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ANIONIC DEPENDENCE OF SODIUM TRANSPORT IN THE FROG SKIN

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SUMMARY

1. Short circuit currents and sodium fluxes were measured in isolated frog skins bathed by Ringer's solutions of different anionic composition.

2. When chloride Ringer's solution bathing both sides of the isolated frog skin is substituted by isoosmolar sulphate Ringer's there was a sharp decrease in the short circuit current.

3. The lowering of the short circuit current could be accounted for by an identical decrease in sodium fluxes, as a good agreement between these two parameters was found in both experimental conditions.

4. This effect on the short circuit current was reproduced when chloride ions were substituted by gluconate ions, but was much less evident when chloride was substituted by bromide or iodide in the Ringer's solutions bathing both sides of the skin.

5. The results suggest that at least some of the sodium transport is anion dependent in a nonspecific way.

INTRODUCTION

The electrical behaviour of the frog skin has been described in terms of an equivalent circuit in which a voltage generator with an internal resistance corresponding to the active sodium transport is shunted by a conduction path for other permeable ions moving passively, mainly chloride[1–4]. Using USSING's technique in which skins bathed on both sides with Ringer's solution and mounted in such a way that the open circuit voltage and short circuit current can be measured, the substitution of chloride by sulphate in the bathing solutions or the treatment of the outside surface by vestigial amounts of copper (Cu_2SO_4, 10^{-5} M)* has been reported to show an increase in the open circuit voltage without appreciable change in the short circuit current[2,5–7]. Thus the sodium transport (equated with the short circuit current) maintained itself independently of these two changes in the bathing fluid composition which, simultaneously with the higher voltage measured across the skin, could be interpreted as due to an increase in the resistance of the shunt path[2].

The following study will show that the short circuit current is not independent of the anionic composition of the bathing fluids and that this effect can be masked if the solutions used are not corrected for the same osmolality.

* Some studies of the effect of copper on the frog skin will be the subject of another paper.

METHODS

Frogs, of the species *Rana ridibunda* Pallas*, were used unless otherwise specified. They were kept in a temperature-controlled room at 6–8°, half immersed in running tap water. Experiments were performed throughout the year, and no appreciable seasonal variation was detected. Frogs were double pithed and the skin dissected and mounted in Ussing-type chambers. Often two symmetrical halves were used. The exposed skin areas were 0.789, 3.14 or 7.06 cm^2. The time elapsing between killing and mounting of the skins did not exceed 10 minutes. The compositions of the Ringer's solutions used are summarized in Table I. The references in the text to the specific Ringer's being employed are made to the main anion used (see table), except in the case of choline Ringer where sodium was substituted by choline.

The composition of the Ringer's solution was frequently checked. Na and K concentrations were measured by flame photometry with the Eppendorf Flame Photometer and Cl by coulometric titration in an Aminco Cotlove Titrator. pH was checked with a Radiometer 4 pH Meter. The aerated Ringer's had a pH of 8.2–8.3. Sometimes bicarbonate was substituted by Tris buffer and the Ringer was titrated to the same pH.

Osmolality was checked with an Advanced Osmometer. The osmolality of the chloride Ringer used was around 0.220 (0.218–0.222). When chloride was substituted by another monovalent anion, the osmolality remained at that value. When chloride was substituted by sulphate, a divalent anion, the osmolality went down to 0.140. To obtain isoosmotic sulphate Ringer, 80 mM of glucose was added (in a few control experiments urea or mannitol was also used).

Throughout the text the isoosmolar sulphate Ringer is designated compensated sulphate Ringer and the hypoosmotic one, noncompensated sulphate Ringer.

The potential of the frog skin was measured through two agar bridges (3 % agar in the Ringer in which the skin was mounted), placed very close to the skin. The agar bridges were linked through saturated KCl to the calomel electrodes connected to a high impedance electrometer (Keithley 610 B). The overall junction potential was less than 0.5 mV and the resistance between two bridge tips was around 70 Ω/cm^2. Short circuit current was measured using two additional bridges placed as far as possible from the skin. The circuit between these was completed by freshly prepared Ag–AgCl–KCl electrodes, a current source and a galvanometer. Skins were mounted and allowed to equilibrate for two to three hours. Open circuit voltages and short circuit currents were measured every five minutes. Experimental protocols were only started when a reasonable steady state of these two parameters was reached. Experimental periods were timed so that a steady state of voltages and currents could be reached, and whenever possible after an experimental period, a control period was allowed with the initial Ringer solution to get a better definition of the baseline corresponding to the time evolution of the skin. Sodium fluxes were measured by the single-labelling technique using symmetrical pieces of abdominal skin. ^{22}Na was used**. Activities were measured in a well-type scintilation detector. Samples were counted to 10000 counts.

* This is the species most frequently found in Portugal and is very similar to *Rana sculenta*.
** Obtained from Amersham, England.

TABLE I

COMPOSITION OF THE RINGER'S SOLUTIONS USED

Values are expressed in mM.

Type of Ringer	*Chloride*	*Sulphate*	*Gluconate*	*Bromide*	*Iodide*	*Sodium*	*Choline*	*Potassium*	*Calcium*	*Bicarbonate*	*Glucose*
Chloride	119	—	—	—	—	114	—	2.4	2.4	2.4	—
Sulphate	—	59.5	—	—	—	114	—	2.4	2.4	2.4	80
Gluconate	—	—	119	—	—	114	—	2.4	2.4	2.4	—
Bromide	—	—	—	119	—	114	—	2.4	2.4	2.4	—
Iodide	—	—	—	—	119	114	—	2.4	2.4	2.4	—
Choline	119	—	—	—	—	—	114	2.4	2.4	2.4	—

TABLE II

STEADY-STATE SHORT CIRCUIT CURRENT VALUES OF SYMMETRICAL PIECES OF SKINS, ONE MOUNTED IN CHLORIDE RINGER AND THE OTHER IN SULPHATE RINGER

Currents expressed in $\mu A/3.14\ cm^2$. Percentages of sulphate currents compared with chloride currents.

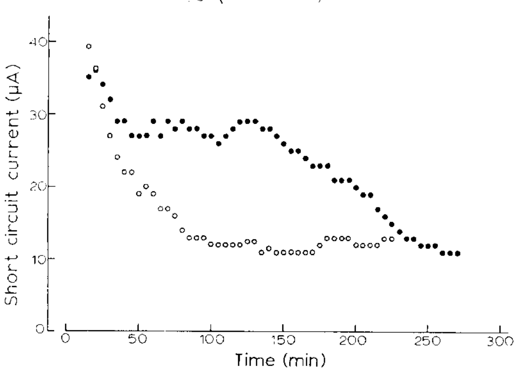
Sulphate Ringer	45	34	28	43	10	20	13	100	25	11
Chloride Ringer	110	138	90	65	18	44	100	125	125	25
%	41	25	31	66	56	45	13	80	20	44

Mean and standard deviation 42% ± 21.

RESULTS

The first results described here were obtained from experiments, performed over a period of two years, to study the time evolution of open circuit voltages and short circuit currents in 60 different skins mounted either in chloride Ringer or in compensated sulphate Ringer. The skins were mounted and readings were made every five minutes during the experiments. There was a considerable scatter in each group but no seasonal variation was detected. The open circuit voltage of skins mounted in compensated sulphate Ringer did not reveal the constant high values described in the literature when compared with the skin mounted in chloride Ringer. The values varied greatly and no definite pattern was found when the two groups were compared. The short circuit current, on the other hand was systematically lower in skins bathed in compensated sulphate Ringer than in skins bathed in chloride Ringer. The individual values obtained were lumped together and the time evolution studied, calculating the means of all measurements taken (zero corresponded to the setting of chambers) and then taking the average of five adjacent mean values and plotting these results at the time corresponding to the central value[8]. Fig. 1 shows the results of these experiments calculated in the above way for short circuit currents. After an initial period the current of the skins in the chloride Ringer tends to settle at higher values than the currents of skins in sulphate Ringer. This is for a period of two to three hours. After that the currents of the skins mounted in chloride Ringer decay with a tendency to reach the steady-state values of the currents of the skins mounted in compensated sulphate Ringer.

In order to confirm the results obtained, paired skins of the same frog were mounted simultaneously, one with chloride Ringer and the other with compensated sulphate Ringer. After having reached a steady state, the short circuit current was always lower in the skins bathed by the compensated sulphate Ringer solution. Steady-state current values obtained with this solution expressed as percentages of current obtained in skins bathed in chloride Ringer gave mean values of 42 % ± 21 (10) (the number of experiments is in brackets) with a minimum value of 13 % and a maximum of 80 % (Table II).



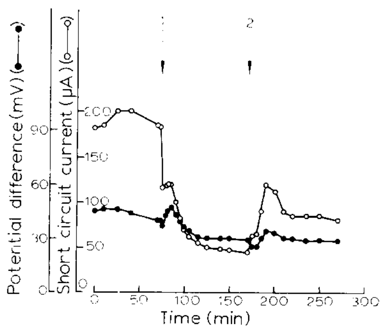


Fig. 1. Time evolution of short circuit current of skins mounted in chloride Ringer's solution (●) and in sulphate Ringer's solution (○). Plot of a moving average of five adjacent mean values of short current expressed in $\mu A/cm^2$.

Fig. 2. Skin mounted in chloride Ringer. Arrow 1. Sulphate Ringer on both sides. Arrow 2. Chloride Ringer on both sides.

In another group of experiments, chloride Ringer and compensated sulphate Ringer were tested on the same piece of skin. Skins were mounted in chloride Ringer and changes in solution were made when currents and voltages reached a steady state. When chloride Ringer was substituted by compensated sulphate Ringer on both sides of the membrane, there was in all experiments a decrease of short circuit current and a less constant change in the open circuit voltages which sometimes increased and sometimes remained constant or even decreased (Fig. 2; Table III).

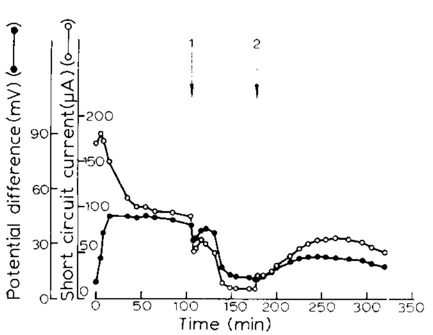
To exclude the possibility that this effect was due either to the brand of sulphate used or was in some way related to the particular nonelectrolyte utilized for correction of the osmolality, a control group of experiments was done in which sulphate from different manufacturers (Merck, B.D.H., May Baker) and several different non-electrolytes (glucose, mannitol, urea) were used (Table III).

TABLE III

CHANGES OF SHORT CIRCUIT CURRENT AND OPEN CIRCUIT VOLTAGE OF EXPERIMENTS IN WHICH CHLORIDE WAS SUBSTITUTED BY ANOTHER ANION ON BOTH SIDES OF THE SKIN

Results were calculated as percentages of experimental periods compared to baseline of control periods ((Experimental/Control) × 100). Means and standard deviation. In parentheses number of experiments.

Ringer	*Short circuit current*	*Open circuit voltage*
Gluconate	22.7 ± 7.5 (7)	64.9 ± 23 (7)
Sulphate glucose	38.7 ± 11 (29)	90.2 ± 38 (27)
Sulphate manitol	55.3 ± 13.3 (6)	109.6 ± 23.8 (6)
Sulphate urea	61.1 ± 16.2 (5)	119.4 ± 27.3 (5)
Iodide	85 ± 43 (11)	129 ± 67 (11)
Bromide	87.6 ± 13.4 (13)	114.5 ± 13.6 (13)
Sulphate (osmolality 0.140)	88.6 ± 20 (11)	153.4 ± 45 (11)



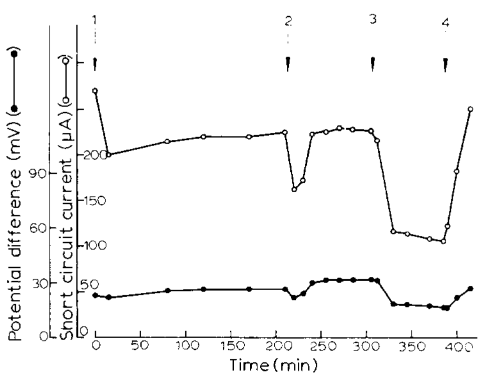


Fig. 3. Skin mounted in chloride Ringer. Arrow 1. Gluconate Ringer on both sides. Arrow 2. Chloride Ringer on both sides.

Fig. 4. Arrow 1. Skin mounted in chloride Ringer. Arrow 2. Sulphate Ringer (osmolality 0.220) on both sides. Arrow 3. Sulphate Ringer (0.140) on both sides. Arrow 4. Chloride Ringer on both sides.

TABLE IV

CHANGES OF SHORT CIRCUIT CURRENT AND OPEN CIRCUIT VOLTAGE OF EXPERIMENTS WHERE SODIUM WAS CHANGED BY CHOLINE IN SKINS BATHED BY CHLORIDE RINGER'S ON BOTH SIDES

Results are expressed as percentages of experimental periods compared with control periods. Means and standard deviation. In parentheses number of experiments.

Ringer	*Short circuit current*	*Open circuit voltage*
Choline	6.9 ± 5.3 (15)	19.6 ± 13 (15)

TABLE V

$^{22}Na^+$ FLUX MEASUREMENTS IN SKINS BATHED IN CHLORIDE RINGER OR SULPHATE RINGER

Results are expressed in µequiv/30 min per 7.06 cm^2. Means and standard deviations. In brackets number of periods. S. S. C., short circuit current.

Influx			*Backflux*			*Net flux*
S. S. C.	*Na^+ flux*	%	*S. S. C.*	*Na^+ flux*	%	%
Chloride Ringer's solution						
2.447 ± 1.2 (25)	2.594 ± 1.25 (25)	108.7 ± 17.3 (25)	2.479 ± 0.79 (26)	0.267 ± 0.11 (26)	11.3 ± 4.8 (26)	97.4
Sulphate Ringer's solution						
2.104 ± 1.25 (23)	2.141 ± 1.13 (23)	105.9 ± 11 (23)	1.838 ± 0.98 (22)	0.153 ± 0.1 (22)	11.4 ± 10 (22)	94.5

Gluconate was also used as a substitute for chloride (Fig. 3), and as this Ringer's solution is almost isoosmolar with the chloride Ringer, no compensation for osmolality was needed. It can be seen in the table in which all the control experiments are summarized that the overall results are the same. This was not the case when the chloride ion was replaced with sulphate without compensation for isoosmolality in the same experimental procedure. In this situation we did not find the changes of short circuit current described above. Sometimes there was a small decrease in the short circuit current but very often it remained unchanged while the voltages increased. The results of eleven such experiments are expressed in Table III. Fig. 4 illustrates one of those experiments. The skin was equilibrated with chloride Ringer which was then changed to uncompensated sulphate Ringer (osmolality 0.140) and finally to compensated sulphate Ringer (0.220). After the first change there was a slight change in the open circuit voltages but none in the short circuit currents; after the second change the typical decrease of the short circuit current could be observed.

The lowering of the short circuit current described above could be explained by the existence of a current component due to an independent active transport of chloride that would disappear in the absence of this ion. To test this hypothesis, sodium was substituted by choline in the chloride Ringer in a group of experiments. In skins in which sodium was substituted by choline, the current and voltages decreased to almost zero, suggesting that there was no independent chloride transport. The results of 15 such experiments are given in Table IV. ^{22}Na fluxes were also measured in paired pieces of skin taken from the same frog. One was used for influxes the other for backfluxes, in 30-min periods and exposed areas of 7.06 cm^2. The results are summarized in Table V and Fig. 5. These results showed a good agreement between

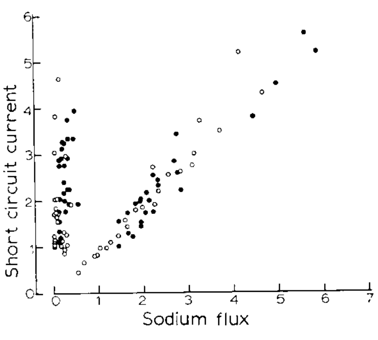
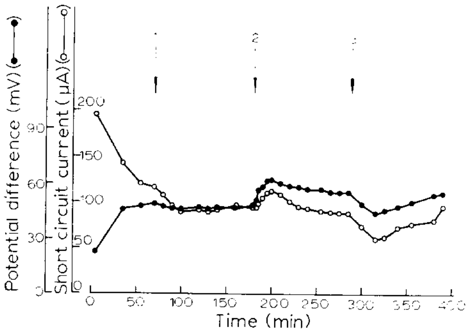


Fig. 5. Plot of short circuit current against sodium fluxes. Both scales expressed in μequiv of Na per 30 min per 7.06 cm^2. Influxes on a slope near unity. Backfluxes near the zero line. ●, NaCl and O, Na_2SO_4.

sodium fluxes and short circuit current in both experimental conditions (chloride and sulphate Ringer). In this group of experiments fluxes were measured in skins immersed from the beginning either in chloride Ringer's solution or in sulphate Ringer's solution. This might explain the overlapping of the results of the two groups. However, it can be seen from Table V that the average value of short circuit currents is higher in skins bathed in chloride Ringer (t test, $P < 0.015$). This difference is much more apparent when short circuit currents are measured using both Ringer's solutions on the same skin. Nevertheless in both conditions, the points fall on the same straight line with a slope near unity. Finally, in order to test whether the effect of the substitution of chloride ion on the short circuit current was specific, bromide and iodide Ringer's solutions were also tested (Figs. 6 and 7). These anions are similar to chloride in regard to their permeability for the frog skin[9]. In these experiments variations of open circuit voltages and short circuit currents were also present but much less important. In Table III the results of these experiments are given.



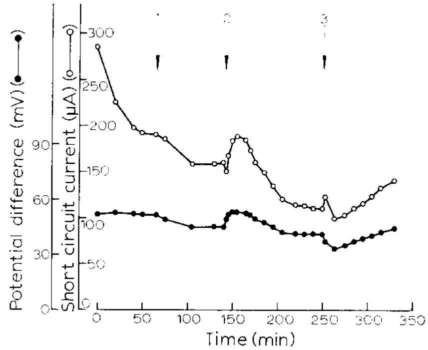
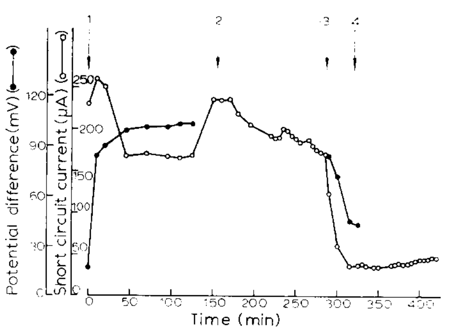


Fig. 6. Skin mounted in chloride Ringer. Arrow 1. Fresh chloride Ringer on both sides. Arrow 2. Bromide Ringer on both sides. Arrow 3. Chloride Ringer on both sides.

Fig. 7. Skin mounted in chloride Ringer. Arrow 1. Fresh chloride Ringer on both sides. Arrow 2. Iodide Ringer on both sides. Arrow. 3. Chloride Ringer on both sides.

All results described above were obtained with *Rana ridibunda* and it seemed important to ascertain whether they could also be obtained in other species. For this purpose we choose to test *Rana temporaria** because it is the species most commonly utilized in other studies of this type. Experiments conducted with *Rana temporaria* always showed, in our hand, the same results that were obtained with *Rana ridibunda*. Two typical experiments are illustrated in Figs. 8 and 9. In one experiment (Fig. 8) it can be seen that, after a period of equilibrium with chloride Ringer, changing of this



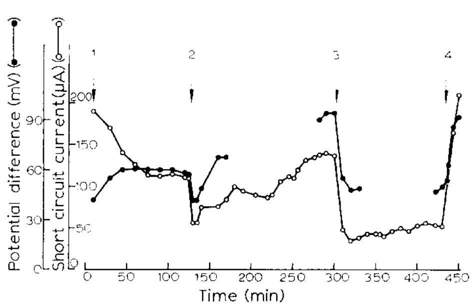


Fig. 8. Arrow 1. Skin mounted in chloride Ringer. Arrow 2. Isotope on inside. Arrow 3. Sulphate Ringer on both sides. Arrow 4. Isotope on inside.

Fig. 9. Arrow 1. Skin mounted in chloride Ringer. Arrow 2. Sulphate Ringer (osmolality 0.140) on both sides. Arrow 3. Sulphate Ringer (0.220) on both sides. Arrow 4. Sulphate Ringer (0.140) on both sides.

* Kindly supplied by Dr. ZERAHN.

TABLE VI

$^{22}Na^{+}$ FLUX MEASUREMENTS IN ONE SKIN BATHED FIRST IN CHLORIDE RINGER AND THEN IN SULPHATE RINGER AND COMPARED WITH THE SHORT CIRCUIT CURRENT

Results are expressed in μequiv/30 min per 7.06 cm^2. S.S.C., short circuit current.

Influx			*Backflux*			*Net flux*
S.S.C.	*Na^+ flux*	%	*S.S.C.*	*Na^+ flux*	%	%
Chloride Ringer's solution						
3.587	3.748	104.5	5.170	0.101	1.95	102.5
3.346	3.509	104.9	5.039	0.091	1.80	103.1
Sulphate Ringer's solution						
0.709	0.806	113.7	1.229	0.089	7.24	106.5
0.802	0.971	121.1	1.371	0.093	6.78	114.3

solution with compensated sulphate Ringer also produced a substantial decrease in short circuit current. Sodium fluxes measured simultaneously again showed a good agreement between fluxes and short circuit current in both experimental conditions (Table VI). When chloride Ringer's solution is substituted by noncompensated sulphate Ringer (Fig. 9) after a transition phase, the increase in open circuit voltage with a small increase in short circuit current was observed. Again with compensated sulphate Ringer an appreciable decrease of these parameters can be observed.

DISCUSSION

Substitution of chloride by sulphate ions in the Ringer's solution bathing both sides of the isolated frog skin preparation produced a substantial reduction in the values of the short circuit current. The higher basal values of short circuit current could be recovered in control periods of chloride Ringer (values of experimental periods expressed as percentages of control periods were 38.7 ± 11). The same type of result can be seen in experiments where skin has not been submitted to mechanical stresses due to changes in bathing solutions. Fig. 1 clearly demonstrates the different behaviours of skins mounted from the beginning either in chloride Ringer or sulphate Ringer's solution.

This was a systematic finding not only in the frog species more frequently utilized, *Rana ridibunda*, but also in the experiments conducted with *Rana temporaria*. *Rana ridibunda* is a species closely related to the species of *Rana sculenta*.

To make sure that the results obtained were due to the particular alteration in the anionic composition of the bathing fluid, special care was taken to insure that no other variable capable of influencing the electrical parameters was introduced. This especially applies to the need to maintain conditions of isotonicity. As a matter of fact, FRANZ *et al.*[14], LINDLEY *et al.*[15], MACROBBIE *et al.*[16] and USSING[17,18] studied the effect of changes in the osmolality of the Ringer's solution when applied to frog skin and got alterations in the thickness of the skin and of the open circuit voltage and short circuit current. Hypotonic solutions bathing the inside of the frog skin, as well as bathing both sides, cause an increase in the short circuit current and a swelling of the skin[16,18], while there is almost no effect when hypotonic solutions bathe the outside face of the skin alone. When uncompensated sulphate Ringer was used for substitution of chloride Ringer's solution on both sides of the frog skin we obtained the results usually described in the literature[1,2,5,7]. In this situation two variables are changed simultaneously and the effect of substitution of chloride ions by sulphate ions in the Ringer's solution is masked by the effect of the hypotonicity of the Ringer. These effects can be separated by using Ringer's solutions with the same osmolality.

The lowering of the short circuit current after substitution of chloride ion by other ions could be explained either by an independent, active, outward chloride transport normally present in the skin, which would disappear in the absence of this ion, or by a direct action of the chloride ion on the sodium transport.

Regarding the first hypothesis, active chloride transport has in fact, already been described. ZADUNAISKY[10,11], in the South American frog, *Leptodactylus ocellatus*, and MARTIN[12], in *Rana sculenta* and *Rana pipiens*, found a net chloride influx. In these cases however, the net chloride transport measured was directed from the outside to the inside solution which is the direction opposite to the one necessary to explain our results. A net chloride outward transport was described by KOEFOED-JOHNSEN *et al.*[13,1], in *Rana temporaria*, when adrenaline was added to the inside solution, and these authors assumed that adrenaline produced this effect by stimulating the secretion of the skin mucous glands. The contribution of this chloride transport for the short circuit current was in this case much less than the one that would be necessary to explain our results. That we are not dealing with a significant chloride transport in our studies can be inferred from the results obtained with choline Ringer, where we got an almost complete reduction of short circuit current and open circuit voltage. Furthermore, in experiments where sodium fluxes were measured, we found a good agreement between short circuit current and sodium net fluxes either in the cases where chloride ions were absent (*e.g.*, compensated sulphate Ringer) or when they were present; that is, low or high currents could always be accounted for by the net sodium fluxes simultaneously measured.

While the first hypothesis can thus be excluded, there does seem to be a direct effect on sodium transport. This means that the active sodium transport or at least some component of it is dependent of the presence of chloride ions in the bathing solutions. That this dependence is not complete can be inferred since sizeable currents can be obtained in the absence of chloride. The experiments in which other anions were used showed that this dependence is not specific for chloride anions, since when we use iodide or bromide instead of chloride we do not get the drastic reduction of the short circuit current observed with sulphate or gluconate.

An ionic interaction of the same type was found by ZADUNAISKY[19] in the rabbit cornea. In this tissue there is a transport of chloride ions from aqueous fluid towards the tear side and sodium ions, though apparently not transported, are necessary for the normal performance of the chloride transport. Our observations showed that in the frog skin sodium transport is in part dependent on the presence of chloride ions in the bathing solutions, but that this dependence is not specific, as already mentioned above, because if we use iodide or bromide in place of chloride ions in the Ringer's solutions, the reduction of short circuit current was much less evident. We might speculate that some step(s) in the transport process is activated in the presence of some anion(s) and that for a particular anion the size of this effect might be dependent on the affinity for it of some activating site. Or perhaps as ZADUNAISKY suggests sodium and the associated anions are both transported in certain regions of the epithelium, the anion being restricted and not showing a complete transepithelial transport, the different effects produced by different anions depending then on the kinetics of their intraepithelial transport by the system.

CONCLUSIONS

1. When chloride ions are replaced by sulphate or gluconate ions in the normal frog Ringer there is always a sharp decrease in the short circuit current and this effect does not appear to depend on the nonelectrolyte used for compensation of osmolality.

2. In the absence of sodium the short circuit current was practically nonexistent so that the fall in short circuit current in the absence of chloride could not be explained by a simultaneous independent active transport of chloride ions in the outward direction.

3. This is confirmed by the good agreement between sodium fluxes and the short circuit current.

4. When chloride ions were substituted by bromide or iodide a smaller decrease of the short circuit current was observed, when compared with the effect of sulphate or gluconate.

5. These results suggest that at least a part of the active transport of sodium is anion dependent in a nonspecific way.

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