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Ion and Water Balance in Isolated Epithelial Cells of the Abdominal Skin of the Frog *Leptodactylus ocellatus*

Edit A. Zylber, Catalina A. Rotunno and Marcelino Cereijido

Department of Biophysics, CIMAE and Department of Physical Chemistry, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina

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Summary. Isolated epithelial cells were obtained from abdominal skin of the frog *Leptodactylus ocellatus* by a trypsination-dissection method. As estimated by nigrosin staining, the amount of damaged cells is only 6.6 ± 0.7 per cent. When washed briefly after incubation the ionic concentrations in these cells were (mM): K^+ 142.0 ± 4.0; Na^+ 15.8 ± 1.8; and Cl^- 57.2 ± 5.3. If they are not washed, the concentration of K^+ remains essentially the same (131.2 ± 1.4 mM) but the Na^+ concentration is much higher (38.5 ± 0.9 mM). It is shown that a large fraction of Na^+ is contained in a compartment that is freely connected with the bathing solution. Ouabain (10^{-4} M) elicits a marked decrease of K^+, a slight decrease of Cl^-, and an increase of Na^+ content. In an equal period, low temperature (3 °C) produces a similar effect, although less marked than ouabain.

Besides maintaining their own ionic balance, epithelial cells translocate a net amount of sodium from one side to another of the epithelium. In spite of the adequate characterization of this transepithelial transport from the macroscopic point of view, the interpretation of how the cells carry out this process is prevented by: (1) our ignorance of the mechanism used by the cells to achieve and maintain their ionic composition, and (2) the anatomical complexity of the skin (several layers of cells, intercellular space, connective tissue, glands, etc.). The present series of studies, starting with the description of a preparation of isolated epithelial cells of frog skin, is aimed at obtaining information on the distribution of ions and water that could shed light on the process of transepithelial movement.

Materials and Methods

Animals

The source of material was the abdominal skin of the South American frog *Leptodactylus ocellatus* (L.). The animals were kept in tanks at room temperature and had free access to running tap water which was continuously gassed with air. Animals of both sexes, during all seasons were used.

Solutions

Unless otherwise stated, the Ringer's used was *Ringer's A* (also referred to as "control Ringer's"), containing (mM): 115 NaCl, 2.4 $KHCO_3$, 1.0 $CaCl_2$, and 2.0 glucose. Other Ringer's solutions used were: *Ringer's B*[1] which had the same composition as *Ringer's A* plus 2% trypsin, *Ringer's B*[2] with only 0.2% trypsin, *Ringer's C* with the same composition as Ringer's A plus 600 mg per cent of bovine albumin. Other solutions used are described in Results. Once gassed as described below they had a pH of 7.4.

Dissection of the Epithelium and Obtainment of the Cell Suspension

Once the animal was pithed the skin was dissected and put on a piece of cork with the outside facing down. A series of incisions of the corion some 5 cm long and at 2 to 3 mm from each other were made with a stainless steel blade, taking care to avoid cutting the epithelium. A second set of incisions, perpendicular to the first were also made. To obtain isolated cells, it is not important to avoid accidental cuttings of the epithelium. This becomes important when one desires to mount the epithelium as a flat sheet to study fluxes or electrical parameters. These measurements are described in the next paper (Rotunno, Zylber & Cereijido, 1973). This was done in a few cases to test the viability of the epithelia through electrical and isotopic measurements. The skin, outer side down, was placed on a tray over a well containing solution A. The inner side of the skin, facing up, was bathed with solution B[1] as depicted in Fig. 1. After 30 to 45 min of incubation at room temperature the skin was washed in Ringer's A and put on a flat glass with the outside facing up. With the aid of a fine forceps, large pieces of epithelium (more than 0.5 cm^2 and occasionally the whole epithelium) could be easily pulled from the skin. The epithelium covering an area of 15 cm^2 was thus collected. The pieces of some 10 to 20 skins were put in 50 ml of Ringer's B[2] at 37 °C. The flask was stirred with a magnetic bar, and continuously gassed with oxygen or air. After 20 min the stirring was stopped, allowed to sediment for 1 min and the supernatant was separated. This procedure was repeated three times. The supernatant was filtered through four pieces of gauze to retain nondisaggregated pieces of epithelium and spun 10 min at $1,500 \times g$ (International Equipment Co., Mass.). The supernatant was discarded and the cells were resuspended in Ringer's C (final volume: 120 ml) in a 1.0-liter Erlenmeyer flask. The suspension was slowly and continuously stirred with a magnetic bar, and gassed with the compressed air with pure oxygen under the desired experimental conditions. With this procedure the formation of foam was prevented.

Duplicate samples of 5.0 ml of the suspension were collected in a Teflon test tube containing 500 μliters of the same Ringer's used during the incubation to which ^{131}I-albumin or ^{14}C-inulin was added. The two liquids were mixed by sucking and blowing with the same pipette and a sample of 5.0 ml was finally withdrawn and put in a Lucite centrifuge tube essentially similar to the one described by Burg and Orloff (1962) (Fig. 1). In some experiments the markers were present throughout the incubation. The suspension was centrifuged for 3 min at $1,500 \times g$ and the cells collected at the bottom, in the narrow part of the tube. The supernatant was separated; 2 to 3 mm of media remaining in the narrow part of the tube above the pellet were removed with a long needle connected to a suction pump and discarded. This reduces the volume and the amount of water and ions in the trapping space that have to be subtracted from the total values of the pellet to calculate the cellular composition. The screws and the piece of Parafilm at the bottom of the tube were removed and the pellet was blown into a small tared Teflon dish of 0.5 cm in diameter. The sample was immediately weighed in a Mettler microbalance with an error of 2 μg. The rate of desiccation was low enough that it was unnecessary to make corrections to zero time. The method afforded about

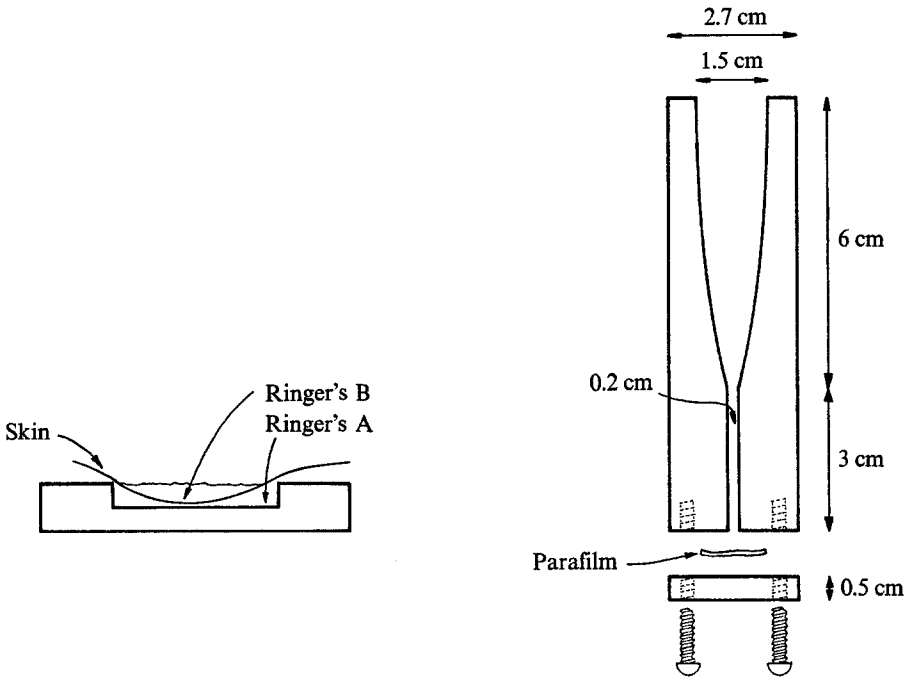

Fig. 1. *Left.* Set up used during trypsination of the skins. After the connective tissue was cut as described in the text the skin is put on a shallow well containing Ringer's A. The inner side is bathed with Ringer's B[1]. *Right.* Schematic representation of the Lucite tube used to collect cells from the suspension

1 mg of cells per cm^2 of epithelium. Samples of the supernatant were diluted for the measurements described below. The samples were dried overnight to constant weight in an oven at 80 °C with a constant circulation of dry air. The dry samples were extracted with 1.0 ml of 0.1 N HNO_3 in a shaker until the pellet was completely disintegrated. Samples were then taken and diluted for analytical determinations.

Nigrosin Staining

Nigrosin staining was used to differentiate live from damaged cells (Kaltenbach, Kaltenbach & Lyous, 1958). The suspension of cells was diluted in a white cell pipette with nigrosin solution (0.5%) as diluent. Cell counting was made in a standard blood counting chamber with Neubauer rulings. Dilutions were made so that at least 50 cells were counted in each of the four large squares of the chamber. Usually the suspension was about 8×10^6 cells/ml^{-1}.

The Spaces of Inulin and Albumin

Distribution spaces were measured by adding 0.1 μC of ^{14}C-inulin, ^{3}H-inulin or 0.3 μC of ^{131}I-albumin per milliliter of Ringer's. The ^{131}I-albumin was previously passed at room temperature through a Sephadex G-25 (Pharmacia Fine Chemicals, N.Y.)

column (100 cm long and 2.5 cm in diameter) pre-equilibrated with Ringer's. Radiochemical purity of the inulin was checked by paper chromatography on Whatman No. 1 using *n*-butanol/ethanol/water (52:18:30) for 12 to 15 hr. Radiochromatograms were read in a 7200 Packard Scanner. The cell suspension was processed as described above. Samples of 250 µliters withdrawn from the bathing solution and from the solution in which the dried cell suspension was extracted were analyzed for ^{14}C or ^{131}I. When ^{14}C-inulin was used the samples were previously neutralized. Samples were collected in liquid scintillation solution (Bray, 1960). They were counted in a three-channel Packard Tri-Carb liquid scintillation counter. Quenching corrections were made by the external standard method. ^{131}I was counted directly in the dryed sample. For this purpose a Nuclear Chicago Automatic Gamma Counter set as a spectrometer in the ^{131}I peak was used.

Sodium and potassium were measured with an EEL flame photometer. Chloride was measured by titration using 1-5-Diphenylcarbazon reagent (Merck) according to the method described by Schales and Schales (1941).

Source of Materials

^{14}C-inulin (N.E.C.164A) and ^{3}H-inulin (N.E.T.-314) having a molecular weight between 5,000 and 5,500 were purchased from the New England Nuclear Corp. ^{131}I-Albumin was obtained from the Atomic Energy Commission of Argentina. Bovine albumin, Fraction V was from Sigma Chemical Co. Trypsin was kindly donated by Roux Ocefa Laboratories of Argentina. Ouabain was from K & K Laboratories, Inc.

Results are given as mean $\pm$ standard error (number of observations).

Results

When trypsin was not of the highest purity, poor preparations were obtained, characterized by relatively low K^+ and high Na^+ content and a large proportion of dead cells. Unsatisfactory results were also obtained in the absence of Ca^{++} and in the presence of EDTA. Under the conditions used, Ca^{++} did not impair the effect of trypsin.

Kaltenbach *et al.* (1958) screened over 50 dyes and found that nigrosin gave the highest contrast between stained and unstained ascites cells, has no evident toxicity, and is a convenient method to differentiate between live cells with unchanged membranes, and cells whose membranes have been modified either experimentally or by death of the cells. We adopted nigrosin staining as one of the tests of viability. By cell counting we found that 6.55 ± 0.74 (6) per cent of cells were stained with nigrosin. This is a comparatively small proportion of damaged cells. The cells in the suspension used appear completely separated from each other.

Table 1 shows the trapped space as determined by the distribution space of albumin and inulin. In two groups of paired observations (p_1 and p_2) inulin gave higher spaces than albumin. This difference led us to explore in

Table 1. Albumin and inulin spaces in pellets of isolated epithelial cells of frog skin

Cell group		% of total water
p_1	^{131}I-Albumin	19.06 ± 3.4 (4)
	^{14}C-Inulin	27.1 ± 2.8 (14)
p_2	^{131}I-Albumin	20.1 ± 1.9 (12)
	^{3}H-Inulin	40.3 ± 3.8 (10)
	^{131}I-Albumin	21.03 ± 0.52 (194)

In p_1 and p_2 the distribution spaces of albumin and inulin were determined simultaneously in the same suspensions of cells. Cells in group p_2 were in contact with the tracer for a longer period than those in group p_1.

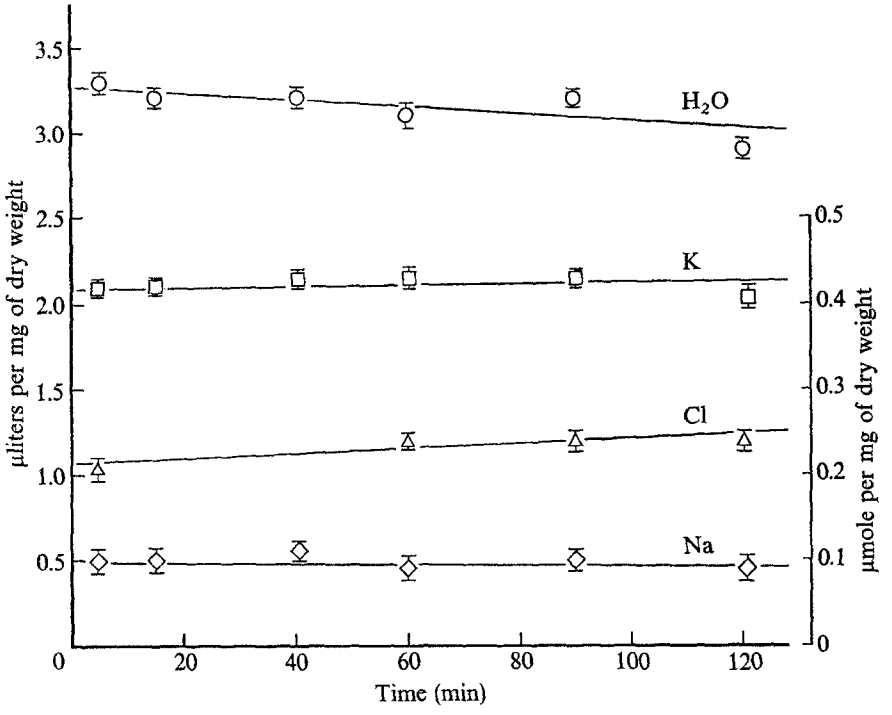

Fig. 2. Water (left ordinate) and ion (right ordinate) content of isolated cells incubated in control Ringer's. (○) water; (□) potassium; (△) chloride; and (◇) sodium. The trapping space was measured with ^{131}I-albumin which was present in the bathing solution throughout the experiment

more detail the nature of the spaces measured by the two markers. The only difference between the two groups is that p_2 was incubated with the markers for a longer period of time.

Fig. 2 shows the cellular content of H_2O, K, Cl and Na as a function of time. In this series of studies ^{131}I-albumin was not added during the sampling

Table 2. Water and ion content of isolated cells using different concentrations of albumin as marker of the trapping space

Albumin	Water (μliter/mg dry wt)	Sodium (mM)	Potassium (mM)
Tracer amount	3.22±0.05	54.3±2.6	130.1±1.4
3 mg/ml	3.38±0.05	59.6±2.6	127.7±1.5
6 mg/ml	3.32±0.06	56.1±4.5	128.6±4.5
9 mg/ml	3.26±0.03	53.6±1.9	128.3±1.9

Table 3. Evaluation of the water and potassium content of isolated cells using inulin as a marker of the trapping space of the pellet

Time	Water	Potassium	
(min)	(μliter/mg dry wt)	Content (μmole/mg dry wt)	Concentration (mM)
0	3.32±0.01	0.462±0.000	139.0±1.0
20	2.89±0.04	0.458±0.000	157.8±1.8
40	2.75±0.02	0.472±0.020	173.3±2.3
120	2.62±0.05	0.463±0.005	175.0±4.2
180	2.54±0.06	0.435±0.018	176.9±3.8

as described in Materials and Methods, but was present in the Ringer's throughout the whole incubation time. This experiment was run both to insure that the quick mixing with albumin during sampling was enough to achieve an adequate distribution, and also to check that, with longer incubation times, albumin does not penetrate the cell compartment to a significative amount as observed with other cells (Giuditta, D'Udine & Pepe, 1971). No difference was observed by using either procedure. Table 2 shows a study where all groups have the same amount of ^{131}I-albumin, but non-radioactive albumin was added in different amounts to compete with possible adsorption of ^{131}I-albumin to the cells. Table 2 shows that the calculated amount of cellular water and the concentrations of Na and K remain constant. This also indicates that there is little or no transfer of ^{131}I from labeled albumin to cellular proteins; otherwise the addition of unlabeled albumin would have competed for ^{131}I adsorption and yielded smaller spaces.

Table 3 shows that the amount of cellular water estimated with inulin at "zero" time (3.32±0.01 μliters/mg dry wt) is similar to the values measured with albumin throughout the experiment (Fig. 2). Since the space one seeks to measure with the markers is the one occupied by the bathing

Ringer's trapped in the pellet, it should be equilibrated even in the sample taken at the initial time. In this respect the similarity of the cellular water initially measured by the two markers suggests that the space subtracted is actually the trapping space.

The amount of cellular water estimated on the basis of the inulin space is not time-independent as in the case of albumin. After the initial distribution in the "trapping space" inulin penetrates at a slower rate into an extra space which is not accessible to albumin. This space though, does not show an indefinite increase with time, but occupies some 23% of the intracellular water as measured with albumin. The nature of this extra space is not obvious. For instance, even if inulin penetrates (and albumin does not) into the damaged cells, their volume (7%) is too small to account for the 23% of extra space occupied by inulin. Of course inulin might enter into a population of damaged cells that are not so altered as to be penetrated by nigrosin, or into a restricted extracellular space which is not accessible to albumin. Yet in these cases the K that would have to be attributed to the nondamaged cells would reach an unlikely high concentration (Table 3). Another possible interpretation of the larger value of the inulin space is that inulin penetrates into a cellular space. Cells of other tissues are known to incorporate inulin. Thus, White and Rolf (1957) report that in liver, the inulin "space" may exceed that total water content. The interpretation that inulin might slowly penetrate into the cells would agree with the fact that even when the amount of K per milligram of dry weight remains constant, its concentration appears to increase (Table 3).

Differences in extracellular spaces due to molecular size were observed in erythrocytes by Maizels and Remington (1959). Thus, large molecules like albumin give apparent values of 1.8 to 2.1%, but those of smaller molecules like inulin give values of about 3%. Differences of this order are too small to explain the difference between the albumin and the inulin spaces observed after 40 to 180 min in the present study. The increase of the inulin space with time might not be due to adsorption of inulin to the surface of the cells as no difference in the water content is seen between determinations made with inulin in tracer amounts and with 1.5 and 3.0 mg of inulin per milliliter (Table 4).

In view of the results described above ^{131}I-albumin was adopted as a marker of the trapping space of the pellet. Its mean value calculated with albumin consistently was 21.03 ± 0.52 (194) per cent of the total water content. This value is relatively low compared with 44.7% (^{14}C-inulin space) found by Macknight, Di Bona, Leaf and Civan (1971), 45 to 62% (^{14}C-inulin) and 45 to 63% (^{14}C-sucrose) found by Gatzy and Berndt (1968) with cells

Table 4. Water and ion content of isolated cells using different concentrations of inulin as marker of the trapping space (120 min of incubation)

Inulin	Water (μliter/mg dry wt)
Tracer amount	2.62 ± 0.05
1.5 mg/ml	2.72 ± 0.13
3.0 mg/ml	2.67 ± 0.08

Table 5. Water and ion content at different times of centrifugation

Centrif. time (min)	H_2O (μliter/mg dry wt)	Na^+	K^+ (μmole/mg dry wt)	Cl^-
3	3.60 ± 0.04	0.134 ± 0.005	0.487 ± 0.000	0.286 ± 0.017
10	3.61 ± 0.08	0.136 ± 0.010	0.490 ± 0.005	0.268 ± 0.005
20	3.44 ± 0.04	0.147 ± 0.000	0.468 ± 0.000	0.294 ± 0.016
30	3.38 ± 0.05	0.140 ± 0.013	0.470 ± 0.007	0.256 ± 0.025

from urinary bladder, and compared also with 81.8 ± 3.7% (^{3}H-manitol) found by Reiser and Christiansen (1971) in cells isolated from rat intestine. The small size of the trapped space of the present preparation may be due to the fact that the cells are completely separated and there is no rest of the intercellular space of the epithelium. Obtaining a small trapped space at low centrifugal force is important in cases such as the distribution of Na ions because one has to subtract a relatively small amount of Na to determine with accuracy the intracellular content.

As mentioned above, the technique used involves 3 min of centrifugation. For technical reasons we could not cut this time shorter. The cells might, in principle, change their concentration during centrifugation. The effect of the centrifugation time was checked in the experiments illustrated in Table 5 where longer times were used. Centrifugation does not seem to disturb the water and ionic composition of the cells. Another potential source of error was that trypsin, besides freeing the epithelial cells, might also alter their permeability. To check this point an experiment was run in which the cells, after isolation, were left 120 min at 37 °C in Ringer's B[2] (0.2% trypsin). Table 6 shows their composition at the end of this period. The contents are slightly lower than those of previous tables, but the concentrations do not differ from the values obtained in control Ringer's (Tables 3 to 5). This indicates that the trypsin used does not alter the ionic balance of the cells. This is in keeping with the observations of Snow and Allen (1970) in tissue

Table 6. Effect of trypsin on isolated epithelial cells

H_2O	3.13 ± 0.06 (4)	μliter/mg dry weight	mM
K^+	0.42 ± 0.00 (4)	μmole/mg dry weight	132
Na^+	0.12 ± 0.00 (4)	μmole/mg dry weight	41
Cl^-	0.26 ± 0.00 (4)	μmole/mg dry weight	83

Table 7. Water and ion content of isolated epithelial cells of frog skin

Water	CR	3.32 ± 0.03 (115)	μliter/mg dry weight
Water	WS	2.80 ± 0.06 (16)	μliter/mg dry weight
Water	WCH	3.32 ± 0.09 (14)	μliter/mg dry weight
Potassium	CR	0.443 ± 0.003 (119)	μmole/mg dry weight
Potassium	WS	0.354 ± 0.015 (20)	μmole/mg dry weight
Potassium	WCH	0.443 ± 0.015 (14)	μmole/mg dry weight
Sodium	CR	0.123 ± 0.004 (105)	μmole/mg dry weight
Sodium	WS	0.061 ± 0.004 (20)	μmole/mg dry weight
Sodium	WCH	0.049 ± 0.004 (14)	μmole/mg dry weight
Chloride	CR	0.26 ± 0.01 (55)	μmole/mg dry weight
Chloride	WS	0.15 ± 0.02 (12)	μmole/mg dry weight
Chloride	WCH	0.26 ± 0.02 (14)	μmole/mg dry weight

CR: incubated in control Ringer's. Washed with ice cold isotonic solution of sucrose (WS) and choline (WCH).

culture of cells from baby hamster indicating that when crystalline trypsin is used, 99% of the cells are impermeable to trypan blue but, when crude extracts of trypsin are used the cells are damaged and lose DNA and RNA. Trypsin itself does not cause cell death (Weiss, 1958) and since it acts best on denatured proteins, can be used to rid cell populations of damaged cells (De Luca, 1965).

Finally, Table 7 summarizes the water and ion content obtained under control conditions at several times during the 2 hr of incubation. The cells have 3.32 μliters of water per mg of dry weight (77% of the cell). A similar value was found by Macknight *et al.* (1971) in cells from the urinary bladder (3.31 μliters). Gatzy and Berndt (1968) report a somewhat higher water content (86%). The cells have a high K^+ (0.443 μmole per mg) and a low Na^+ (0.123 μmole per mg) content which amounts to 0.566 μmole per mg, i.e., well in excess of Cl^- ions (0.26 μmole per mg) which is the main anion present in the bathing solutions. Table 8 shows the ion concentrations calculated with the values of ion content in cells bathed in control Ringer's (Table 7: CR).

Table 8. Concentration of ions in isolated epithelial cells of the frog skin

Ion	Concentration (mM)
K^+	131.2 ± 1.4 (82)
Na^+	38.5 ± 0.9 (105)
Cl^-	81.2 ± 3.0 (55)

If chloride is distributed passively, the electrical potential difference between the cell and the Ringer's ($\Delta\psi$) is given by

$$\Delta\psi = -\frac{RT}{F} \ln \frac{[Cl]_{Ringer}}{[Cl]_{cell}}. \quad (1)$$

In this calculation we assume that the fact that a fraction of the Cl^- influx across the entire skin is actively transported (Zadunaisky & Candia, 1962; Zadunaisky, Candia & Chiarandini, 1963) does not disturb the passive distribution of Cl^- between the cells and the inner bathing solution. The concentration of chloride in the cells is 81.2 (Table 8). Eq. (1) gives

$$\Delta\psi = -58 \log \frac{117}{81.2} = -9.2 \text{ mV}. \quad (2)$$

This electrical potential difference is considerably lower than the one recorded with glass microelectrodes in the cells of the undissociated epithelium by Cereijido and Curran (1965): 18.2 ± 1.7 mV. However, the concentrations listed in Table 8 might not reflect the actual concentration in the normal cells. This stems from the fact that by briefly washing the cells (1 min of resuspension of the pellet, plus 3 to 4 min of centrifugation) they lose a considerable amount of their ion content (Table 7). Washing with an ice cold isotonic solution of choline does not modify the water, potassium and chloride content, but it significantly ($p < 0.001$) lowers the Na content. Washing with an isotonic solution of sucrose near 0 °C, however, decreases the content of water (by 0.52 μliter) and of the three ions (K by 0.089; Na by 0.062; Cl by 0.11 μmole/mg dry weight). It may also induce the loss of some other anion since the sum of lost K^+ plus Na^+ is 0.151 μmole/mg, but the loss of Cl^- is only 0.11 μmole/mg. The osmolarity of the solution lost is $0.302/0.52 = 581$ mOsm; i.e., more than twice the osmolarity of the sucrose solution used (which, in turn is isotonic with the Ringer's used during the incubation). Fig. 3 shows the time course of cell suspensions incubated in Ringer's where NaCl was replaced by an equivalent amount

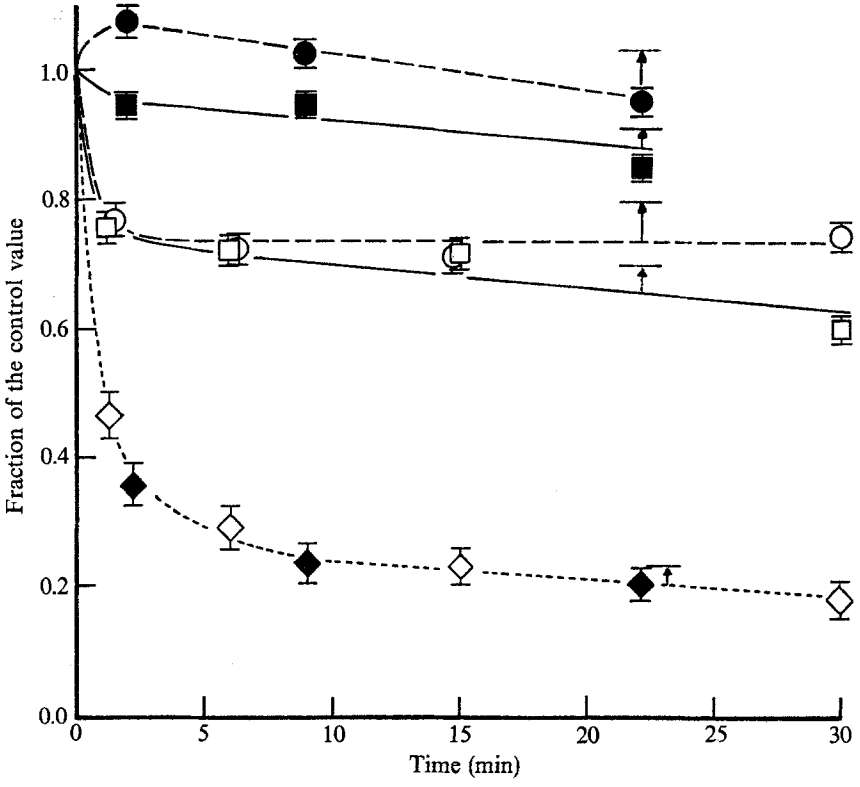

Fig. 3. Water and ion content of epithelial cells incubated in Ringer's in which all the NaCl was replaced with choline (full symbols) or sucrose (open symbols). Symbols have the same meaning as in Fig. 2. The temperature was 2 °C. The values are expressed as fraction of the water and ion content in control Ringer's at room temperature. The arrows point the correction for the ion and water contained in the nigrosin-stained cells assuming that they have the same composition as the control Ringer's

of sucrose (open symbols) and choline chloride (closed symbols) at 2 °C. The only difference between these solutions and the ones regularly used to wash the cell samples in the procedure described in Materials and Methods, is that in the present case they also have 2.4 mM KCO_3H, 1.0 mM $CaCl_2$ and 2.0 mM glucose; i.e., they are Ringer's. The curves consist mainly of two parts: a fast one and a slow one. The slow parts of the different curves have essentially the same slope (except the curve of water in sucrose Ringer's). The slow component might be attributed to the effect of cold, and can be easily extrapolated at zero time to obtain the values of ion and water content. The cells lose more K in cold sucrose Ringer's than in cold choline Ringer's. In sucrose Ringer's water follows essentially the same pattern as K. As expected on the basis of Table 7 there is a fast initial loss of Na.

Table 9. Ion concentrations in isolated epithelial cells washed after incubation

Washing solution	K (mM)	Na (mM)	Cl (mM)
Sucrose	135.5±3.3 (16)	23.3±1.5 (20)	57.2±5.3 (11)
Choline chloride	142.0±4.0 (14)	15.8±1.8 (14)	83.5±4.7 (14)

Although the amount of Na washed with choline is generally somewhat larger than the amount washed with sucrose, in the study of Fig. 3 both solutions washed the cells to the same extent and with the same time course. As judged by the nigrosin method the cells in the studies of Fig. 3 had 7% damaged cells. The arrows at 25 min in Fig. 3 indicate the shift in the values of the measured parameters if one corrects for the ions and water contained in the 7% of damaged cells. This correction assumes that albumin does not penetrate into dead cells. It is evident that, even when damaged cells could contribute to the variations of Na content observed in Fig. 3, they could not, by themselves, account for the Na lost. Therefore, the possibility exists that these cells have a Na compartment which can be easily washed. The possibility also exists that this compartment is not inside the cytoplasm. Farquhar and Palade (1965) have pointed out that in the epithelium the cells are surrounded by a material having the characteristics of an ion exchanger. If the cells used in the present preparation carry part of an amphoteric exchanger attached to their surface, it may conceivably show the behavior found in Fig. 3. If one calculates the ion concentration on the basis of the ion content of washed cells (Table 7) and corrects also for the 7% of damaged cells, one obtains the values listed in Table 9. The comparison of concentrations in Tables 8 and 9 shows that the concentration of K is changed slightly (131.2 *vs.* 135.5 and 143.0 mM). The concentration of Na^+ is the one reduced most markedly by the washing procedure (38.5 *vs.* 23.3 mM in sucrose) indicating that the washed space must contain a high proportion of this ion. The observation that choline lowers still further the concentration of Na^+ (23.3 *vs.* 15.8 mM) suggests that part of the washed Na^+ could have been adsorbed to some exchanger.

The washing with choline chloride does not vary appreciably the concentration of Cl^- (81.2 *vs.* 83.5 mM). However, the washing with sucrose reduces the concentration to 57.2 mM. If one considers the concentrations in the washed cells and uses 57.2 mM as the concentration of Cl^-, Eq. (1) gives an electrical potential difference of -18 mV between the cell and the bathing Ringer's. This value is in keeping with the value obtained by

Table 10. Composition of the moulting

	Content		Concentration (mM)
H_2O	4.40	μliter/mg dry weight	
K	0.34	μmole/mg dry weight	78
Na	0.38	μmole/mg dry weight	87
Cl	0.73	μmole/mg dry weight	166

Cereijido and Curran (1965) using microelectrodes: −18.2 mV. The agreement between these figures suggests that the fact that the skin of the frog used may exhibit an active transport of Cl^- of about 15% of the Na flux, does not displace cell chloride from passive equilibrium. Cereijido and Curran (1965) have shown that Na transport across also may vary in an ample range without disturbing the intracellular electrical potential.

The origin of the 6 to 7% of damaged cells in our preparation might not be entirely ascribed to the isolation procedure. The normal epithelia *in vivo* also possess a certain proportion of cells undergoing a process of descamation or moulting in which their composition changes. Table 10, for instance, shows the ionic composition of the cells in the moulting layers, and it is evident that they have more water, Na^+ and Cl^-, and less K than normal cells. It may be noticed that in these cells the amount of Cl^- contained is roughly equal to the sum of Na^+ plus K^+. This is not observed in control cells.

Effect of Ouabain

Ouabain inhibits Na transport in frog skin by acting on the Na pump (Koefoed-Johnsen, 1957; Zadunaisky *et al.*, 1963; Curran & Cereijido, 1965). Fig. 4 shows the effect of 10^{-4} M ouabain on the ionic balance of the cells. Water content decreases slightly after 110 min. Sodium increases and potassium shows a marked decrease that exceeds the gain of Na^+. The chloride content is reduced. The variations in Na and K content are similar to those obtained in the isolated epithelium of the frog skin (Aceves & Erlij, 1971), in the toad bladder (Herrera, 1968) or in the isolated cells of toad bladder (Macknight *et al.*, 1971). Since ouabain is regarded as a specific inhibitor of a pumping mechanism involving an ATPase system, the results in this section indicate that the ionic distribution of the epithelial cells depend to a great extent on an ATPase system, and that the technique used to separate the cells has not damaged such mechanism.

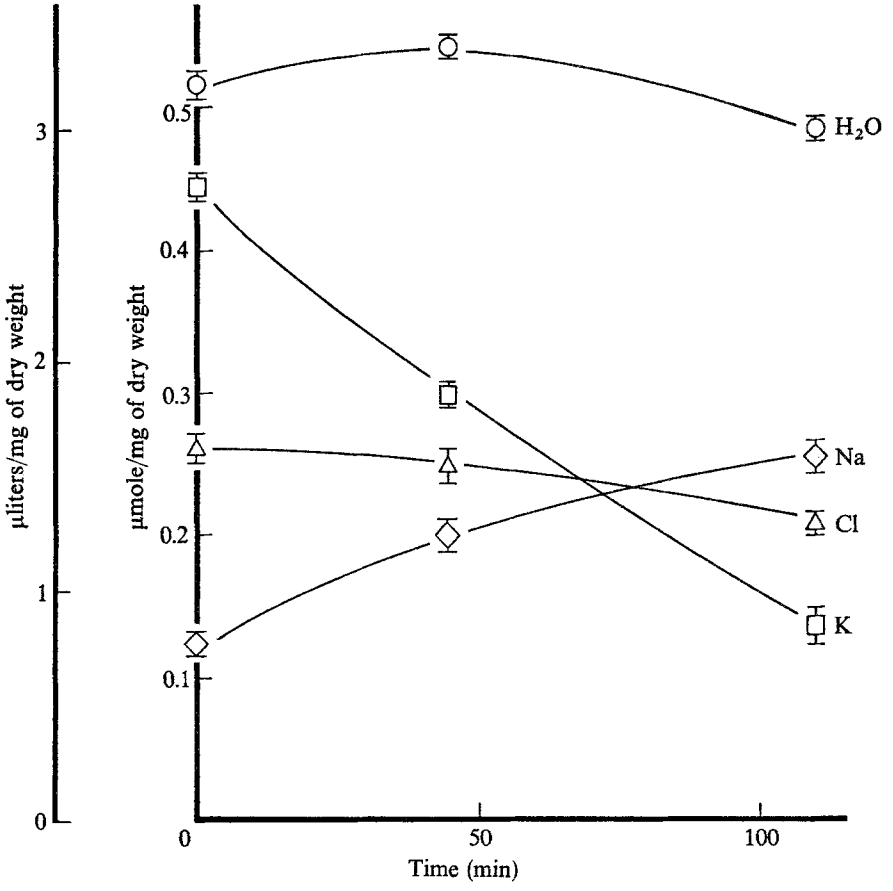

Fig. 4. Effect of ouabain 10^{-4} M on the water and ion balance of isolated epithelial cells. Ouabain was added at zero time. Symbols have the same meaning as in Fig. 2

Effect of Low Temperature (3 °C)

The results on ouabain shown above may be taken to indicate that the ionic distribution depends on the metabolism of the cells through the supply of ATP. Therefore, the incubation of the cells at low temperature is expected to produce a similar effect to that of ouabain. Fig. 5 shows that, in fact, cold decreases K content and increases Na content. The effect on K though, is less marked than in the case of ouabain. This could be due to different degrees of inhibition elicited by the two factors, or else to a decrease of K permeability produced by cold. The evolution of the Na and Cl content in the second part of the experiment may be due to penetration of NaCl.

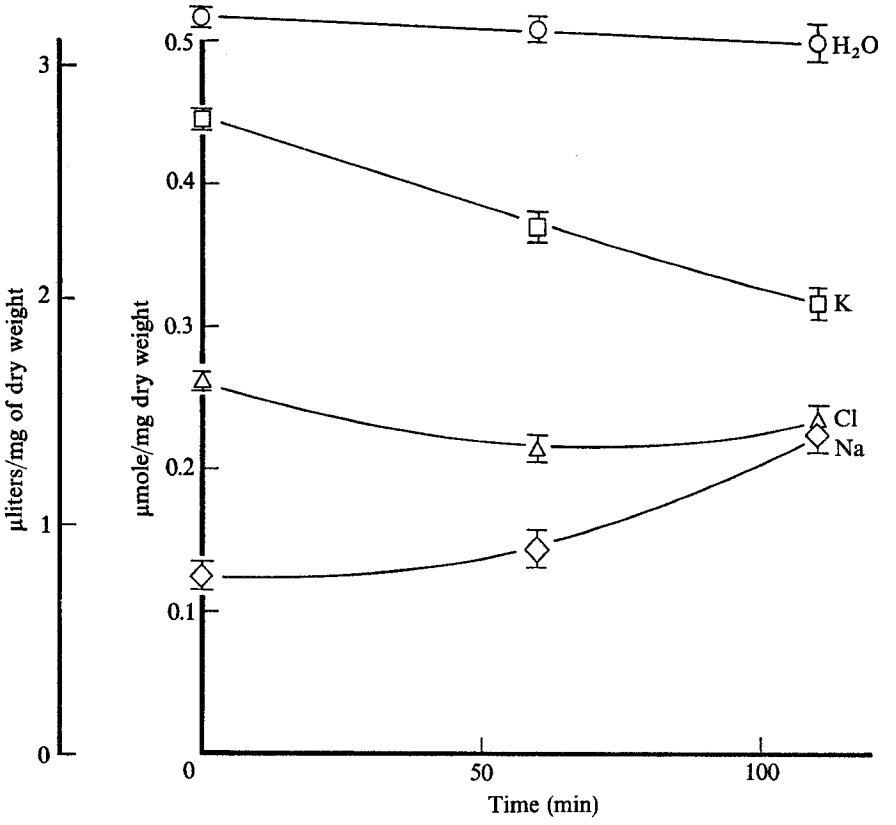

Fig. 5. Water and ion content of isolated epithelial cells incubated at 3 °C. Symbols have the same meaning as in Fig. 2

Discussion

As mentioned in the Introduction, the purpose of the present work is to obtain a preparation of isolated epithelial cells that would permit studies of the mechanisms maintaining their ionic balance, and to use the information to obtain further insight in the process of ion transport across the epithelium.

On the basis of the different tests performed the cells exhibit acceptable levels of K, Na and Cl concentration, and maintain them for a period of at least 2 or 3 hr. The evaluation of the trapping space is reliable, and the relatively small portion of the total volume that it occupies ($21.03 \pm 0.52\%$, 194 determinations) has the advantage of requiring the subtraction of small quantities of ions and water to calculate the intracellular composition. When the cells are quickly washed after incubation they lose water and

ions. We failed to correlate the change in composition with the small population of damaged cells (Fig. 3). It may be noticed, for instance, that although washing with choline chloride solution leads to a considerable drop in Na content [from 0.123 ± 0.004 (105) to $0.049 \pm 0{,}004$ (14) μmole/mg dry weight] it does not disturb the high K content of the cells [0.443 ± 0.003 (119) *vs.* 0.443 ± 0.015 (14) μmole/mg dry weight]. If one assumes that the fraction of damaged cells is much larger than estimated by the nigrosin method, so that it could accommodate the amount of "washable sodium" at the concentration of the Ringer's, then the amount of cellular water would be so reduced that the K concentration would appear as having improbably high values. On the other hand, if the volume of the cytoplasmatic compartment is calculated simply as the total water minus the water in the trapping space, then the concentration of K found in the cells will be 131.2 ± 1.4 (82) mM (Table 8), 135.5 ± 3.3 (16) and 142.0 ± 4.0 (14) mM (Table 9). The satisfactory state of the cells is not only revealed by the distribution and constancy of the ionic level, but also by their response to factors known to disturb membrane processes associated to metabolism such as ouabain and cold.

All but the outermost cell layer of the epithelium are surrounded by an intercellular space which is closed at the outer border. Even the outermost cell layer has most of its surface in contact with the intercellular space. The cellular borders in contact with this intercellular space are thought to constitute what is functionally known as the "inner facing barrier." Therefore, the population of isolated cells studied in the present work are mainly surrounded by "inner facing barrier." One of the properties assigned to this barrier is to possess a Na and a K pump (or perhaps a Na-K pump) and to have a low Na and a high K permeability. The kigh K and low Na content of the population of cells studied, as well as their response to ouabain would agree with such view. In this respect, if the fractions of washable ions (Na^+ and Cl^-) and the extra space of inulin belong to intracellular compartments it would mean that the treatment with trypsin has the peculiarity of making the cells (at least some of them) very permeable to Na^+, Cl^- and inulin without disturbing the permeability to K^+. Should this be the case, trypsin would remove the factor that confers to the "inner facing barrier," its low Na permeability (as compared to the "outer facing barrier").

A more remote possibility is the following: the cells coming from the outer layer must have a fraction of their surface constituted by "outer facing barrier." Since this barrier is assumed to have a high and passive Na permeability, those cells in our preparation which come from the outer

layer may, in principle, constitute the cellular compartment where washable Na^+ and Cl^- come from.

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