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Effect of $FeCl_3$ on Ion Transport in Isolated Frog Skin

Thomas U.L. Biber, Terry L. Mullen, and John A. DeSimone

Department of Physiology, Medical College of Virginia, Richmond, Virginia 23298

Summary. The effect of addition of $FeCl_3$ to the media bathing the isolated skin of *Rana pipiens* was studied by measuring short-circuit current, transepithelial potential, and resistance, and by determining the influx and efflux of sodium (J^{Na}_{13} and J^{Na}_{31}, respectively) and the influx and efflux of chloride (J^{Cl}_{13} and J^{Cl}_{31}, respectively) across the epithelium. With normal Ringer's solution on both sides of the skin, addition of 10^{-3} M $FeCl_3$ to the external medium resulted in nearly complete inhibition of active Na transport (J^{Na}_{13} decreased from 1.30 ± 0.14 to 0.10 ± 0.04 μeq/cm^2 hr ($N=8$)) and in appearance of active chloride transport in outward direction due to an 80% increase in J^{Cl}_{31}. Average ($J^{Cl}_{31} - J^{Cl}_{13}$) obtained from means of 8 skins in 6 consecutive control and last 3 experimental periods was -0.17 ± 0.04 and 0.38 ± 0.05 μeq/cm^2 hr, respectively. $FeCl_3$ added to external medium also induced substantial net chloride movement in outward direction when external medium contained Na-free choline chloride Ringer's or low ionic strength solution. Under the latter condition net Na movement was virtually eliminated by external $FeCl_3$. After addition of $FeCl_3$ to serosal medium there was delayed inhibition of J^{Na}_{13} but no change in chloride fluxes. Immediate and profound changes in Na and Cl transport systems seen after external application of $FeCl_3$ indicate charge effects of Fe^{3+} on surface of apical cell membranes, possibly close to or in ion channels.

According to the model put forward by Koefoed-Johnsen and Ussing [20] for NaCl transport across isolated frog skin, sodium enters the epithelial cell along an electrochemical gradient by simple diffusion. Indirect and direct measurements of sodium uptake, however, have revealed that the permeability coefficient for Na at the outwards facing barrier decreased with increasing Na concentration [4, 9], a finding which is clearly not in accord with the concept of simple diffusion. In addition to the saturation of Na entry at higher Na concentrations, it was found that lithium ions competitively inhibit Na uptake [4]. Recent studies have indicated that charged sites serve as filters or binding sites for the translocation of Na

Table 1. Effect of external $FeCl_3$ on ion fluxes, I_0 and R

Condition	Measurement	Control	Experimental	
I	J^{Na}_{13}	1307 ± 13	140 ± 48	114 ± 15
	J^{Na}_{31}	29 ± 1	42 ± 3	44 ± 3
	J^{Cl}_{13}	1037 ± 31	1190 ± 19	1200 ± 23
	J^{Cl}_{31}	867 ± 17	1570 ± 19	1580 ± 23
	I_0 [a]	1598 ± 29	395 ± 17	393 ± 24
	R	1575 ± 21	1445 ± 52	1393 ± 7
II	J^{Na}_{13}	203 ± 1		54 ± 2
	J^{Na}_{31}	29 ± 3		49 ± 4
	J^{Cl}_{13}	131 ± 2		228 ± 5
	J^{Cl}_{31}	101 ± 7		1350 ± 13
	I_0	160 ± 7		968 ± 65
	R	5431 ± 127		1013 ± 47
III	J^{Cl}_{13}	920 ± 12	963 ± 16	918 ± 6
	J^{Cl}_{31}	942 ± 14	1187 ± 23	1204 ± 124
	J^{Ch}_{13} [b]	44 ± 5		63 ± 11
	J^{Na}_{31} [b]	24 ± 3		76 ± 9
	I_0	47 ± 4		304 ± 34
	R	1888 ± 249		1227 ± 208

Control and *Experimental* refer to control and experimental phase of experiment before and after addition of $FeCl_3$, respectively. Control data were calculated from averages of 6 control periods. *Condition I:* Normal Ringer's on both sides; experimental data on left and right obtained from averages in last 4 and last 3 experimental periods, respectively. *Condition II:* "Low ionic strength solution" in external bath; experimental data from averages in last 4 experimental periods. *Condition III:* Choline Ringer's in external bath; experimental data on left and right from averages in all and last experimental periods, respectively. Fluxes and I_0 in neq/cm^2 hr, R in Ωcm^2.

[a] Control I_0 in group used for J^{Na}_{13} measurement was 1235 ± 31 neq/cm^2 hr.

[b] $n=30$ for control and $n=40$ for experimental.

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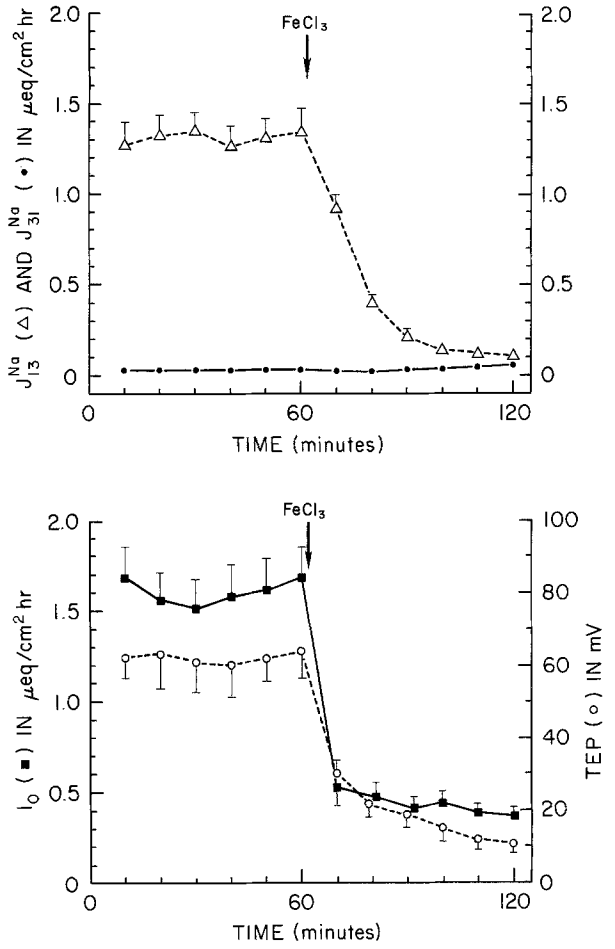

Fig. 1. Effect of external $FeCl_3$ (10^{-3} M) on transepithelial Na influx (J_{13}^{Na}), transepithelial Na efflux (J_{31}^{Na}), short-circuit current (I_0), and transepithelial potential (TEP) of isolated frog skin exposed to external Na-Ringer's solution. $n=8$ for all points representing Na flux measurements and $n=16$ for all points indicating electrical parameters. For details, *see* text

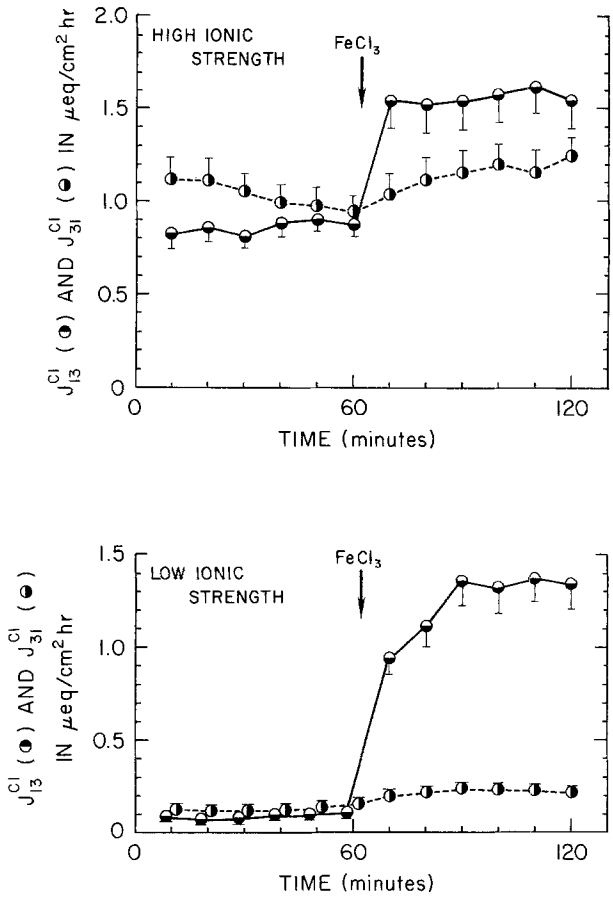

Fig. 2. Effect of external $FeCl_3$ (10^{-3} M) on transepithelial Cl influx (J_{13}^{Cl}) and on transepithelial Cl efflux (J_{31}^{Cl}) of isolated frog skin exposed to external Na-Ringer's solution (upper panel) or to external "low ionic strength" solution (lower panel). $n=8$ for all points. For details, *see* text

across the external surface of frog skin [1, 6, 28, 31]. To further analyze the importance of charge interaction on active transport processes, we studied the effects of external Fe^{3+}, an ion which is very electropositive because of its high charge and small radius, on Na transport in the isolated skin of *Rana pipiens*.

Materials and Methods

Circular pieces of skin were dissected from the abdominal skin of *R. pipiens* (sacrificed by double pithing) and mounted between two halves of new chambers which had been specially designed to reduce edge damage [5]. The methods for voltage-clamping the tissue and for measuring the fluxes were identical to the ones described in detail previously [5]. Except for brief (< 5 sec) interruptions for changing solutions, the tissue remained short circuited and the short-circuit current (I_0) was recorded continuously. In each experiment 6 consecutive measurements were made before the application of $FeCl_3$ (control points) and another 6 consecutive determinations were carried out after addition of $FeCl_3$ (experimental points). Fluxes are 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 where it is not clear from the context the number of observations is added in parenthesis.

Results

Since preliminary experiments indicated that addition of 10^{-3} M $FeCl_3$ to the external medium caused large changes in transepithelial potential (TEP) and short-circuit current (I_0), this concentration was used to explore electrical parameters and ion fluxes under three conditions: (i) when normal frog Ringer's (NaCl

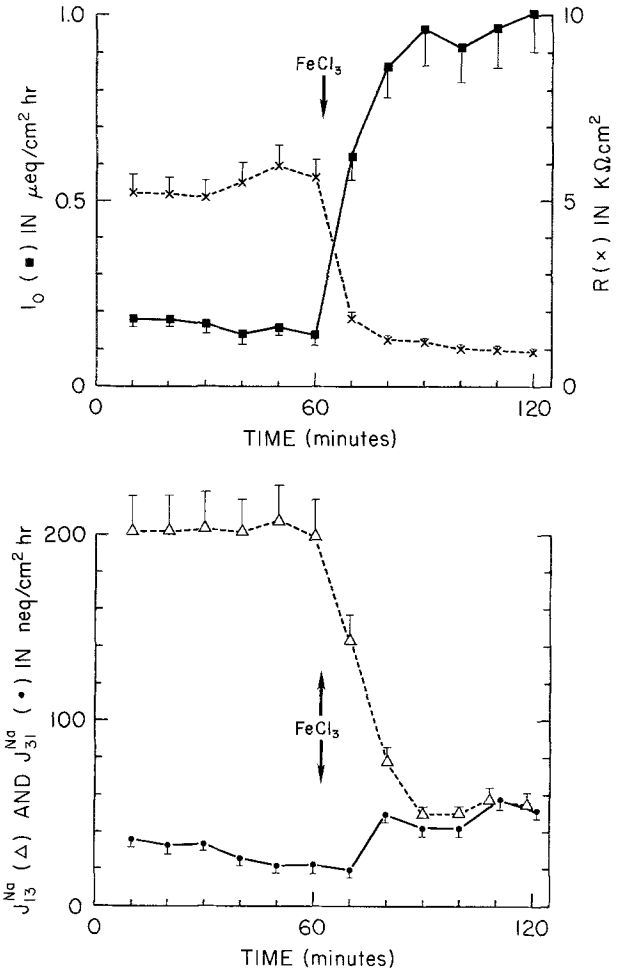

Fig. 3. Effect of external $FeCl_3$ (10^{-3} M) on short-circuit current (I_0), transepithelial resistance (R), transepithelial Na influx (J_{13}^{Na}) and transepithelial Na efflux (J_{31}^{Na}) of isolated frog skin exposed to external "low ionic strength" solution. $n=16$ for all points which represent electrical parameters and $n=8$ for all points indicating Na flux measurements. For details, *see* text

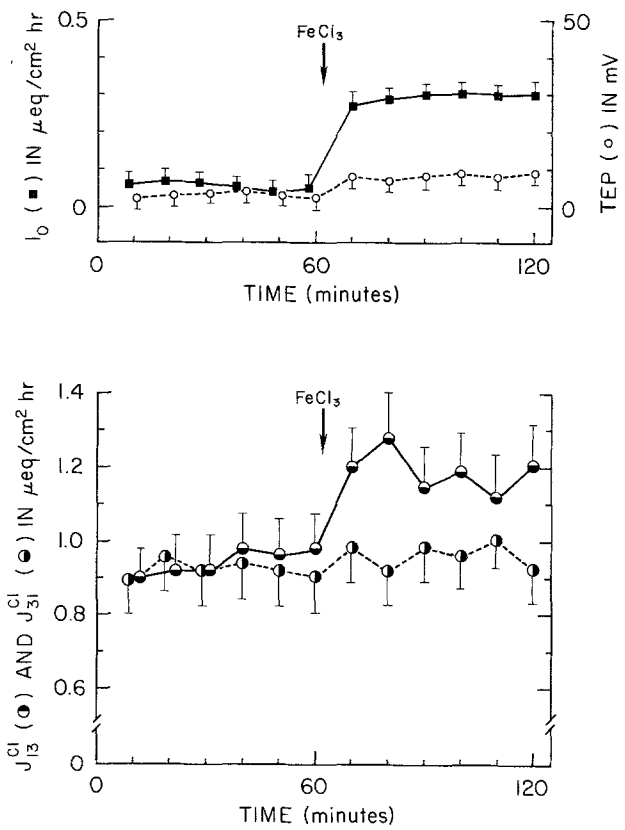

Fig. 4. Effect of external $FeCl_3$ (10^{-3} M) on short-circuit current (I_0), transepithelial potential (TEP), transepithelial chloride influx (J_{13}^{Cl}), and transepithelial chloride efflux (J_{31}^{Cl}) of isolated frog skin exposed to external Ringer's solution containing choline chloride instead of NaCl. $n=16$ for all points representing electrical parameters and $n=8$ for all points indicating chloride fluxes. For details, *see* text

Ringer's) bathed both sides of the frog skin, (ii) when the frog Ringer's in the external bath was replaced by a "low ionic strength" Ringer's solution, and (iii) when the frog Ringer's in the external bath was replaced by Na-free choline Ringer's solution. The results are presented separately for each of these conditions.

With normal Ringer's on both sides of the skin the addition of $FeCl_3$ to the external bath resulted in a pronounced reduction of the transepithelial Na movement as shown in the upper panel of Fig. 1. Inhibition of Na influx (J_{13}^{Na}) by 93% and a moderate increase in Na efflux (J_{31}^{Na}) resulted in a decrease of active Na transport by 96% to 70 ± 36 neq/cm² hr in the last 3 experimental periods. The bottom panel of Fig. 1 illustrates the sharp drop in I_0 and TEP, but there was only a very small decrease in resistance (R in Table 1). Comparison of I_0 and Na flux data listed in Table 1 indicates that, after exposure to $FeCl_3$, most of the current cannot be accounted for by Na fluxes. The upper panel of Fig. 2 indicates a sharp increase in Cl efflux (J_{31}^{Cl}) and only a slight change in Cl influx (J_{13}^{Cl}). During the control periods, the average J_{13}^{Cl} was significantly greater than the average J_{31}^{Cl} ($P<0.001$). After $FeCl_3$ addition net movement of Cl was reversed and proceeded outward. The difference between J_{31}^{Cl} and J_{13}^{Cl} was 380 ± 46 neq/cm² hr for the last 4 experimental periods, very close to the I_0 value listed on Table 1 for the same time span.

In a second series of experiments the normal external Ringer's solution (115 mM NaCl, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$) was replaced with a "low ionic strength" Ringer's solution consisting of 5 mM NaCl,

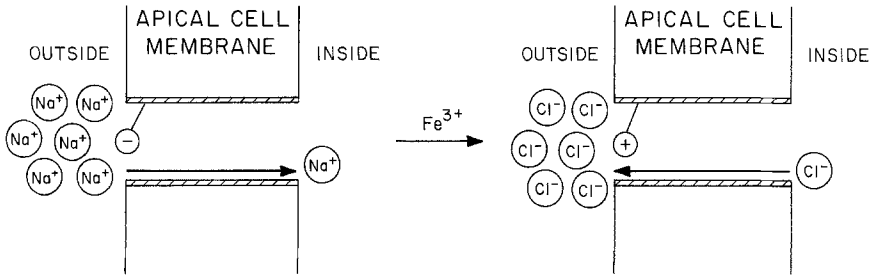

Fig. 5. Drawing depicts schematically possible mode of action of $FeCl_3$ on apical cell membrane of epithelial cells of frog skin. Sodium entry into cells is assumed to be mediated by fixed negative charges at the apical border. These charges determine the structure of the double layer in the vicinity of the site of sodium entry. After Fe^{3+} addition to external solution, the double layer structure is altered and the ion selectivity changes. For details, *see* text

Table 2. Relative self-energies

Metal	$W_{Relative}$
Fe^{3+}	13.4
La^{3+}	7.4
Mn^{2+}	4.8
Cd^{2+}	3.9
Na^+	1

220 mM mannitol, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$. The internal solution was not changed (i.e., was normal Ringer's solution), and therefore large Na and Cl gradients existed under both control and experimental conditions. As shown on the lower panel of Fig. 3, there was a large decrease in J_{13}^{Na} and a modest increase in J_{31}^{Na} after addition of $FeCl_3$. J_{31}^{Na} which, in presence of $FeCl_3$, reached the same level as in the previous set of experiments was not different from J_{13}^{Na} (Table 1). From the upper panel of Fig. 3 it can be seen that $FeCl_3$ induces a large increase in I_0 and decrease in R despite the fact that net Na transfer was essentially zero. A plot of the Cl fluxes *vs.* time on the lower panel of Fig. 2 shows that J_{31}^{Cl} increased dramatically whereas J_{13}^{Cl} rose only by a small amount. If one compares, on Table 1, the flux data obtained under these conditions (Part *II*) one finds (i) that under control conditions J_{13}^{Cl} exceeds J_{31}^{Cl} by 30 neq/cm^2 hr, J_{13}^{Na} is larger than J_{31}^{Na} by 174 neq/cm^2 hr, and I_0 accounts for the combined net fluxes of Na and Cl, and (ii) that after addition of $FeCl_3$ I_0 increases to the level of net Cl movement of 1122 neq/cm^2 hr.

In a third series of experiments, normal NaCl Ringer's solution in the external bath was replaced with choline Cl Ringer's solution (115 mM choline chloride (ChCl), 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$) and J_{13}^{Cl}, J_{31}^{Cl}, J_{13}^{Ch}, J_{31}^{Na}, I_0 TEP and R were measured before and after addition of $FeCl_3$ to the external bath. Figure 4 represents plots of J_{13}^{Cl}, J_{31}^{Cl}, I_0 and TEP *vs.* time. J_{31}^{Cl} and I_0 increased immediately after $FeCl_3$ addition whereas TEP and J_{13}^{Cl} remained relatively unchanged. From part *III* of Table 1 it can be seen (i) that J_{13}^{Cl} is not different from J_{31}^{Cl} during the control period but that the average J_{31}^{Cl} obtained during the experimental periods is significantly larger than the corresponding value measured for J_{13}^{Cl}, (ii) that the increase in I_0 seen after exposure to $FeCl_3$ is accounted for by the net outward movement of Cl, (iii) that $FeCl_3$ causes a sizable drop in R, and (iv) that after $FeCl_3$ application the charges transferred by J_{13}^{Ch} and by J_{31}^{Na} cancel each other out as these fluxes run in opposite directions.

In an additional series of experiments $FeCl_3$ (final concentration of 10^{-3} M) was added to the serosal bath under the conditions used for the first set of experiments (normal Ringer's solution on both sides). This resulted in a 26 and 27% decrease in J_{13}^{Na} and I_0, respectively, at the end of the experiment. The inhibitory effects of serosal $FeCl_3$, however, proceeded at a much slower rate when compared to the inhibition observed after addition to the external solution. Such a slower effect may simply reflect the presence of a longer diffusion pathway for Fe^{3+} on the serosal side. The I_0 was $94.1 \pm 2.0\%$ and $92.2 \pm 8\%$ of J_{13}^{Na} during the control and experimental periods, respectively ($n=48$). The presence of serosal $FeCl_3$ caused a gradual increase in skin resistance throughout the experimental phase (from 1509 ± 171 to $3323 \pm 344\ \Omega cm^2$, $n=48$) and a transient increase in TEP. A small but significant increase in J_{31}^{Na} could be observed after addition of $FeCl_3$ but unidirectional Cl fluxes remained unchanged.

Discussion

The addition of Fe^{3+} to the external medium causes a nearly complete inhibition of active Na transport across the skin. The reduction in net Na transport is almost exclusively the result of a decrease in transepithelial influx of Na (J_{13}^{Na}). The transepithelial efflux of Na (J_{31}^{Na}) which is a tiny fraction (1/46) of J_{13}^{Na}

under control conditions increases only by a small amount. The changes in short-circuit current (I_0) and in Na and Cl fluxes occur immediately after addition of external $FeCl_3$, whereas application of $FeCl_3$ to the serosal medium causes no changes in Cl fluxes and a much less extensive and delayed response of I_0 and of Na fluxes. It therefore seems reasonable to conclude that Fe^{3+} added to the external solution acts on the outside surface of the skin and that the observed decrease in J_{13}^{Na} is the consequence of an inhibition of J_{12}^{Na}, i.e., of the Na entry across the apical (outwards-facing) cell membranes of the epithelial cells. The existence of a close relationship between J_{13}^{Na} and J_{12}^{Na} has been clearly demonstrated by direct measurements of J_{12}^{Na} carried out under a wide variety of conditions, including conditions which resulted in large changes of J_{13}^{Na} [2, 3, 4, 7, 11, 22].

In the first series of experiments in which normal Ringer's solution was present on both sides of the skin, the $FeCl_3$-induced inhibition of J_{13}^{Na} is accompanied by a drop in I_0. However, I_0 does not decrease as much as J_{13}^{Na} and I_0 continues at a considerable level (about 400 neq/cm^2 hr) so that most of the I_0 cannot be accounted for by the minimal residual net movement of Na (about 70 neq/cm^2 hr). The persistence of I_0 can be explained by changes in Cl fluxes: After addition of $FeCl_3$ the relatively modest but significant inward active Cl transport (i.e., from external to serosal bath) is transformed into a very substantial and sustained outward Cl transport. The net outward movement of Cl appears immediately after addition of $FeCl_3$ with J_{13}^{Cl} remaining essentially at the control level. Given the limitations of the experimental determinations the net movement of Cl accounts for that portion of I_0 which during the experimental periods cannot be explained by the remaining movement of Na. By the end of the experiment the I_0 is practically identical to the amount of charges transferred by the net outward movement of Cl.

When $FeCl_3$ is added to an external medium made up of "low ionic strength" solution J_{31}^{Cl} increases much more than J_{13}^{Cl}, and we again observe the appearance of net outward Cl movement. Again there is a significant, albeit small, net inward movement of Cl during the control period, but this time the net inward movement proceeds against a steep concentration gradient. The net outward Cl transfer observed after $FeCl_3$ addition moves along a concentration gradient although the flux ratio J_{31}^{Cl}/J_{13}^{Cl} is much smaller than the concentration ratio for chloride ($[Cl]_{serosal}/[Cl]_{external}$). Clearly, the $FeCl_3$-induced active Cl transport described in the first set of experiments cannot be the result of exchange diffusion. In addition, the possibility of an activation of a major exchange diffusion component by $FeCl_3$ does not seem to be supported by the observation that in the experiments with low external Cl concentration J_{31}^{Cl} increases after $FeCl_3$ treatment to values which are nearly the same as those measured in the first set of experiments in presence of high external Cl concentration. I_0 increases about fivefold after addition of $FeCl_3$ to the external "low ionic strength" solution. By following the usual assumption that I_0 reflects identical changes in net Na transport, one would have concluded that $FeCl_3$ under these conditions stimulates active Na transport. But measurements of Na and Cl fluxes show that this is not the case: net Na transport is virtually abolished after addition of $FeCl_3$ and I_0 is practically identical to net outward Cl movement by the end of the experiment. The active transport in outward direction observed after application of $FeCl_3$ in the third series of experiments demonstrates that $FeCl_3$ induces net outward Cl transport even when no Na is present in the external medium. Here again, the I_0 measured after $FeCl_3$ treatment is equivalent to net Cl transfer.

Active Cl transport in outward direction has been observed in skins of *Rana temporaria* after application of adrenaline to the serosal side [19, 21]. In contrast, serosal addition of $FeCl_3$ causes no changes in Cl fluxes. Furthermore, the events triggered by adrenaline, especially the time course of the current response and the changes in Na fluxes, are so different from the ones observed after $FeCl_3$ in this study that there appears to be no connection between the action of the two agents. In addition, in a recent study of the adrenaline effect it was concluded that the adrenaline-induced current change is the result of changes in Na rather than in Cl fluxes [29].

The I_0 recorded in the first and second series of experiments represents initially net movement of Na and at the end net movement of Cl, and it appears that within the technical limitations of these flux measurements no other ions besides Na and Cl contribute to I_0 during the entire course of the experiment. This means that we can analyze the events in terms of Na and Cl currents. In the first set of experiments the large sodium current observed during the control period is transformed during the experimental periods into a more modest Cl current. In the second set of experiments the initially small Na current changes into a large Cl current after $FeCl_3$ treatment. The time course of I_0 and of other electrical measurements (TEP, R) indicates that both processes, the inhibition of net inward Na transport and the activation of net outward Cl transport, proceed towards completion at about the same rate. This apparent synchronization of Na and Cl transport systems suggests that a common event underlies the changes in both systems. In view of its charge characteristics, Fe^{3+} is

most likely to act over a charge effect on or close to the external surface of the epithelial cells. Hence, the inhibitory action on Na transport might arise from the binding of Fe^{3+} to negatively charged groups associated with a carrier or a charged ion channel site in the outer cell membranes and/or from modifications in general surface charge. Studies by Biber and Mullen [6], Singer and Civan [28], and Zeiske and Lindemann [31] have indicated that charged sites may serve as filters or binding sites for the translocation of Na across the external surface of the frog skin.

It is as yet unknown by which mechanism Fe^{3+} acts on the apical cell membranes and brings about an inhibition in Na entry (J_{12}^{Na}) and, at the same time, a promotion of Cl exit (J_{21}^{Cl}). The appearance of an outwardly directed active Cl transport may be the result of a transformation of existing Na pathways across the apical cell membrane into Cl pathways. The decrease in membrane resistance observed in the presence of "low ionic strength" solution may, however, also imply the availability of new transport sites. A sharp decrease in the effect observed below 1 mM $FeCl_3$ suggests that Fe^{3+} may be binding to an assembly of surface transport sites on the outside surface of the skin. The transformation of the apical cell membranes from a Na-transporting system to a Cl-transporting system may be observed only after a certain fraction of the transport sites are bound by Fe^{3+}. In this case the fixed surface charge could be a key determinant in the transport of Na and indeed in the transport of other ions as well. If the normal Na transport site in the apical cell membrane carries a negative charge, substitution by a fixed positive charge will certainly alter the local electrostatic potential near the site so as to create a double layer of predominantly negative ions [12]. This in itself may be sufficient to convert the external cell surface into an anion selective membrane (Fig. 5). The presence of the positive bound ions on the external surface may also bias the cellular potential profile favoring the outward movement of Cl across the apical cell membrane[1]. Intracellular potentials were not measured in the frog skin under these conditions, but the development of a positive serosal potential in the Fe^{3+}-treated skins exposed to external choline chloride is consistent with changes in the potential gradient across the apical cell membranes[2].

If the mere reversal in the polarity of the charge of the sites at the apical surface can accomplish the conversion of the skin from a cation into an anion transporting system then it may be possible that surface charge density alone is responsible for the identity of a transport site in terms of it being either cationic or anionic. The role of the surface charge and its influence on membrane transport and surface reactions is widely acknowledged and has been extensively discussed elsewhere [8, 12, 26].

In a detailed study, Biber and Mullen [6] showed that Na transport is inhibited by monovalent alkali cations and that the order of inhibition increases with decreasing ion crystal radii in accordance with Eisenman's sequences *X* or *XI*. This observation suggests the presence of a strong anionic electric field sufficient to overcome the hydration forces of the alkali cations. The effect of multivalent ions and transition metal ions is more difficult to assess largely because interactions with other molecules may also involve complex ion formation including the stoichiometric binding of water [10, 14]. There have been several investigations of the effects of heavy metals such as La^{3+} [13, 17, 30], Mn^{2+} [15] and Cd^{2+} [18] on transport properties across the skin. These studies each included measurements of I_0, but in some cases the current carriers were not identified directly using tracer flux determinations. A notable feature of these studies is the fact that in each case except that of Fe^{3+} the I_0 is increased by the presence of the metal. Again assuming the presence of a strong anionic site on the membrane, a strong electrostatic attraction between Fe^{3+} ions and this site can be expected. The attraction may be far stronger than for La^{3+}, Mn^{2+} or Cd^{2+}. This conjecture is supported by the relative electrostatic self-energies of these ions in the dehydrated state. Using the ion crystal radii of Pauling [25], the self-energy, *W*, in an infinite dielectric me-

[1] It should be pointed out here that the site of active Cl transport cannot be located without additional information about the driving forces which affect Cl movement either across the serosal or across the apical cell membrane. The two most simple situations are the following: On one hand, the changes occurring in the apical cell membrane after $FeCl_3$ treatment could simply result in passive net movement of Cl from the cells into the external medium along an electrochemical gradient. This would require (i) that the intracellular Cl concentration is greater than can be accounted for by the electrochemical gradient (*see* footnote 2), and (ii) that there is an active Cl uptake across the basolateral cell membrane. On the other hand, the intracellular Cl concentration may not be above the equilibrium value, in which case one would have to postulate that the active transport process is located in the apical, rather than in the basolateral, cell membrane.

[2] If one accepts as valid measurements the values of about 36 mM for intracellular Cl concentration [27] and of 90–100 mV, cell interior negative, for the potential gradient across the apical cell membrane [16, 23], then one must conclude that the intracellular Cl concentration is much higher than predicted from the equilibrium due to the electrochemical potential difference. Replacement of NaCl in external medium by choline Cl causes a reversal of the Na gradient across the apical cell membrane with the result that the potential gradient across this border of the cell becomes even steeper [24]. Now if, as the experiments indicate, treatment with $FeCl_3$ causes a decrease in Na permeability and an increase in Cl permeability at the apical cell border, then one would expect a depolarization of the apical cell membrane under these conditions. This, in turn, would tend to increase the transepithelial potential difference (i.e., serosal side more positive).

dium can be found from the relation

$$W=(z^2e^2)/(2\varepsilon r)$$

where z is the valence of the ion in question, e is the electric charge in electrostatic units, ε is the dielectric constant, and r is the crystal radius. Values for self-energies of several ions relative to the self-energy of Na are given in Table 2. The high energy for Fe^{3+} indicates the possibility of a strong interaction with anionic sites, provided that the expected large hydration forces can be overcome. If this is possible, one might anticipate that Fe^{3+}-binding results in a qualitative change toward anionic rather than cationic transport.

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