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Influence of Extracellular Cl Concentration on Cl Transport Across Isolated Skin of *Rana Pipiens*

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Summary. The effect of changes in Cl concentration in the external and/or serosal bath on Cl transport across short-circuited frog skin was studied by measurements of transepithelial Cl influx (J_{13}^{Cl}) and efflux (J_{31}^{Cl}), short-circuit current, transepithelial potential, and conductance (G_m). J_{13}^{Cl} as well as J_{31}^{Cl} were found to have a saturating component and a component which is apparently linear with Cl concentration. The linear component of J_{31}^{Cl} appears only upon addition of Cl to external medium, and about 3/4 of this component does not contribute to G_m. The saturating component of J_{31}^{Cl} is only 5% of total J_{31}^{Cl} with 115 mM Cl in the serosal medium. Replacement of 115 mM Cl^- in external medium by $SO_4^=$, NO_3^-, HCO_3^- or I^- results in 87–97% reduction of J_{31}^{Cl}, whereas replacement with Br^- has no effect. As external Cl concentration is raised in steps from 2 to 115 mM, J_{13}^{Cl} and J_{31}^{Cl} increase by the same amount but J_{13}^{Cl} is persistently 0.15 μeq/cm²hr larger than J_{31}^{Cl}. These results indicate that at least 3/4 of linear components of J_{13}^{Cl} and J_{31}^{Cl} proceed via an exchange diffusion mechanism which seems to be located at the outer cell border. The saturating component of J_{13}^{Cl} is involved in active Cl transport in an inward direction, and there is evidence suggesting that Cl uptake across outer cell border, which proceeds against an electrochemical gradient, is electroneutral but not directly linked to Na.

An increasing body of evidence indicates that all the chloride movement across isolated frog skin proceeds via a transcellular rather than extracellular route. For example, it was found that the transepithelial chloride fluxes change after addition of amiloride to the external medium (Candia, 1978; Kristensen, 1978). Yet, it is known that amiloride blocks selectively the entry of Na from the external medium into the epithelial cells. The interpretation of the amiloride effect on Cl fluxes might be either that amiloride acts directly on Cl penetration across the outward-facing cell membrane of the epithelial cells (common mechanism shared with Na?) or that it has a less direct effect; for example, by changing the electrochemical gradient across the outward-facing cell membrane. A few years ago, when we started using new flux chambers specifically designed to minimize edge-damage, we carried out an investigation of transepithelial effluxes (i.e., fluxes from serosal to external side) of Na. But occasionally we simultaneously measured Cl effluxes. To our amazement we found that after addition of ouabain to the serosal solution the ratio of Na efflux over Cl efflux (J_{31}^{Na}/J_{31}^{Cl}) remained completely unchanged despite a dramatic increase in Na efflux (Biber & Mullen, 1977). Since the Na efflux measurements led us to conclude that essentially the entire efflux of Na proceeds through the cells, possibly by interacting with the active transport process at the serosal side of the cell, the observation of an identical response of Na and Cl efflux suggested that most of the Cl efflux passes through the cells. It seemed, therefore, to be of interest to study the kinetics of transepithelial Cl fluxes by changing the concentration of Cl ions and of other ions in the bathing solutions.

The experiments were carried out under short-circuit conditions, and the results indicate that the transepithelial fluxes in both directions (Cl influx as well as Cl efflux) are composed of at least two components: a saturating component and a component which is linear with the Cl concentration. A large part (about 75%) of the linear component has

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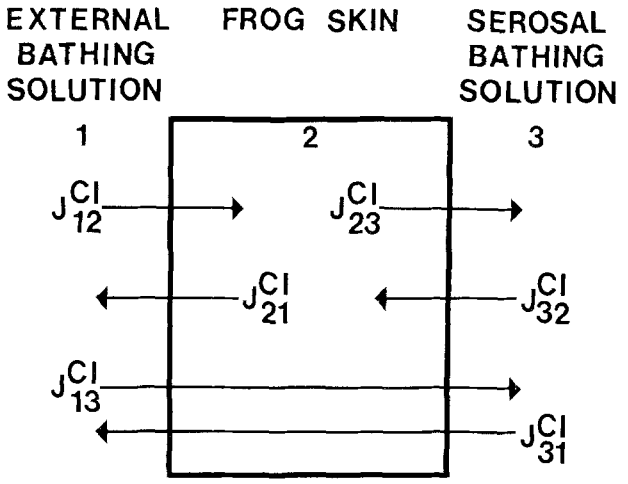

Fig. 1. Three-compartment model for movement of chloride across frog skin

characteristics of an exchange diffusion process. In addition, the results indicate that the active Cl movement in an inward direction passes via the saturating component of the Cl influx. Movement of Cl in an inward direction across the outward-facing cell membrane appears to proceed against an electrochemical gradient for Cl but does not seem to involve an electroneutral NaCl transfer mechanism.

Materials and Methods

Frogs (southern variety of *Rana pipiens*) were purchased from a commercial source in Connecticut (Connecticut Valley) throughout the year.

After double pithing, the abdominal skin was carefully removed and circular pieces were cut 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, 1976). The mounted frog skin with an 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 (TEP) across the skin was measured by calomel half-cells which were connected to the solution by agar bridges. A second pair of bridges led to AgAgCl electrodes for applying the short-circuit current (I_0). TEP and I_0 were determined by using an automatic clamping device (WP Instruments) which is adjustable for changes in fluid resistance between the TEP bridges. The I_0 was monitored continuously by a recorder (Gould Brush Model 220).

Fluxes of Cl^- were measured by adding ^{36}Cl to one side of the skin and measuring the 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 determined to be negligible. The "cold" bathing medium was entirely emptied and replaced at 10-min intervals, and during this 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 chloride was measured by chloridometry (Buchler Model 4-2008). Osmolality and pH were determined by utilization of an osmometer (Advanced Instruments Model 3D) and pH meter (Radiometer Model PHM 64), respectively.

Flux measurements were made only under short-circuit conditions and were started only after achievement of a steady state. Fluxes are expressed in terms of the 3-compartment model shown in Fig. 1, in which compartments *1*, *2*, and *3* are the external bathing solution, cell compartment, and serosal bathing solution, respectively. J_{ij} 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 parentheses.

Unless otherwise mentioned, the serosal bathing solution was made up by "normal" NaCl Ringer's solution (standard Ringer's solution) containing 115 mM NaCl, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$.

Results

In a first series of experiments, J^{Cl}_{13} and J^{Cl}_{31} were measured under conditions in which the Cl concentration in the external medium was changed from 0 to 115 mM by substituting Na_2SO_4 plus mannitol by NaCl. Throughout the experiments, the serosal medium consisted of Ringer's solution with 115 mM NaCl. The results are listed in Table 1. Although the Cl influx, J^{Cl}_{13}, rises with increase in external Cl concentration, it is clear that the increase in J^{Cl}_{13} is not proportional to the increase in Cl concentration. In other words, the measurements of J^{Cl}_{13} indicate a substantial decrease in Cl permeability with increasing Cl concentration in the external medium.

With no Cl present in the external medium there is a small flux of Cl in an outward direction (J^{Cl}_{31}). This efflux cannot be due to an exchange diffusion process. As the Cl concentration in the external medium is increased one can also observe an increase in J^{Cl}_{31}, although, as mentioned above, the serosal Cl concentration remains constant at 115 mM during these procedures. A comparison between J^{Cl}_{13} and J^{Cl}_{31} reveals two interesting points. First, at external Cl concentrations higher than 2 mM, the increases observed for J^{Cl}_{13} and for J^{Cl}_{31} are remarkably similar for each given increase in external concentration. Second, the average for J^{Cl}_{13} is consistently larger than the average for J^{Cl}_{31} at each level of external Cl concentration from 1 through 115 mM. The difference between the means for J^{Cl}_{13} and J^{Cl}_{31} at each of the 6 external Cl concentrations from 1 to 115 mM averages at 0.145 ± 0.01 μeq/cm^2hr. Since the Cl fluxes were obtained under short-circuit conditions, in the absence of an osmotic gradient and with a Cl concentration in the external medium which is lower or equal to the Cl concentration in the serosal medium, such a net inward movement of Cl is consistent with an active transport process. As a consequence of

Table 1. Effect of external Cl concentration on transepithelial Cl fluxes

External Cl concentration (mM)	Cl influx (J_{13}^{Cl}) in μeq/cm²hr	Cl efflux (J_{31}^{Cl}) in μeq/cm²hr	J_{13}^{Cl}/J_{31}^{Cl}
0.0	–	0.02±0.01	
1.0	0.15±0.02	0.03±0.01	5.0
2.0	0.23±0.02	0.05±0.01	4.6
6.0	0.35±0.08	0.18±0.04	1.9
12.0	0.44±0.08	0.28±0.06	1.6
25.0	0.58±0.12	0.44±0.08	1.3
115.0	1.11±0.21	1.01±0.19	1.1

The external Cl concentration was increased by substitution of NaCl for Na_2SO_4 and mannitol. $N=8$ for all points.

progressively larger fluxes in both directions, the flux ratio decreases from 5.0 to 1.1 with an increase in external Cl concentration (Table 1), although the differences between the averages for J_{13}^{Cl} and J_{31}^{Cl} remain relatively constant. This difference between Cl influx and Cl efflux is highly significant at lower external Cl concentration but, due to higher variability of the fluxes, not at higher Cl concentrations. However, the persistence of a very similar difference between the averages for J_{13}^{Cl} and J_{31}^{Cl} throughout the entire range from 1 to 115 mM suggests that active Cl transport in an inward direction continues at about the same rate over the entire range.

The data listed in Table 1 are shown in Fig. 2 in which the chloride influx (J_{13}^{Cl}) and the chloride efflux (J_{31}^{Cl}) are plotted against $[Cl]_{ext}$, the chloride concentration in the external medium. The position of the points for J_{13}^{Cl} suggests that J_{13}^{Cl} is made up of two components, a saturating component which reaches saturation at low external Cl concentration and a linear component which, with increasing external Cl concentration, contributes more to J_{13}^{Cl} so that the line representing J_{13}^{Cl} straightens out at higher Cl concentrations when the saturating component reaches more stable values. Accordingly, the Cl influx, J_{13}^{Cl}, would be given by:

$$J_{13}^{Cl}=\frac{J_{13}^{m}[Cl]_{ext}}{K'_{Cl}+[Cl]_{ext}}+\alpha[Cl]_{ext} \quad (1)$$

in which J_{13}^{m} and K'_{Cl} are the maximal influx and "apparent Michaelis constant" for a saturating component, $[Cl]_{ext}$ is the Cl concentration in the external medium, and α is a permeability coefficient for the linear component. The solid line in Fig. 2 shows the best fit (least squares) of Eq. (1) to the experimental points for J_{13}^{Cl}. The values of the constants in Eq. (1) providing this fit were $J_{13}^{m}=0.45$ μeq/cm²hr, $K'_{Cl}=2.15$ mM, and $\alpha=0.0058$ cm/hr. The fit to the points, assuming $\alpha=0$, was much less satisfactory than that in Fig. 2. An additional indication that the results cannot be described by a single saturating component was obtained when the data were plotted as J_{13}^{Cl} against $J_{13}^{Cl}/[Cl]_{ext}$ (Woolf, 1932; Hofstee, 1959; Dowd & Riggs, 1965) as shown in Fig. 3. The relationship is clearly nonlinear, but if a linear component given by $0.0058\ [Cl]_{ext}$ is subtracted from each point, the resulting points fall on a straight line. The correlation coefficient reaches a peak (0.992) with a value of 0.0058 for α and a decrease or increase in α causes a decline in the correlation coefficient. According to Dowd and Riggs (1965), this method provides the most sensitive indicator for failure of data to conform to simple Michaelis-Menten kinetics.

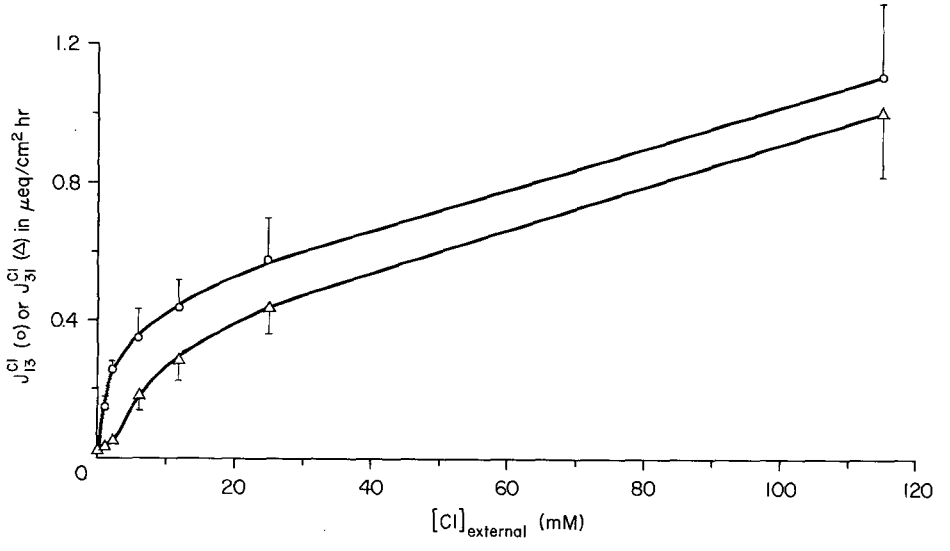

Fig. 2. Transepithelial Cl influx (J_{13}^{Cl}, open circles) and transepithelial Cl efflux (J_{31}^{Cl}, triangles) plotted against the Cl concentration in the external solution ($[Cl]_{external}$). Data taken from Table 1

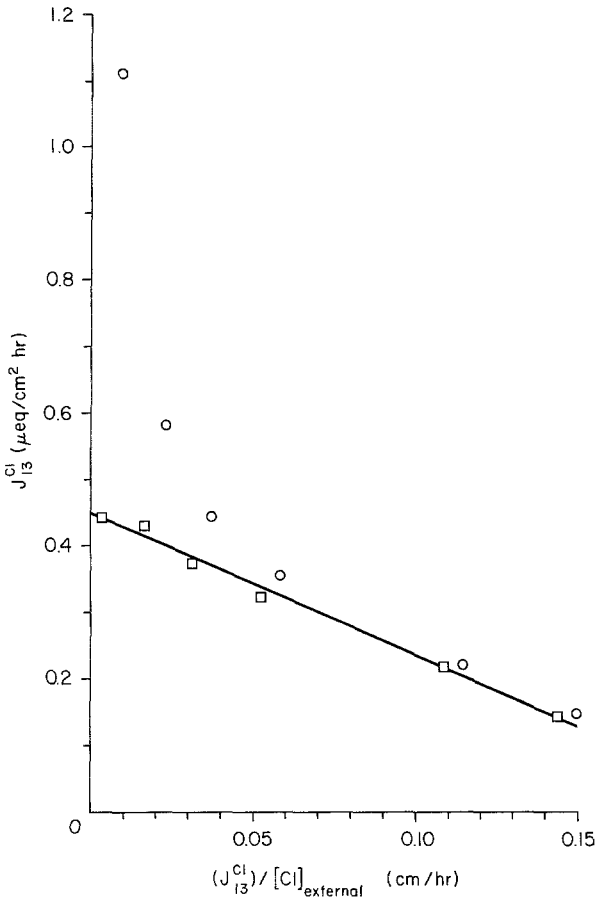

Fig. 3. Cl influx (J_{13}^{Cl}) plotted against the Cl influx divided by the Cl concentration in the external medium ($J_{13}^{Cl}/[Cl]_{external}$) before (○) and after (□) subtraction of the linear component (0.0058 $[Cl]_{external}$)

Therefore, the best explanation for the kinetics of Cl influx is given by a saturating component and a component linear with external Cl concentration.

In this series of experiments the short-circuit current increased progressively as the external Cl concentration was raised from 1 to 115 mM. The average short-circuit current measured at each level of external Cl concentration was 0.744 ± 0.075, 0.772 ± 0.080, 0.812 ± 0.084, 0.912 ± 0.091, 1.090 ± 0.123, and 1.316 ± 0.134 μeq/cm²hr at an external Cl concentration of 1, 2, 6, 12, 25 and 115 mM, respectively ($N = 16$ for each average). Also, the conductance of the epithelium increased with an increase in external Cl concentration. The average conductance obtained at each level of external Cl concentration was 0.22 ± 0.02, 0.25 ± 0.03, 0.28 ± 0.03, 0.35 ± 0.03, 0.48 ± 0.05, and 0.68 ± 0.07 mmho cm^{-2} at an external Cl concentration of 1, 2, 6, 12, 25 and 115 mM, respectively ($N = 16$ for each average).

In a second set of experiments Cl efflux was measured when the Cl concentration in the serosal medium was varied from 1 to 115 mM at 3 fixed levels of external Cl concentration. The results of these experiments are summarized in Table 2. The effluxes listed in the 3 columns represent the values obtained at an external Cl concentration of 0, 5 and 115 mM, respectively. In each column (i.e., at each given external Cl concentration) one can observe an increase in J_{31}^{Cl} as the serosal Cl concentration is raised from 1 to 115 mM. On the other hand, if one compares on the 6 lines the Cl effluxes obtained at a given serosal Cl concentration one can observe that, except for one instance (2 mM serosal Cl concentration at 0 and 5 mM external Cl concentration), J_{31}^{Cl} becomes progressively larger as the external Cl concentration is increased. The difference between the effluxes obtained at the 3 levels of external Cl concentration becomes much more apparent when the data listed in Table 2 are plotted as J_{31}^{Cl} *vs.* the serosal Cl concentration (Fig. 4). With no Cl present in the external medium, J_{31}^{Cl} saturates at low serosal Cl concentration, apparently by following simple Michaelis-Menten kinetics (circles in Fig. 4). As the external Cl concentration is increased to 5 and 115 mM (squares and triangles, respectively, in Fig. 4) the relationship between J_{31}^{Cl} and serosal Cl concentration changes progressively towards a linear relationship between J_{31}^{Cl} and serosal Cl concentration. Thus, the position of the points for the 3 external Cl concentrations indicate that Cl efflux is made up of a single saturating component when the external Cl concentration is zero, but that Cl efflux is the result of two components, a saturating and a linear one, when the external solution contains chloride. Therefore, analogous to the situation for chloride influx, the chloride efflux, J_{31}^{Cl}, would be given by:

$$J_{31}^{Cl} = \frac{J_{31}^{m}[Cl]_{ser}}{K_{Cl}'' + [Cl]_{ser}} + \alpha[Cl]_{ser} \qquad (2)$$

where J_{31}^{m} and K_{Cl}'' are the maximal efflux and "apparent Michaelis constant" for the saturating component, respectively. $[Cl]_{ser}$ is the Cl concentration in the serosal medium, and α is the permeability coefficient for the linear component. The solid lines in Fig. 4 show the best fit of Eq. (2) to the experimental points, and the values of the constants providing this fit were $J_{31}^{m} = 0.055$ μeq/cm²hr, $K_{Cl}'' = 4.70$ mM, and $\alpha = 0$ when no external chloride was present. With an external Cl concentration of 5 mM the values for the constants were $J_{31}^{m} = 0.056$ μeq/cm²hr, $K_{Cl}'' = 5.11$ mM, and $\alpha = 0.00112$ cm/hr, and with an external Cl concentration of 115 mM the values for the constants amounted to $J_{31}^{m} = 0.053$ μeq/cm²hr, $K_{Cl}'' = 4.65$ mM and $\alpha = 0.0088$ cm/hr. The chloride effluxes listed in

Table 2. Effect of serosal concentration on Cl efflux

Serosal Cl concentration (mM)	Cl efflux in μeq/cm²hr at external Cl concentration of		
	0 mM	5 mM	115 mM
1	0.009±0.001	0.010±0.001	0.018±0.002
2	0.018±0.002	0.018±0.002	0.034±0.003
6	0.027±0.003	0.037±0.004	0.083±0.008
12	0.039±0.004	0.053±0.005	0.145±0.021
25	0.049±0.005	0.077±0.008	0.266±0.040
115	0.053±0.005	0.179±0.027	1.065±0.210

The serosal and external Cl concentration was increased by substitution of NaCl for Na_2SO_4 and mannitol. $N=8$ for all points.

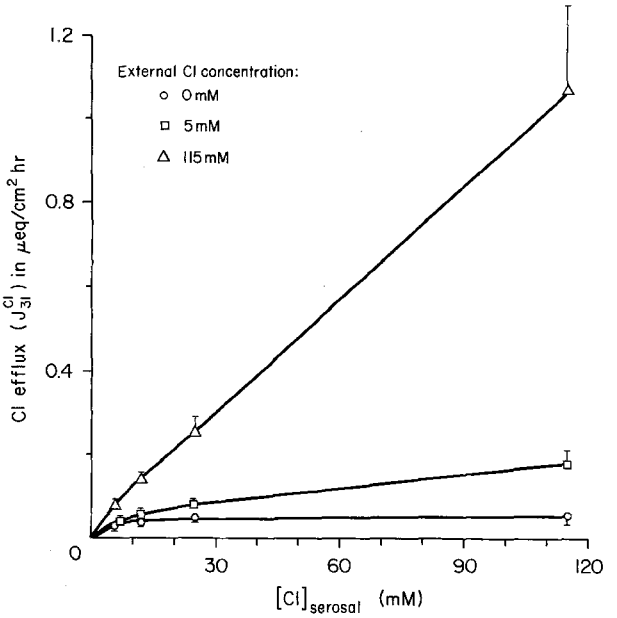

Fig. 4. Cl efflux (J_{31}^{Cl}) plotted against the Cl concentration in the serosal medium ($[Cl]_{serosal}$). Cl efflux was determined when the Cl concentration of the external medium was either 0 mM (○), or 5 mM (□) or 115 mM (△). Data taken from Table 2

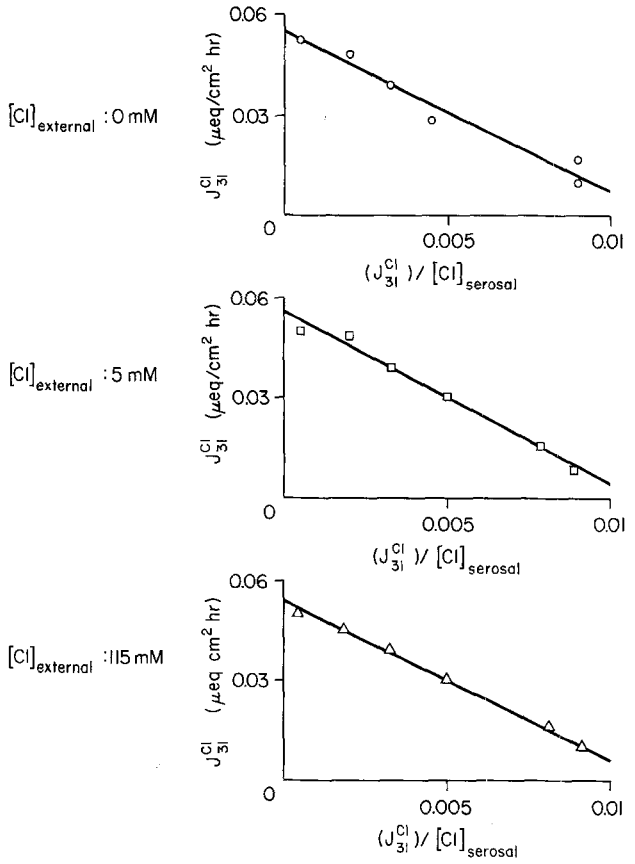

Fig. 5. Cl efflux (J_{31}^{Cl}) plotted against Cl efflux divided by the Cl concentration in the serosal medium ($J_{31}^{Cl}/[Cl]_{serosal}$). *Upper panel:* Cl efflux with no Cl present in external medium. No correction for linear components. *Middle panel:* 5 mM Cl present in external medium. Cl efflux after subtraction of linear component (0.00112 $[C]_{serosal}$). *Lower panel:* 115 mM Cl present in external medium. Cl efflux after subtraction of linear component (0.0088 $[Cl]_{serosal}$)

Table 2 are plotted in Fig. 5 as J_{31}^{Cl} against $J_{31}^{Cl}/[Cl]_{ser}$ after subtraction of 0.00112 $[Cl]_{ser}$ and of 0.0088 $[Cl]_{ser}$ for the linear components at an external Cl concentration of 5 and 115 mM, respectively. No linear component was subtracted from J_{13}^{Cl} for the experiments in which Cl was not present in the external medium. For each of the plots the points are located close to a line and the correlation coefficients are 0.967, 0.991 and 0.998 for an external Cl concentration of 0, 5 and 115 mM, respectively. This indicates that after subtraction of a linear component at external Cl concentrations of 5 and 115 mM a saturating component of the Cl effluxes remains which conforms to simple Michaelis-Menten kinetics. Moreover, the slopes and intercepts of the 3 plots shown in Fig. 4 are remarkably close (*see also* values for J_{31}^{m} and K_{Cl}'' mentioned above). This suggests that the saturating component of J_{31}^{Cl} remains unaltered when the external Cl concentration is increased and that J_{31}^{Cl} becomes larger with an increase in external Cl concentration because of an increase in the linear component (i.e., the value of α becomes progressively larger).

The conductances listed in Table 3 were obtained during the flux measurements of the second series of experiments (Table 2). A greater increase in conductance can be observed when the serosal Cl concentration is increased with 115 mM Cl present in the external medium and a much more modest increase in conductance can be observed with an increase in

Table 3. Effect of serosal Cl concentration on conductance

Serosal Cl concentration (mM)	Conductance in mmho/cm² at external Cl concentration of		
	0 mM	5 mM	115 mM
1	0.22+0.02	0.24+0.02	0.35±0.04
2	0.21±0.02	0.26±0.03	0.33±0.03
6	0.23±0.02	0.27±0.03	0.36±0.04
12	0.28+0.03	0.28±0.03	0.46±0.05
25	0.27±0.02	0.30±0.03	0.48±0.05
115	0.23±0.02	0.31±0.03	0.61±0.06

The serosal and external Cl concentration was increased by substitution of NaCl for Na_2SO_4 and mannitol. $N=8$ for all points.

serosal Cl concentration when the external Cl concentration is reduced to 5 mM. There is no significant change in conductance when no Cl is present in the external medium. It appears, therefore, that the increase in conductance coincides with the appearance of a linear component for J_{31}^{Cl}. For each group of experiments carried out at a given external Cl concentration there was no significant change in short-circuit current as the serosal Cl concentration progressed from a lower to a higher level. In view of this we may assume that there was no substantial change in Na fluxes with an increase in serosal Cl concentration.

In a third series of experiments, J_{31}^{Cl} was measured when the NaCl in the NaCl Ringer's of the external medium (115 mM NaCl, 2.5 mM $KHCO_3$, 1.0 mM $CaCl_2$) was substituted by one of the following: 115 mM $NaHCO_3$, 115 mM $NaNO_3$, 115 mM NaI, or 57.5 mM Na_2SO_4 plus 57.5 mM mannitol. In this series of experiments, as well as in all the following experiments, the serosal medium contained throughout the experiments normal NaCl Ringer's solution. The results of these experiments are shown in Fig. 6. The control values for the different groups averaged from 1.00 to 1.06 μeq/cm²hr in the last period before the substitution. J_{31}^{Cl} dropped precipitously in all groups after chloride removal from the external solution. The averages for J_{31}^{Cl} during the last experimental period for the groups with Na_2SO_4, $NaHCO_3$, $NaNO_3$ and NaI replacement were 0.06 ±0.01, 0.13±0.01, 0.03±0.01 and 0.07±0.01 μeq/cm²hr, respectively.

In a fourth series of experiments the effect of addition of Br to the external medium on J_{31}^{Cl} was studied by substitution of 115 mM NaBr for the 115 mM NaCl contained in the standard NaCl Ringer's solution, as well as by substitution of 115 mM Br for 57.5 mM Na_2SO_4, plus 57.5 mM mannitol in a solution which originally contained 57.5 mM Na_2SO_4, 57.5 mM mannitol, 2.5 mM $KHCO_3$ and 1.0 mM $CaCl_2$. The results of these experiments are shown in Fig. 7. Replacement of NaBr for NaCl causes only a small and statistically insignificant decrease in J_{31}^{Cl}. Substitution of NaBr for Na_2SO_4 causes an abrupt increase in J_{31}^{Cl} to the same level as the one observed when NaCl is replaced by NaBr. It seems, therefore, that the presence of the bromide ion in the external medium has an effect on J_{31}^{Cl} which cannot be distinguished from the one exerted by the chloride ion.

In a fifth series of experiments the external surface of the skin was first exposed to a solution containing 15 mM NaCl, 2.5 mM $KHCO_3$, 1 mM $CaCl_2$ and 200 mM mannitol. Then, the 200 mM mannitol was replaced by either 100 mM $NaNO_3$ or, 100 mM $NaHCO_3$ or, 100 mM NaI or 50 mM Na_2SO_4 plus 50 mM mannitol. In this way the effect of various anions on J_{31}^{Cl} could be tested during continued presence of 15 mM NaCl in the external medium. The results shown in Fig. 8 indicate that addition of $NaNO_3$, $NaHCO_3$, and of Na_2SO_4 to 15 mM NaCl causes no changes in J_{31}^{Cl}. However, addition of NaI results in an inhibition of J_{31}^{Cl} from an average of 0.28 ±0.003 μeq/cm²hr in the last control period to an average of 0.004±0.001 μeq/cm²hr in the last experimental period. This suggests that, in contrast to the other anions tested here, iodide counteracts the increased level of J_{31}^{Cl} which is observed in presence of 15 mM NaCl in the external medium.

During the control period of the last series of experiments, the external medium contained 200 mM mannitol, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$, together with 15 mmol/liter of either NaCl or KCl or choline chloride. Then, for the experimental part of the experiment, the mannitol was substituted by increasing the concentration of either NaCl or KCl or choline chloride to 115 mM. The measurements of J_{31}^{Cl} obtained under these conditions indicate that the chloride efflux values for all groups are very similar during the last control period (the averages range from 0.30 to 0.36 μeq/cm²hr) and that J_{31}^{Cl} increases at about the same rate to final values which are not significantly different, regardless of which of the cations is present. The averages for the last experimental period are 0.91±0.19, 1.00±0.15 and 0.86 ±0.10 μeq/cm²hr for the NaCl, KCl and choline chloride groups, respectively (Fig. 9). The increase in J_{31}^{Cl}, observed with an increase in external Cl concentration, seems not to be dependent on the presence of Na, at least at the higher concentrations of external Cl tested in these experiments. However, it should be mentioned here that this may not be the case at a lower range of external Cl concentrations.

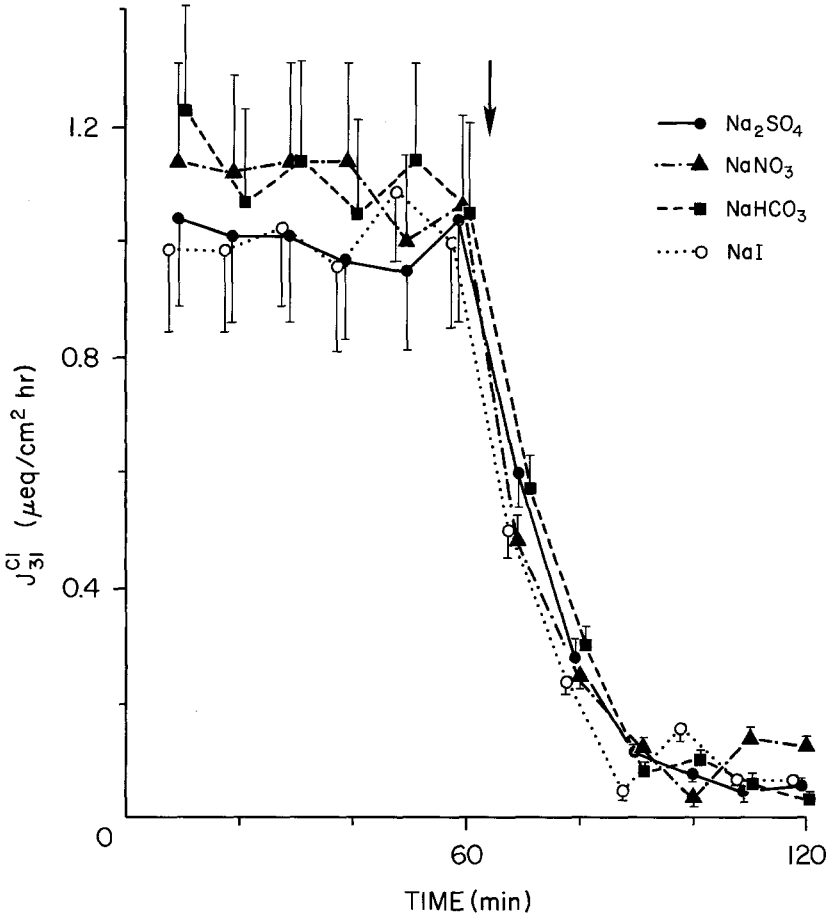

Fig. 6. The effect of the substitution of 57.5 mM Na_2SO_4 plus 57.5 mM mannitol (●), 115 mM $NaNO_3$ (▲), 115 mM $NaHCO_3$ (■) or 115 mM NaI (○) for 115 mM NaCl in the external medium on the transepithelial efflux of Cl (J_{31}^{Cl}). $N=8$ for each point

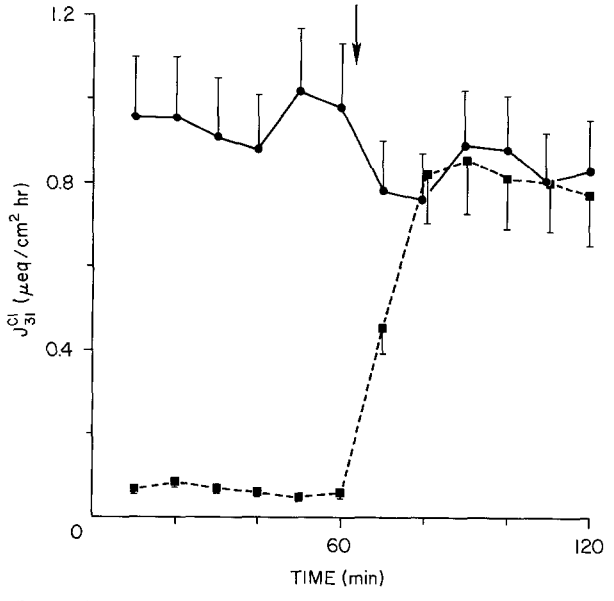

Fig. 7. The effect of substitution of 115 mM NaBr for 115 mM NaCl (●), or 57.5 mM Na_2SO_4 plus 57.5 mM mannitol (■) in the external medium on the transepithelial efflux of Cl (J_{31}^{Cl}). $N=8$ for each point

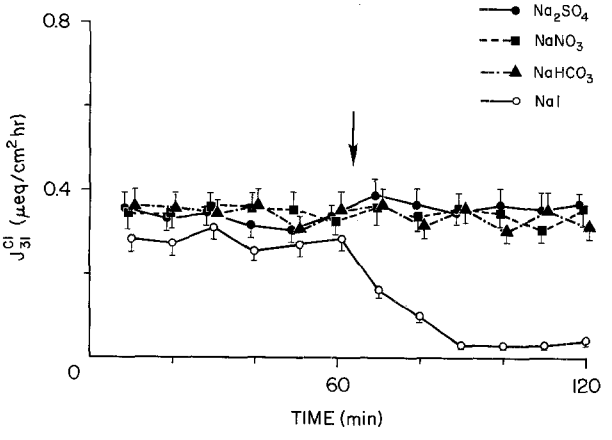

Fig. 8. The effect of the substitution of 50 mM Na_2SO_4 plus 50 mM mannitol (●), 100 mM $NaNO_3$ (■), 100 mM $NaHCO_3$ (▲), or 100 mM NaI (○) for 200 mM mannitol in the external medium on the transepithelial efflux of Cl (J_{31}^{Cl}). The external medium used initially (i.e., during the first 60 min) was composed of 200 mM mannitol, 15 mM NaCl, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$. $N=8$ for each point

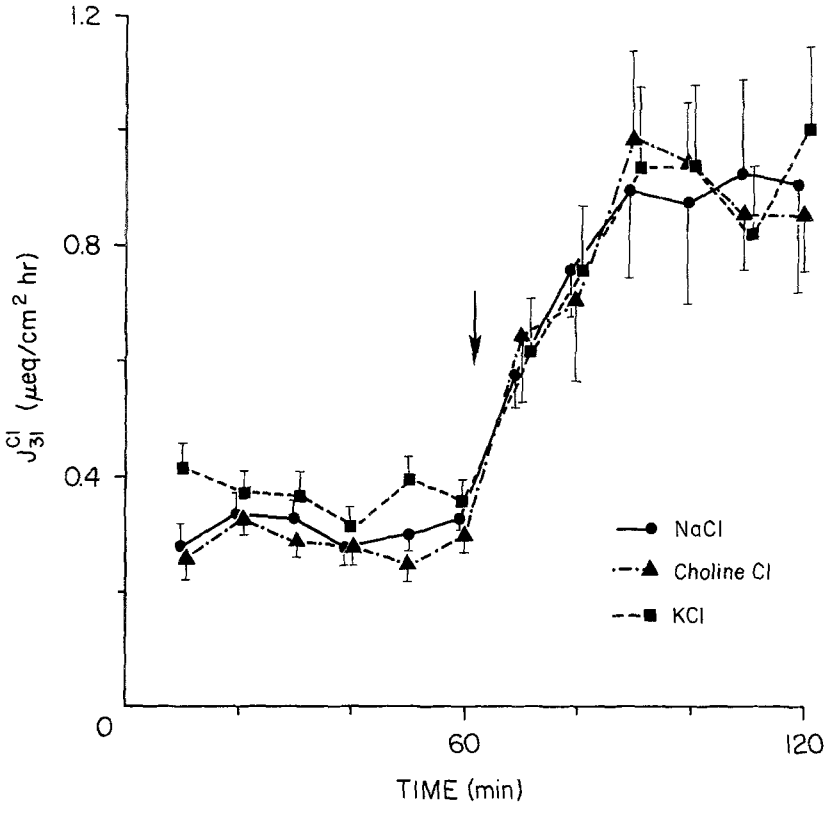

Fig. 9. The effect of an increase in NaCl (●), choline Cl (▲) or KCl (■) concentration in external medium from 15 to 115 mM. Initially (i.e., during the first 60 min) the external medium contained 15 mM of the test salt, 200 mM mannitol, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$. The increase in concentration of the test salt was achieved by substitution of 100 mM of test salt for 200 mM mannitol. $N = 8$ for each point

Discussion

The data provided by this study indicate that both transepithelial Cl fluxes, the influx as well as the efflux, are made up of two components, a saturating component and a linear one which is proportional to the Cl concentration in the bathing medium. The linear component of these transepithelial fluxes has several apparent characteristics usually attributed to an exchange diffusion process. First, with each of the four increases in chloride concentration of the external medium, which were carried out at a chloride concentration higher than 2 mM, the increase in chloride influx was accompanied by an increase in chloride efflux which was nearly identical although the chloride concentration in the serosal solution remained unchanged during these procedures (Table 1 and Fig. 2). A plot of the change in J_{13}^{Cl} against the change in J_{31}^{Cl} for these four increases in external Cl concentration can be characterized by a regression line which has a slope close to one (0.94), an intercept not different from zero, and a correlation coefficient of 0.999. Second, an increase in external Cl concentration coincided with the appearance of a linear component for J_{31}^{Cl} (Fig. 3). Third, replacement of 115 mM Cl^- in the external medium by equivalent amounts of either NO_3^- or HCO_3^- or I^- or by 57.5 mM $SO_4^=$ results in a 87 to 97% inhibition of J_{31}^{Cl}. Fourth, the observations of an increase in J_{31}^{Cl} when Na_2SO_4 plus mannitol in the external medium are replaced by NaBr and of the persistence of a large J_{31}^{Cl} when 115 mM NaCl in the external medium is replaced by 115 mM NaBr would be consistent with an exchange mechanism which accepts Br^-. On the other hand, some of the observations made in this study seem to fit less satisfactorily into the concept of exchange diffusion for this component of the chloride fluxes. The component is linear, and there is no evidence for saturation as one would expect for an exchange process. It could be argued, however, that the concentration range within which the experiments were conducted is too narrow and saturation at higher concentrations might have remained undetected in these experiments. Another observation which seems to be not in favor of an exchange diffusion process is concerned with changes in transepithelial conductance. In the second series of experiments, when no Cl was present in the external medium there was no increase in conductance as the serosal Cl concentration was increased, but there was a small increase in conductance with 5 mM Cl in the external medium and a more substantial increase with an external Cl concentration of 115 mM (Table 3). The increase in conductance coincides with the appearance of a small and large linear component at 5 and 115 mM external Cl, respectively. An increase in conductance from 0.22 ± 0.02 to 0.68 ± 0.07 mmho cm^{-2}, very much like the one listed in

Table 3 in the column for 115 mM external Cl concentration, could be observed in the first series of experiments in which the external Cl concentration was increased from 1 to 115 mM in presence of a serosal Cl concentration fixed at 115 mM.

It is generally thought that the exchange diffusion mechanism has nonconductive properties (*see*, e.g., Cabantchick, Knauf & Rothstein, 1978), and in view of the observation that the conductance increases with an increase in Cl fluxes one might suspect that at least part of the linear component is not connected with an exchange diffusion process. Therefore, we will examine in more detail the relationship between conductance changes and the linear component. On one hand, we can obtain from flux measurements an estimate of the total membrane conductance, G_m, and of the conductance of the n^{th} ion, G_n, by using the following relationship (Hodgkin, 1951):

$$G_m = \frac{F^2}{RT} \sum_0^n Z_n^2 J_n \tag{3}$$

and

$$G_n = \frac{Z_n^2 F_n^2 J_n}{RT} \tag{4}$$

where Z_n represents the valency of ion n, F the Faraday number, R the gas constant, T the absolute temperature, and J_n the flux of ion n. On the other hand, we can also measure the total membrane conductance directly from electrical measurements by determining the current-voltage relationship of the membrane:

$$G_m = \frac{\Delta I}{\Delta V} \tag{5}$$

where G_m is the conductance determined by a change in transmembrane current, ΔI, for a given change in transmembrane potential difference, ΔV. G_m was determined by measurements of transepithelial current and voltage difference for each condition of the second series of experiments (Table 3) and for each condition of the first series of experiments (*see* text, Results). Since G_m represents the sum of all ion currents (Eq. (3)), we have to consider the flow of the Cl as well as the Na ion since the conductance of the frog skin epithelium is determined by these two ions. This means that we can obtain an estimate of the Na conductance under conditions when the Cl fluxes are zero or close to zero (i.e., at lowest Cl concentrations in Tables 1, 2 and 3) since under these conditions the total conductance is close to the Na conductance. Furthermore, we can use the short-circuit current recorded in these experiments as an indicator for the presence or absence of changes in Na fluxes (and in Na conductance) since the relationship between short-circuit current and Na fluxes is known from Na flux determinations. With "standard" Ringer's in the external solution, the Na influx, J_{13}^{Na}, was found to be practically identical with the short-circuit current (Cruz & Biber, 1976). The Na efflux, J_{31}^{Na}, on the other hand, is only a small fraction of the values obtained for J_{13}^{Na} (Biber & Mullen, 1976, 1977), except after application of ouabain or of dinitrophenol. In addition, with low (15 mM) or high (115 mM) Cl concentration present in the external solution it was found that changes in short-circuit current reflect, within experimental error, identical changes in J_{13}^{Na} (Biber & Mullen, 1980).

In the second series of experiments with 115 mM Cl in the external medium present, the increase in J_{31}^{Cl} is accompanied by an increase in conductance (last column in Tables 2 and 3, respectively). The relationship (least squares) between G_m and G_{Cl}, the Cl conductance calculated from J_{31}^{Cl} (Eq. (4)), is

$$G_m = 0.366 + 0.221\, G_{Cl} \tag{6}$$

for the averages at the 6 serosal concentrations (correlation coefficient 0.920). Since the intercept of 0.37 represents the conductance in absence of chloride conductance, this value can be used as an estimate for the Na conductance. As no difference could be detected between the average short-circuit current observed at different serosal Cl concentrations, there is no sign that the Na conductance is altered and the observed changes in conductance must be assigned to a change in Cl conductance. Actually, measurements of unidirectional Na fluxes taken in separate experiments but under identical conditions indicate that replacement of Na-sulfate Ringer's by NaCl Ringer's on the serosal side causes no significant change in the relationship between J_{13}^{Na} and short-circuit current and an increase in J_{31}^{Na} so small that it accounts at most for a conductance increase of 0.02 mmho/cm^2 (*unpublished observation*). From this it must be concluded that the actual increase in Cl conductance is only some 20% of the one predicted from the increase in J_{31}^{Cl}. A similar discrepancy between the measured increase in G_m and the one predicted from the rise in J_{31}^{Cl} could be observed in the first series of experiments. After correction for an increase in Na conductance with rising external Cl concentration, the increase in total conductance turns out to be only 27% of the one predicted from the increase in J_{31}^{Cl} (correlation coefficient 0.987 for 6 pairs of observations). From all this it appears either that the largest part of the linear component (i.e., 73 to 80%) has nonconductive properties and only the small remaining portion is associated with conductive characteristics or that the entire linear component is due

to a process linked to a low conductivity (e.g., an exchange process with a coupling ratio different from one).

The saturating components of Cl influx and efflux saturate at low Cl concentrations (K'_{Cl} for influx about 2 mM and K''_{Cl} for efflux about 5 mM). Since active inward movement of Cl can be observed at low external Cl concentrations, it must be connected with the saturating component of J^{Cl}_{13}. Under short-circuit conditions and with NaCl Ringer's solution bathing both sides of the skin, the intracellular potential was measured to be -73 ± 2 mV (Nagel, 1976) and large changes in extracellular Cl concentration on both sides of the skin were found to cause "little or no change" in intracellular potential under short-circuit conditions (Helman, Nagel & Fisher, 1979). Nagel specifically reported that the intracellular potentials observed with NaCl Ringer's solution were not different from the ones observed with Na_2SO_4 Ringer's solution (Nagel, 1977). Estimates of intracellular Cl concentration obtained with electron microprobe analysis under short-circuit conditions with NaCl Ringer's on both sides of the tissue indicate that the cytoplasmic Cl concentration is 36.5 ± 5 mM (Rick et al., 1978). Very recent measurements done with Cl-selective microelectrodes give no indication for cytoplasmic compartmentalization since the intracellular Cl activities obtained with this method are in general agreement with estimates of Cl concentration based on microprobe analysis (W. Nagel, *personal communication*). It appears, therefore, that the intracellular Cl activity is several times higher than the equilibrium value under the conditions outlined above.

From this it follows that a site of active transport for the net inward Cl movement must be located in the outward-facing cell membrane of the epithelial cells. The net movement of charges which could be attributed to the observed net movement of Cl would tend to reduce the short-circuit current by a substantial fraction. However, simultaneous determinations of Na fluxes across the outward-facing cell membranes of the epithelial cells and of short-circuit current indicate that net charge movement and net movement of Na are virtually identical (Mullen & Biber, 1978). But if net charge transfer is fully accounted for by net movement of Na, then one must postulate a mechanism for net movement of Cl which does not involve net charge transfer[1]. One possible explanation for this situation would be an anion exchange mechanism such as a chloride-bicarbonate exchange across the outward-facing cell membranes.

Cereijido and co-workers recently investigated in detail the chloride transport across the skin of the frog *Leptodactylus ocellatus* (Ques-von Petery, Rotunno & Cereijido, 1978; Rotunno, Ques-von Petery & Cereijido, 1978; Rodriguez-Boulan et al., 1978). Several years ago, Zadunaisky and associates found that significant amounts of chloride are transported in an inward direction across the skin of this frog (Zadunaisky & Candia, 1962; Zadunaisky, Candia & Chiarandini, 1963; Zadunaisky & Fisch, 1964). The experiments done by Cereijido and co-workers confirm this, and their data show that net chloride movement across the skin of *L. ocellatus* is not only an order of magnitude greater than the net chloride movement reported here (Table 1 and Fig. 1), but also exceeds the total chloride influx or efflux measured in this study on the skin of *Rana pipiens*. Furthermore, Cereijido and co-workers observed in *L. ocellatus* much higher rates of J^{Cl}_{31}. In addition, they observed that J^{Cl}_{31} exhibited saturation with a J^{m}_{31} at 0.70 μeq/cm²hr when the external Cl concentration was raised by addition of up to 99 mM choline chloride to 1 mM NaCl. On the other hand, they noted much higher rates of J^{Cl}_{31} with a J^{m}_{31} at 2.65 μeq/cm²hr when the external Cl concentration was raised in presence of a constant high level of external Na of 100 mM, substituting Na_2SO_4 by NaCl. This is quite different from the observations reported here for *R. pipiens*. First, the presence of different cations (Na, K, choline) did not have an effect on the magnitude of J^{Cl}_{31} (Fig. 9). Second, in *R. pipiens*, J^{Cl}_{31} does not exhibit saturation at high external Cl concentration (Figs. 2 and 4) since the increase in external Cl concentration seems to induce a linear component of J^{Cl}_{31}. Other differences between the Cl transport systems in the two species can be noted in the relationship between G_m, the total tissue conductance, and G_{Cl}, the Cl conductance calculated from J^{Cl}_{31}. Thus, for example, in contrast to what was observed in *R. pipiens*, the change in G_m observed in the presence of external Na in *L. ocellatus* is not smaller than the change in G_{Cl} predicted from changes in J^{Cl}_{31}. Therefore, the argument in favor of an exchange diffusion cannot be used here for *L. ocellatus*. On the other hand, Cereijido and co-workers did observe changes in J^{Cl}_{31} which were not accompanied by a change in G_m when the skin of *L. ocellatus* was exposed at the external surface to a very low Na concentration. From this they concluded that an exchange diffusion mechanism is present at low external Na concentration (Ques-von Petery et al., 1978). The reason for the differences between the Cl transport systems of these two species is not known. One might raise the question whether or not

[1] Simultaneous measurements of short-circuit current and of Na fluxes are sufficiently precise that a difference in the order of magnitude of net Cl flux (i.e., 0.15 μeq/cm²hr) should have been detected. However, the determinations of Na and Cl fluxes were made in separate experiments, and it should be pointed out that simultaneous determinations of Na fluxes, Cl fluxes, and short-circuit current would provide superior means to rule out the possibility that net Cl movement contributes to net charge transfer.

differences in the active transport system alone could also be the source of apparent differences of the other components of Cl transport.

As mentioned above, the linear component has properties which are expected from an exchange diffusion process. However, Kristensen's observation (Kristensen, 1978) on *Rana temporaria* that ratios of Cl fluxes across the skin change in agreement with the Ussing flux ratio equation (Ussing, 1949) when the frog skin is voltage clamped at different potentials seems to rule out the possibility that exchange diffusion is substantially involved in Cl transport. Kristensen therefore concluded that the Cl fluxes across the frog skin, in contrast to those across the toad skin, cannot be due to an exchange diffusion process. On the other hand, Idzerda and Slegers (1976) observed in *R. temporaria* that, depending on the group of frogs examined, the Cl flux ratio was not or was in agreement with the flux ratios predicted by the Ussing flux ratio equation, and they concluded that "significant" to "extreme" exchange diffusion was present in many of the skins they investigated. They commented that their results could be explained "by a 1:1 exchange mechanism working in parallel with a diffusion pathway." Furthermore, they pointed out that some of the Cl flux ratios obtained earlier by Linderholm (Linderholm, 1952) were also lower than those predicted by the flux ratio equation. Alvarado et al. (Alvarado, Dietz & Mullen, 1975) measured Cl fluxes in isolated skin of *R. pipiens* when dilute solutions of NaCl, KCl, K_2SO_4 or choline chloride were present in the external medium. Since they observed flux ratios which were lower that those predicted by the flux ratio equation and since for a given change in transepithelial potential J_{31}^{Cl} changed much less when, instead of K_2SO_4, KCl was present in the external solution, they concluded that a sizable part of the Cl flux was due to exchange diffusion. The reasons for the differences between these observations is not known. Differences in seasonal properties, in the maintenance of animals and – in our view, most important – in experimental techniques could be responsible for these discrepancies. In fact, it has been shown that differences in techniques for mounting the skin in the flux chambers can have profound effects on transepithelial ion fluxes. For example, in a previous study we have compared fluxes measured in new chambers, specially designed to avoid edge damage where the tissue is held in the chamber, with fluxes obtained in conventional chambers (Biber & Mullen, 1976). We found that the transepithelial efflux of Na (J_{31}^{Na}) obtained in conventional chambers was up to 20 times higher than the one measured in the new chambers, and, even more disturbing, the J_{31}^{Na} measured in conventional chambers gave no indication of the saturation of J_{31}^{Na} observed in the new chambers. From this we concluded that the edge damage caused by mounting tissue in conventional chambers may be associated with an artificial leakage pathway across the tissue. It might be expected that fluxes across such an artifactual shunt pathway may have characteristics of a diffusional flux. It is therefore conceivable that the presence of different degrees of edge damage could be the source for the observed differences. On the other hand, other factors such as true differences in transport mechanisms between *R. temporaria* and *R. pipiens* could be responsible for at least part of the observed differences.

Experiments conducted previously in our laboratory support the view that Cl transfer across the skin proceeds through the cells (Biber & Mullen, 1977). These studies were mainly concerned with measurements of J_{31}^{Na} and led to the conclusion that the Na efflux across the skin passes via a transcellular route. Among the major observations were the findings (1) that, in contrast to nonelectrolyte fluxes, Na efflux exhibits saturation kinetics and (2) that Na efflux increases several-fold after application of ouabain with an increase both in V_{max} and in apparent Michaelis-Menten constant. The ouabain-induced increase in J_{31}^{Na} followed a complicated time course (initial spike-like increase before reaching a steady state). In the same study we found that J_{31}^{Cl} also increased after ouabain and that when J_{31}^{Cl} and J_{31}^{Na} were measured simultaneously, the ratio of J_{31}^{Cl} over J_{31}^{Na} remained constant despite the large changes and the complicated time course of the individual fluxes. This suggests strongly that J_{31}^{Cl} passes through the cells rather than between the cells. Candia (1978) as well as Kristensen (1978) observed changes in Cl fluxes after application of amiloride, known to act selectively on the external cell membrane. On the basis of their observations, both investigators came to the conclusion that Cl transport across the skin proceeds via a transcellular pathway.

The following four observations made in this study provide additional support for transcellular passage of Cl: First, the linear component has properties difficult to reconcile with extracellular passage of Cl (changes in external bath cause changes in Cl efflux). Second, the saturating component of J_{31}^{Cl} seems to be connected with active transport of Cl across the external cell membrane. Third, when no Cl is present in the external medium, J_{31}^{Cl} is very small and exhibits saturation kinetics ($J_{31}^{m}=0.055$ μeq/cm²hr; $K_{Cl}''=4.70$ mM). Fourth, in the presence of low external Cl concentration (15 mM) J_{31}^{Cl} is reduced to a small fraction of J_{31}^{m}, namely, to 0.003 μeq/cm²hr when I^- is added to the external medium. From all this we conclude that most, if not all, transepithelial Cl transfer proceeds across the cells and not via extracellular shunt pathway.

The recent description of a Cl shunt pathway through mitochondria-rich cells (Voute & Meier, 1978) opens an entirely new range of possibilities for Cl fluxes across the epithelium. On one hand, it seems reasonable to think that all the functionally intact epithelial cells are in some way connected with the transepithelial transfer of Cl. On the other hand, it seems possible that the different components of the chloride fluxes proceed through different pathways given by different cell types. Another possibility would be that the entire transepithelial Cl flux passes exclusively through a special pathway (mitochondria-rich cells?).

In summary, the experiments indicate that the Cl fluxes across the skin of *Rana pipiens* are made up of two components, a saturating one and a component apparently linear with the Cl concentration in the bath from which the flux originates. The largest part (about 3/4) of the linear component has characteristics of an exchange diffusion process. As judged by the rapid and reversible action of changes in the external medium, this process is located in the outward-facing cell membrane. The linear component of J_{31}^{Cl} disappears when Cl^- in the external medium is replaced by $SO_4^=$, NO_3^- or HCO_3^- or I^- and returns when Br^- is added. The saturating component of J_{31}^{Cl} appears to be connected with active Cl transport in an inward direction. This transport involves a mechanism capable of moving Cl against an electrochemical gradient across the outward-facing cell membrane. This mechanism appears not to be a NaCl transfer system, but another electroneutral transfer which possibly operates via $Cl\text{-}HCO_3$ exchange.

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