or shrinking.

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