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Ion Activities in the Lateral Intercellular Spaces of Gallbladder Epithelium Transporting at Low External Osmolarities

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Summary. The ion activities in the lateral spaces of the unilateral preparation of the gallbladder of *Rana catesbiana* were measured by double-barrelled ion-selective microelectrodes. The bladders were bathed in a saline solution with a low osmolarity (62 mOsm) containing, in mM: 27 Na^+, 27 Cl^-, 2 K^+, 1 Ca^{++}, 4 HCO_3^-. Working at reduced osmolarities had the advantage of an increased volume transport and of widened intercellular spaces. The reference barrel recorded an electrical potential of +2.7 mV in the spaces; they contained a solution similar to the external solution. The electrodes recorded a Na^+ concentration of 27 mM, a K^+ concentration of 1.7 mM, a Ca^{++} concentration of 0.69 mM and a Cl^- concentration of 28.5 mM. In the spaces there was a lower resistance between the tip of the electrode and the serosal bath than that recorded with the tip in the lumen, and injection of fluorescent dye (11 Å diameter) via the electrodes did not stain the cells. The concentrations in the secretion were similar to those in the spaces. The intracellular compartment had an apparent K^+ concentration of 95 mM, and the concentrations of Na^+ and Cl^- were both about 5 mM. These data indicate that when the gallbladder is bathed with hypotonic solutions and is transporting fluid at approximately three or four times the normal rate, there are no significant osmotic gradients between the lumen and the lateral spaces. It is suggested that transcellular transport of water is implemented by a combination of high osmotic permeabilities across both mucosal and serosal cell membranes and low reflection coefficients (for K^+ salts) at the serosal cell membranes.

Key Words ion-selective microelectrodes · lateral intercellular spaces · osmolarity · water transport · epithelia · cellular osmolarity

Introduction

The theory for transcellular water transport across leaky epithelia is based on three assumptions: i) cell cytoplasm has an osmolarity which is higher than the osmolarity of the luminal solution causing influx of water into the cell; ii) solution in the lateral intercellular spaces has an osmolarity which is higher than the solution in the cell causing flux of water into the spaces; iii) hydrostatic pressure of a few cm of water causes the solution in the spaces to flow into the serosal compartment.

The purpose of this paper was to estimate the concentrations of Na^+, K^+ and Cl^- in the lateral intercellular spaces and thereby to estimate the osmolarity in this compartment. Similarly, information was gathered on the osmolarity of the cellular compartment.

The ion concentrations were measured by ion-selective microelectrodes in gallbladders of the bullfrog. These gallbladders were adapted to transport solutions of low osmolarities (62 mOsm). This had several advantages: i) The rate of transepithelial transport was higher than at normal osmolarities (210 mOsm). ii) The lateral spaces were wider. iii) The ion-selective microelectrodes are more sensitive at low ionic strengths.

The present measurements confirm the expectation that the cell is hyperosmolar to the luminal solution. Thus water can move from the lumen into the cell by simple osmosis. The solution in the lateral intercellular spaces, however, had the same osmolarity, within a few mOsm, as the luminal solution. This suggests that *simple* osmosis across both the mucosal and the serosal cell membrane is not a general mechanism for transepithelial transport of water unless the reflection coefficient for some ion or salt across the serosal membrane is low.

Several fresh-water organisms, like the hydra and some amoebas, appear to have osmotic gradients across their cell membranes similar to the epithelial cell adapted to transport at low osmolarities in this study. Perhaps all these cells are in an osmotic steady state because the membranes have a low reflection coefficient for K^+ ions or a K^+ salt.

Materials and Methods

Mounting of the Tissue and Solutions

Mature, healthy and feeding bullfrogs (*Rana catesbiana*, supplied from Nasco) were decapitated and pithed. The gallbladder was removed within 3 min and the exterior rinsed in saline (in mM): Na^+ 115, K^+ 2, Ca^{++} 1, Mg^{++} 1, HCO_3^- 25, Cl^-

94, SO_4^{--} 1, saturated with 5% CO_2, 95% O_2, pH 7.4, 210 mOsm. The gallbladder, still containing bile, was fastened with tissue-glue (isobutyl 2-cyanoacrylat monomer, Ethicon) onto a Plexiglas plate (1 mm thick) over a round hole of a diameter of 6 mm. The gallbladder was then opened and rinsed on both sides with normal saline and excess tissue was removed. Openings, 1 mm long, separated by 100 μm, were cut in the mesothelium by means of a hypodermic needle. Thus a flat layer of epithelial cells was obtained, with the same degree of stretch as found in the intact animal, and in which the hydrostatic resistance of the mesothelium had been reduced (Bundgaard & Zeuthen 1982). The tissue was then immersed for 30 min in a saline solution where the osmolarity had been reduced to 115 mOsm by removing NaCl from the saline described above. Finally the tissue was placed for 30 min in (in mM) Na^+ 27, K^+ 2, Ca^{++} 1, Mg^{++} 1, HCO_3^- 4, Cl^- 27, SO_4^{--} 1, saturated with 1% CO_2, 99% O_2, pH 7.4, 62 mOsm. This saline solution was used for all the experiments.

After adaptation to low osmolarities the tissue was placed in chamber for microscopic observation (previously described, Zeuthen, 1982). The tissue was placed at an angle of 20° from the horizontal plane of a 40× water immersion lens combined with Nomarski optics. The microscope was also equipped with an epifluorescence unit. All microscopic equipment was from Zeiss Optical Co. (Nomarski condenser no. 465273, objective no. 5180741, epifluorescence no. 488032-9902).

Transepithelial currents could be applied and the fractional resistance recorded. This resistance was defined as the voltage evoked between the tip of the electrode and the serosal bath, divided by the voltage evoked across the whole tissue. Only the mucosal side of the tissue was perfused. The serosal surface was bathed in its own secretion exposed to air saturated with water vapor. This is the so-called sweating or unilateral preparation. The secreted solution could be collected from the serosal side for weighing and analysis as previously described (Zeuthen, 1982); samples of solution were held under oil and probed with microelectrodes.

Microelectrodes

All microelectrodes were double-barrelled. In order to avoid contamination, the reference barrels of the microelectrodes were filled with the bath solutions given above; they had impedances of 10^9 to 10^{10} Ω. However, the intracellular electrical potentials were obtained in separate experiments in which the reference barrels were filled with 2 M KCl.

The other barrel was filled with either a fluorescent dye when the lateral spaces were stained, or with liquid ionexchanger when ion activities were recorded. The electrodes were similar in terms of glass type, dimensions and tip diameters as those used in a recent publication (Zeuthen, 1980; *see also* Zeuthen, Hiam & Silver, 1974; and Zeuthen, 1982). These electrodes have tip diameters of approximately 0.3 μm and the two barrels had resistances of 40 to 200 MΩ when filled with 2 M KCl and measured in physiological salines.

Electrodes for Staining

Fluorescein-sodium (Uranin, $C_{20}H_{10}O_5Na_2$) has a molecular weight of 376 and a diameter of 11 Å. The electrodes were filled with a 1% solution. Negative current pulses of up to 10 nA lasting 0.3 sec were passed every second through the electrode. The current carried the fluorescein dye from the tip of the electrode. The ejected dye was excited by blue light from the epifluorescence unit (450 to 490 nm) and analyzed via a yellow filter (510 to 520 nm).

Table 1.

Electromotive forces (corrected electrode potential) measured in the lateral spaces[a]

E_{LIS} [mV]	E_{Na^+} [mV]	E_{K^+} [mV]	$E_{Ca^{++}}$ [mV]	E_{Cl^-} [mV]
2.7±0.5	0.0±0.7	−4.7±0.8	−4.8±0.5	+1.48±0.5
$P<0.01$	N.S.	$P<0.01$	$P<0.01$	$P<0.02$

Concentrations[b] in the lateral spaces

	C_{Na^+} [mM]	C_{K^+} [mM]	$C_{Ca^{++}}$ [mM]	C_{Cl^-} [mM]
	27.±0.68	1.66±0.06	0.69±0.03	28.6±0.67
	N.S.	$P<0.01$	$P<0.01$	$P<0.05$
(35,8)	(23,5)	(14,4)	(18,4)	(16,4)[c]

[a] Relative to lumen (Na^+ 27 mM, K^+ 2 mM, Ca^{++} 1 mM, Cl^- 27 mM), corrected for Ca^{++} influence; $E_{Na^+,Ca^{++}} = -2.0$ mV ±0.12 ($n=29$): $E_{Na^+,true} = E_{Na^+,measured} - E_{Na^+,Ca^{++}} = (-2.0\text{ mV}\pm0.7) - (2.0\text{ mV}\pm0.12) = 0.0\text{ mV}\pm0.7$.

[b] These are apparent concentrations which equal the true concentrations if the activity coefficients are the same in the mucosal solution (about 0.86, Robinson & Stokes, 1959) as in the spaces.

[c] The brackets are number of observations and number of animals. Statistics (*t*-test) apply for both the electromotive forces and the concentrations in comparison with luminal values.

The Ion-Selective Electrodes

These electrodes had properties which have been described previously (Zeuthen et al., 1974; Zeuthen, 1980; Hansen & Zeuthen, 1981; Zeuthen, 1982). The K^+ ion exchanger was Corning 477317, the Na^+ exchanger was the one described by Güggi, Oehme, Pretsch and Simon (1976) supplied by Science Trading, to which 3% vol/vol of K^+ exchanger was added. This decreased the response time to about 1 sec, without destroying the selectivity to K^+ which was 0.01. (For details, *see* Bindslev & Hansen, 1981.) The Ca^{++} exchanger was a generous gift to the laboratory from Prof. W.Simon. The Cl^- exchanger was Corning 477315. The electrodes had sensitivities and selectivities described previously. The Ca^{++} electrodes had a sensitivity of 29 mV for a 10-fold change in the Ca^{++} concentration within the range of Ca^{++} concentration used.

All electrode measurements were corrected for non-Nernstian behavior and influence from other ions. The resulting potentials, the electromotive force, are presented in Tables 1 and 2. Potential measurements are translated into apparent concentrations which equals the true concentration if the activity coefficient at the point of measurement is the same as in the test solutions, about 0.86 (Robinson & Stokes, 1959). All values are mean ±SEM.[1]

[1] According to Waddell and Bates (1969) it may be incorrect to present data as activity (mM) ±standard error of mean. They argue that the recorded potential, which approximates the electromotive force, might have a normal distribution and only relates to the activity via a logarithmic transformation; the derived activities might not therefore be normally distributed. I have circumvented this problem by presenting statistics and significances both for the corrected electrode potential (the electromotive forces) and for the derived concentrations. The differences between the two evaluations are small and have little or no influence on the conclusions.

Table 2.

Electromotive forces (corrected electrode potential) measured in the secreted solution[a]

E_s	E_{Na^+} [mV]	E_{K^+} [mV]	$E_{Ca^{++}}$ [mV]	E_{Cl^-} [mV]
3.7±0.4	1.2±2.1	−12.8±1.4	−16.5±2.1	1.8±0.3
$P<0.01$	N.S.	$P<0.01$	$P<0.01$	$P<0.01$

Concentrations in the secreted solution

	C_{Na^+} [mM]	C_{K^+} [mM]	$C_{Ca^{++}}$ [mM]	C_{Cl^-} [mM]
	28.3±2.5	1.24±0.06	0.33±0.03	29.0±1.4
	N.S.	$P<0.01$	0<0.01	N.S.
(15,3)	(33,4)	(17,4)	(20,4)	(4,3)

[a] Relative to lumen (Na^+ 27 mM, K^+ 2 mM, Ca^{++} 1 mM, Cl^- 27 mM) corrected for Ca^{++} influence; $E_{Na^+,Ca^{++}} = -14.3$ mV±0.8 (5); *see* text in Table 1.

Correction for the Tip-Potential Dependence on NaCl-Concentrations

Any hyperosmolarity in the lateral spaces is most likely made up by additional NaCl. The reference barrels, filled with the low osmolarity test solution, were sensitive to changes in external concentrations. For example, the electrodes recorded a potential which was 17.9 mV more positive if the external NaCl-concentration was increased 10-fold; thus an erroneous reference potential would be recorded in the lateral spaces if the NaCl-concentration (osmolarity) in these spaces was different from the NaCl-concentration (osmolarity) of the luminal solution.

Due to simultaneous measurement with the ion-selective electrodes and the reference barrel, any influences from difference in NaCl concentration could be assessed:

$$E_{ref} = E_{LIS} + 17.9 \text{ mV} \log \frac{NaCl_{LIS}}{NaCl_{lumen}} \quad (1)$$

$$E_{Na} = E_{LIS} + 58 \text{ mV} \log \frac{Na^+_{LIS}}{Na^+_{lumen}} \quad (2)$$

or if the Cl^- concentration is proportional to the Na^+ concentration:

$$E_{Na} = E_{LIS} + 58 \text{ mV} \log \frac{NaCl_{LIS}}{NaCl_{lumen}}. \quad (2a)$$

E_{ref} is the potential recorded by the reference barrel, E_{Na^+} the potential recorded by the Na^+-sensitive barrel (corrected for non-Nernstian behavior). E_{LIS} is the true potential in the lateral intercellular space. Equations (1) and (2a) are equations with two unknowns which can be solved algebraically. As it turns out (*see* Table 1 and p. 117) the correction, the second term in Eq. (1), is of the order of only 1 mV for all ions.

Correction for Ca^{++} During Na^+ Reading

The Na^+ ion exchanger is sensitive to Ca^{++} (Bindslev & Hansen, 1981): less Ca^{++} will give a reading which can be misinterpreted as there being less Na^+. The Na^+ exchanger was tested in salines similar to the test solution (62 mOsm) but in which the Ca^{++} concentration, $[Ca^{++}]$, was varied from 1 to 0 mM

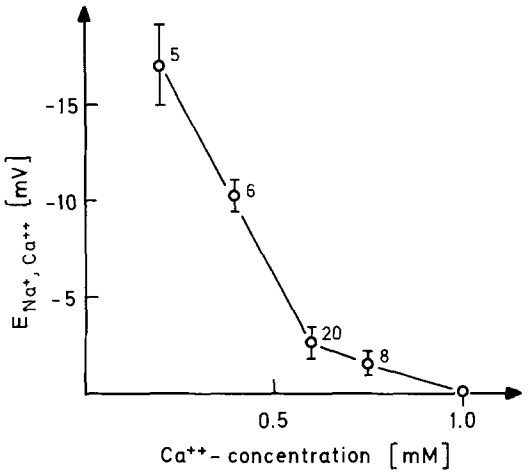

Fig. 1. Change in potential of a Na^+-sensitive electrode when Ca^{++} concentration is varied in the medium. The solution contained (in mM): Na^+ 27, K^+ 2, Mg^{++} 1, Cl^- 27, SO_4^{--} 1, HCO_3^- 4, but Ca^{++} was varied from 1 mM (equal to the test solution) to 0 mM ±SE. For example, in the lateral spaces, where the Ca^{++} concentration was 0.69 mM (*see* Table 1) the reading of the Na^+ electrode will be about 2 mV lower compared to the case where the Ca^{++} concentration was the same in the lumen and in the spaces, 1 mM

(Fig. 1). In the range 1.0 to 0.6 mM the change $E_{Na^+,Ca^{++}}$ was linear and could be expressed as

$$E_{Na^+,Ca^{++}} = \alpha(1.0 \text{ mM} - [Ca^{++}]) \quad (3)$$

where $\alpha = -6.52$ mV $mM^{-1} \pm 0.38$ ($n=29$). In the range 0.4 to 0.2 mM the dependence was best described by

$$E_{Na^+,Ca^{++}} = 33.3 \text{ mV } mM^{-1} \times [Ca^{++}] - 23.7 \text{ mV} \quad (4)$$

where the correlation is described by $r^2 = 0.85$. The true Na^+ potential (or electromotive force), $E_{Na^+,true}$ was obtained as the difference of the measured electrode potential E_{Na^+} and $E_{Na^+,Ca^{++}}$, the latter being determined by the actual Ca^{++} concentration (*see* Fig. 1, and Eqs. 3 & 4).

Positioning

The microelectrodes were positioned by means of a Huxley-micromanipulator (Huxley, 1961) equipped with stepping motors. The electrodes could be moved in steps of 0.2 μm in three perpendicular directions by remote control. The position of the tip of the electrode was recorded digitally. Outside the tissue the tip of the electrode was visible when viewed via the Nomarski optics (Fig. 1*A*). With the plane of the tissue at an angle of 20° with the focal plane lateral spaces were brought into focus. Stretches of lateral spaces two to four cell diameters in length and with a direction which matched the forward direction of movement of the electrode were chosen. As the electrode moved in the focal plane, the tip could be guided into the spaces. In about 5% of cases the advance of the electrode resulted in a valid penetration of the lateral intercellular space.

The criteria for a valid recording were: (i) The coordinates of the chosen lateral space coincided with the coordinates of the tip of the electrode. (ii) Injection of dye via the electrode caused no staining of the cell cytoplasm. (iii) The electrical resistance between the tip and the serosal bath decreased when the tip entered the lateral space. (iv) The spontaneous electrical

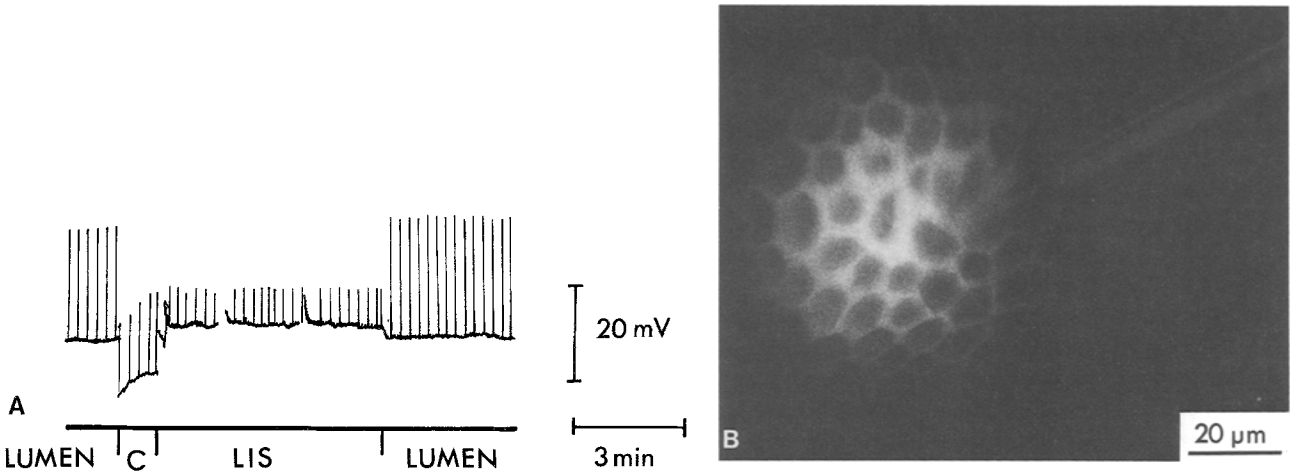

Fig. 2. *A.* The tip of the electrode was positioned in the lateral intercellular space (LIS) as estimated from i) the decrease in the fractional resistance (measured as a voltage evoked by a transepithelial current every 20 sec), and ii) the recording of a small positive potential. Fluorescent dye could be ejected from the electrode. Two such ejections are shown in the figure as indicated by the interruption of the trace. *c* indicates a cell which is penetrated transiently during the advance of the electrode. *B.* The fluorescent dye in the lateral spaces was excited via the epifluorescence unit of the microscope. Only lateral spaces are seen to be stained. The double-barrelled electrode is seen in the right aspect of the picture

potential recorded in the spaces was positive relative to the mucosal solution. These criteria are discussed further on pp. 117 and 119.

Results

General Properties of the Gallbladder at Low External Osmolarities

When the unilateral preparation of the gallbladder was bathed on the mucosal side in a solution of 62 mOsm ± 1.7 (11) it produced a secretion on the serosal side which had an osmolarity of 62 mOsm ± 1.1 (11). The rate of volume transport (J_v) was 89 µl h^{-1} $cm^{-2} \pm 10$ (11). The resistance of the tissue was 203 $\Omega cm^2 \pm 29$ (11), and the transepithelial potential differences was +3.7 mV (Table 2 for statistics) serosa positive.

Anatomy

The gallbladders were arranged with the same degree of stretch found in the animal. At normal osmolarities the cell layer was virtually flat when viewed in the Nomarski optics; i.e., the variation in height was less than 5%. Adaptation to low osmolarities caused foldings to appear in the cell layer. When examined in sections fixed for light microscopy it could be estimated that the cell layer was flat for about 20 cell diameters (the apical cell diameter was 7.1 µm ± 0.4 ($n=10$) measured in the living tissue). These flat areas were limited by a groove about 3 cell diameters deep. The parallel grooves increased the apparent surface area of the tissue by about 20 to 30%.

The lateral intercellular spaces were distended at low osmolarities. If we assume that the cells have a circular cross-section with a diameter of 7 µm at the luminal end, a diameter of 4 µm at the serosal end and a height of 20 µm, then the lateral intercellular spaces comprise some 39% of the tissue volume of the epithelial layer at low osmolarities (*see also* Bundgaard & Zeuthen, 1982).

Observations with the Staining Electrode

Two types of penetration into the lateral intercellular spaces could be distinguished.

1) Initially the electrode tip was intracellular as indicated by a negative potential, but as advance of the electrode tip continued (typically 10 µm) the tip left the cell and entered the lateral intercellular space. This was evident from (i) the abrupt change in the fractional resistance (Figs. 2*A*, & 3), (ii) the observation of a small positive potential relative to the mucosal solution of +3 mV (Fig. 2*A*), and (iii) when fluorescent dye was injected through the tip of the electrode only the lateral spaces were stained (Fig. 2B). This pattern of staining was observed in 8 spaces in 4 tissues; no backflux of dye into the mucosal solution was observed.

2) The penetration was characterized as in (1) but the electrical potential measured by the reference barrel remained negative, −2 to −10 mV and ejection of dye from the tip of the electrode caused not only the lateral spaces to be stained but also one or more cells.

When the staining was discontinued the fluo-

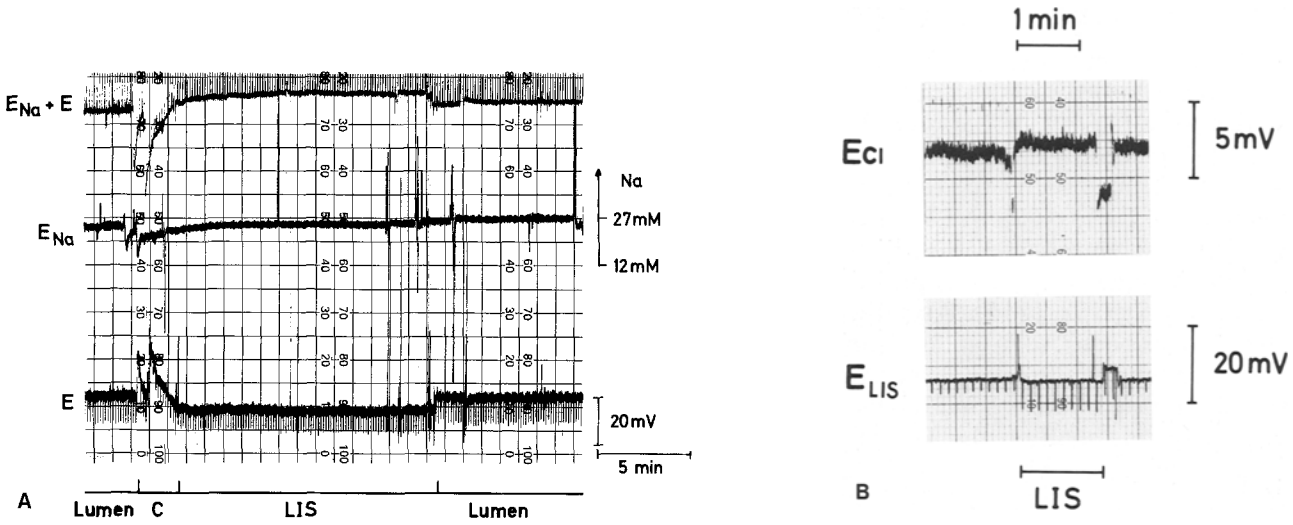

Fig. 3. *A*. The tracings obtained by a Na^+-sensitive double-barrelled microelectrode in the lateral intercellular space. *E* is the electrical potential, $E_{Na}+E$ the potential recorded by the ion-selective barrel and E_{Na} the potential obtained by subtraction. Scale corrected for non-Nernstian behavior. A cell was penetrated during the advance of the electrode (*c*). A transepithelial current of about 10^{-5} A was passed every 10 sec. *B*. As in *A* but here Cl^- was recorded. Please note the different scale on the ordinate

rescence of the lateral spaces was reduced to 20% within 10 sec.

The Electrical Potentials and the Concentrations in the Intercellular Spaces

Even if the tip of the electrode was aimed at a lateral intercellular space the tip had to transverse the intracellular compartment. 70% of the recordings in the spaces were initiated by periods of low Na^+, high K^+ or low Ca^{++}, values indicative of leakage of ions from the penetrated cell (Fig. 3*A*). This period was on average 10 sec for the reference barrel and 20 sec for the ion-selective barrels. In 30% of the cases the potentials in the spaces were obtained after an abrupt change from cellular values (Fig. 3*B*).

If a cell remained permanently damaged it would continue' to take up dye from the staining electrode and a negative potential would be recorded in the spaces. Consequently, the first criterion for a successful penetration was: 1) The reference barrel should record a positive potential. Other criteria were: 2) The fractional resistance should decrease when the tip was positioned in the lateral intercellular space and should remain constant. 3) The recorded values of concentrations should remain constant (periods of up to 15 min were tested). 4) The lateral intercellular spaces should remain expanded as viewed via the Nomarski optics.

When these criteria were met I obtained the potentials from the Na^+, K^+, Ca^{++} and Cl^- electrodes and the electrical potential in the lateral spaces, which are seen in Table 1. The corresponding concentrations are also shown.[1] The electrical potential was positive by 3 mV, the Na^+ concentration was the same as in the lumen, 27 mM, and Cl^- was slightly higher, 28.5 mM. The concentrations of K^+, 1.7 mM, and Ca^{++}, 0.7 mM, were slightly lower than in the lumen.

Accuracy of the Na^+ Determinations

The liquid ion exchanger for Na^+ is the most convenient tool for making sharp and fast Na^+-sensitive microelectrodes. Unfortunately two sources of error influence these measurements; the influence from Ca^{++} and the effect of osmolarity and NaCl concentration on the reference electrode.

Due to the fairly accurate measurement of Ca^{++} concentration in the spaces, however, the error from Ca^{++} does not add significantly to the error on the determination of the Na^+ concentration (*see* Table 1). In the secretion, however, the uncertainty in the determination of Ca^{++} concentration causes standard error of the mean for the Na^+ concentration to increase by 20%.

The sensitivity of the reference barrel to external NaCl concentrations can be corrected for using Eqs. (1) and (2a). The correction gives a lower value for Na^+ concentrations in the spaces, 24 mM, compared to the uncorrected value, 27 mM. Other corrected values are: Electrical potential in serosa +3.8 mV, the concentration of Cl^- 30 mM, K^+ 1.6 mM and Ca^{++} 0.6 mM.

The Intracellular Potentials

The electrical potential of the cellular compartment was recorded with electrodes whose reference barrels were filled with 2 M KCl. It was 62.7 mV ±2.1 (17 recordings in 4 animals). (*See* Zeuthen (1981, 1982) for criteria for cell penetration.) The potentials of the ion-selective barrels were obtained when the reference barrels were filled with the test solution (62 mOsm). The potential of the K^+ barrels was 93.5 mV ±2 (15 recordings in 4 animals) which corresponds to a concentration of 93 mM of K^+ ions inside the cells. If the external solution was equilibrated with N_2 and the tissue was superfused with this solution for 30 min, the electrical potential fell reversibly to 37 mV ±2 (3 cells) and K^+ ions attained equilibrium across the cell membrane – equivalent to an intracellular concentration of 11 mM. The intracellular potential of the Na^+ barrels in oxygenated tissues was 40.0 mV ±6.0 (5 recordings in 2 animals) which corresponds to an intracellular concentration of 6 mM. The potential measured intracellularly with the Cl^--selective barrel was 42.5 mV ±2.0 (10 recordings in 2 animals). This corresponds to an intracellular Cl^- concentration of 5 mM.

The Fractional Resistance

The ratio in the lateral spaces was on average 0.56±0.04 (33 recordings in 15 animals). If we correct for the resistance of the connective tissue the value is about 0.42. In the cells the uncorrected value was 0.50±0.05 (17 recordings in 5 animals).

The Impedance of the Reference Barrel

The impedance and the coupling between the two barrels did not change significantly when the electrode was placed in the lateral intercellular spaces.

The Concentrations and Potentials of the Secretion

These are compiled in Table 2. The standard error of the mean for the potential of the Na^+ electrodes is large, partly due to the larger variation in and low value of the Ca^{++} concentration; the Na^+ electrode is much more sensitive to variation in Ca^{++} at low Ca^{++} levels than at high Ca^{++} levels (*see* Materials and Methods, p. 115 and Fig. 1).

Discussion

This paper reports the ion concentrations in the lateral intercellular spaces and in the cells of the bullfrog gallbladder epithelium adapted to low external osmolarities (62 mOsm). A summary of the major findings is given in Table 3.

Table 3. Mean values of concentrations and potentials in bullfrog gallbladder epithelium[a]

	E [mV]	Na^+ [mM]	K^+ [mM]	Cl^- [mM]	Ca^{++} [mM]	mOsm
lumen		27	2	27	1	62
cells	−60.6	6	95	5	~0	87[b]
spaces	+ 2.7	27	1.66	28.6	0.69	62
serosa	+ 3.7	28	1.2	29	0.33	62

[a] For errors, number of observations and significance, *see* Tables 1 and 2 and Results, p. 117.
[b] Calculated by assuming the activity coefficient and osmotic coefficient are similar to a 95 mM solution of KCl (*see* Discussion, Zeuthen, 1982).

Tissue Viability

As judged from the transport characteristics and electrical parameters the tissue appeared to be healthy: the absorption was isotonic with the luminal solution. Similar findings from numerous other experiments utilizing a wide range of osmolarities have been documented (Diamond, 1964a; Case, Harper & Scratcherd, 1968; Maddrell, 1969; Hill & Hill, 1978; Whittembury, de Martinez, Linares & Paz-Aliaga, 1980; Zeuthen, 1982). The rate of volume transport, 89 $\mu l\ hr^{-1}\ cm^{-2}$, was 3.4 times the value found at normal osmolarities, 26 $\mu l\ h^{-1}\ cm^{-2}$ (Bindslev, Tormey & Wright, 1974). This agrees with the observation (*see* references above) that the rate of volume transport is inversely proportional to the osmolarity.

The tissue resistance was 203 Ωcm^2 ±29 (11) which is about twice the value determined at normal osmolarities, 113 Ωcm^2 (Bindslev et al., 1974). At low osmolarities one would expect the tissue resistance to increase in proportion to the increase in resistance of the bathing solutions which is a factor of three. In the present case, however, the width of the lateral spaces increased which causes a reduction of the transepithelial resistance. Furthermore, the junctions may become more leaky at low osmolarities (Diamond, 1979). Therefore the relatively modest increase in resistance seems explainable. The transepithelial potential difference was +3.7 mV, serosa positive (Table 3). This is only slightly larger than the value determined at normal osmolarities, +3.0 mV (Bindslev et al., 1974). The magnitude of the potential will depend on the resistance of the lateral spaces and the tight junctions since the current generated by the Na^+ pump returns via this pathway. The change in os-

molarity will cause a change in these resistances as discussed above. Finally, the intracellular potential, −63 mV, the intracellular concentrations, K^+ 108 mM, Na^+: 6 mM, Cl^-: 5 mM, were also indicative of healthy cells (Table 3).

Localization of the Electrode Tip

The electrode tip was localized in the lateral intercellular spaces when the following criteria were fulfilled: i) The coordinates of the electrode tip coincided with the coordinates of the chosen lateral space (*see* Materials and Methods, p. 115). ii) Injection of dye via the electrode caused staining of the lateral space only (Fig. 2*B*). iii) The electrical resistance between the tip and the serosal bath decreased when the tip entered the lateral intercellular space. iv) The spontaneous electrical potential recorded in the spaces was positive.

The high rate of volume transport sustained at low osmolarity caused a widening of the lateral intercellular spaces. With the osmolarity used, 62 mOsm, the spaces had a width of up to 5 μm (Fig. 1) with the volume of the spaces constituting up to 39% of the volume of the epithelium. Thus with an accuracy of 0.2 μm in tip positioning and the resolution obtained with Nomarski optics (~0.5 μm) it was possible to position the tip.

The localization was confirmed by injection of fluorescein dye (Fig. 2*B*). Fluorescein has a diameter of 11 Å. It could therefore be ejected from glass electrodes of the same dimensions as those used for the measurements of ion concentrations. The damage done to the tissue by the two types of electrodes must be similar. When a small positive potential was recorded relative to the lumen and when the voltage-divider ratio was decreased relative to the lumen (*see below*) the dye stained lateral intercellular spaces only.

The localization of the electrode tip was also confirmed by electrical measurements. When a current was passed across the epithelium, a voltage was induced across the tight junction, lateral spaces and connective tissues. With reference to the serosal bath, the microelectrode in the lateral spaces recorded an induced voltage which was 40% of the voltage induced in the mucosal solution (Fig. 3). This is compatible with the data obtained by Simon et al. (1981) on *Necturus* gallbladder; they estimated that between 13 and 54% of the paracellular resistance was located in the spaces. In the same tissue Curci and Frömter (1979) found that almost all the resistance was located in the junctional region. When comparing the data from the bullfrog and *Necturus* it should be borne in mind that due to the oblique angle at which the electrode was advanced in the present study, the tip of the electrode was located in the top half of the lateral intercellular spaces. In the experiments on *Necturus* the electrode was advanced perpendicular to the mucosal surface and the location of the tip was closer to the serosal compartment.

Validation of the Recordings from the Lateral Intercellular Spaces

Although it is relatively certain that the tip of the electrode is located in the lateral intercellular space it remains to discuss to what extent leakage of ions from a damaged cell perturbs the ion concentrations at the point of measurement: even if it is found that no fluorescent *dye* penetrates from the spaces into cells it is no guarantee against *ions* exchanging via an artificial leak between the penetrated space and the penetrated cell. This is an important consideration because it is difficult to design a control experiment. For example, if the tissue was treated with ouabain, the lateral space could collapse and the geometry of the tissue in the 'control' situation would be different from the experimental situation.

There are, however, several indications that the ion concentrations measured in the lateral intercellular spaces (Tables 1 and 3) are not influenced by leakage of ions from the neighboring cells:

1) The K^+ concentration was lower than the luminal concentration and close to that found in the secretion.

2) The Na^+ concentration was the same in the spaces as in the secretion.

3) The electrical potential measured in the spaces (2.7 mV) was not incompatible to the serosal potential 3.7 mV (mucosal solution grounded), when the resistance ratio is taken into account.

If a significant leakage of ions existed one would expect the K^+ concentration to be higher in the spaces, not lower than the luminal concentration. It is also difficult to imagine a leak from a cell into the spaces which would cause the Na^+ concentration in the spaces to be exactly equal to the concentration in the mucosal and serosal solution. The finding that the electrical potential in the space was almost identical to the serosal solution suggests that there is no electrical leak between the cell and the space.

Comparison with Other Studies

Simon et al. (1981) have measured the concentrations of Na^+, K^+ and Cl^- and Curci and Frömter (1979) have measured K^+ in the lateral spaces of

Necturus gallbladder transporting at normal external osmolarities (205 mOsm). Based on the potentials recorded by ion-selective and conventional microelectrodes, which were of the order 0.6 to 3.2 mV with standard errors up to 100%, they concluded that there *might* be sufficient hypertonicity in the spaces to mediate a transepithelial transport of water by simple osmosis. On the other hand the authors were careful to point out that any systematic error in the recordings would seriously impair their conclusions.

There are two major differences between their study and the present one. (i) They found a *higher* K^+ concentration in the spaces whereas I have found a lower K^+ concentration compared to the external solutions. (ii) They recorded a slightly negative potential in the spaces, whereas I recorded a positive potential which was similar to that of the transepithelial potential. As the ratio of the volume of the lateral space relative to the volume of the cells is about 9% in the *Necturus* gallbladder preparation (Spring & Hope, 1979) and as high as 39% in the present study, it can be seen that any leakage of ions from the cells will be more serious in the *Necturus* gallbladder preparation because the ions will not be diluted to the same degree as in the bullfrog preparation. Electrode penetration in the *Necturus* preparations may have been influenced by leakage of K^+ from the neighboring cells.

The solution in the lateral spaces of rabbit small intestine has been analyzed by means of electron-probe (Gupta, Hall & Naftalin, 1978). The high values of K^+ concentrations obtained, 40 mM (which was about 3 times the luminal values) suggests that cytoplasmic contents might have contaminated the measurements.

The present study is at variance with the data obtained from salivary glands of *Caliphora* where Wall, Oschman and Schmidt-Nielsen (1970) determined a space hypertonicity of 30 mOsm. Epithelial cells may be hypertonic to the surrounding media (Zeuthen, 1982, and present results); thus any leakage would result in increased osmolarity.

An indirect estimation of the hypertonicity of spaces was attempted by Machen and Diamond (1969). They attributed the transepithelilal potential entirely to a diffusion potential across the junctions and estimated a space hypertonicity of about 10 mOsm. They made the erroneous assumption that the transport of Na^+ and Cl^- across the whole epithelium was entirely neutral. The exit step for Na^+ across the serosal membrane has been shown to be electrogenic (*see* discussion by Baerentsen et al., 1982), and the potential across the junctions cannot be attributed to diffusion alone.

Relation Between the Secretion and the Ionic Composition of the Spaces

The similarity in composition and lack of gradient in potential between the final secretion and the solution in the spaces (Tables 2 and 3) suggest that the coupling between the salt and water has largely taken place before the salt and water reach the lateral intercellular spaces, i.e. the coupling takes place in the serosal membrane and/or the junctional complexes. The small but significant differences between the K^+ and Ca^{++} concentrations could be explained if there were sources and sinks for these ions in the connective tissue.

Diamond (1964*b*) also observed that the K^+ concentration in the secretion produced by the unilateral gallbladder preparation was lower than the concentration in the luminal solution and that the Cl^- concentration was higher.

Mechanisms of Transepithelial Transport of Water

The data suggest that transepithelial transport of water cannot take place via the transcellular route unless either of the following are true:

1) the L_p's of the membranes are high – of the order of 10^{-2} cm sec^{-1} osm^{-1} and the data from the intracellular compartment (Table 3) are incorrect or misinterpreted: the osmolarity in the cell must be about 52 mOsm.

2) The data in Table 3 are correct. The L_p's are similar to those determined in *Necturus* gallbladder at normal osmolarities (Spring et al., 1980; Zeuthen, 1982) being about 10^{-3} cm sec^{-1} osm^{-1}. The reflection coefficient for K^+ ions or a K^+ salt is low across the serosal membrane.

Regarding 1): With a mucosal membrane, L_p, of 10^{-2} cm sec^{-1} osm^{-1} and a water transport rate of 89 µl cm^{-2} hr^{-1} the cell interior would need to be hyperosmolar by 2.5 mOsm in order to explain the influx of water. For water to move across the serosal membrane by osmosis, the osmolarity in the spaces should be higher than $62 \pm 2.5 = 64.5$ mOsm. If the osmolarity is proportional to the Na^+ concentration an osmolarity of 64.5 mOsm in the spaces would be detected by the Na^+ electrodes as a potential of 1 mV ($= 25.3 \ln 64.5/62$). This is within the resolution of the method. If the L_p was lower, say by a factor of 10, then the osmolarity in the spaces should have been

at least 25 mOsm higher than in the lumen.[2] This should have been detected by the Na^+ electrode (and Cl^- electrode) as a potential of 9 mV relative to the lumen. Finally, if interpretation no. 1 is correct the ion concentration data inside the cell must be in error: the efflux of water from the cell into the spaces cannot proceed against the osmotic gradient.

Regarding 2): With a mucosal membrane L_p of 10^{-3} cm sec^{-1} osm^{-1} the observed influx of water (89 µl cm^{-2} hr^{-1}) would be explained if the cell was hyperosmolar to the lumen by 25 mOsm – quite in agreement with the data and interpretations given in Table 3. But if the cell is hyperosmolar to the secretion the question remains: how can a cell by hyperosmolar to its surroundings by as much as 25 mOsm? Or, as the cell apparently is in a steady state, how can the efflux of water proceed against its chemical gradient?

Osmotic Equilibrium Across Cell Membranes

The hypothesis that the cell cytoplasm is hypertonic to the external solutions at low external osmolarities is based on two lines of evidence: Recordings by K^+ sensitive microelectrodes in (i) *Necturus* gallbladder (Zeuthen, 1982) and the present measurement (Table 3). A discussion of the translation of activities into osmolarities is given in Zeuthen (1982). (ii) From direct measurement of freezing point depression by a Ramsay osmometer (Zeuthen, 1981).

It is well established that many types of cells are in osmotic equilibrium with their bathing medium (Macknight & Leaf, 1977). It is also well established that many cells are not. This applies, e.g., to the fresh water amoeba (Schmidt-Nielsen & Schrauger, 1963; Riddick, 1968) and to the fresh water hydra. This is an animal which consists largely of two layers of water-permeable epithelia, the salt content of which is roughly 40 mOsm hyperosmotic to the internal and external bathing media (Steinbach, 1963; Benos et al., 1977). The animal has no nephridium. The localization and orientation of ion gradients, active transport and transport of water are the same in these fresh water species and in the gallbladder (*compare* House, 1974, and Benos et al., 1977). Furthermore the morphology is similar (Bundgaard & Zeuthen, 1982) in terms of wide, expanded lateral intercellular spaces.

It has been suggested in previous experiments (Zeuthen, 1982) that the reflection coefficient of the membrane for K^+ ions or some K^+ salt was significantly less than one. If this were the case the intracellular K^+ salt would not exert its full osmotic pressure across the membrane and an osmotic steady state could exist even if the intracellular compartment were hypertonic to the extracellular compartment. It is interesting that when isolated contractile vacuoles (Hopkins, 1945) and renal proximal tubules (Dellasega & Grantham, 1973) are made to swell and shrink osmotically in NaCl solutions they respond initially as perfect osmometers. If they are made to swell and shrink in solutions made hyper- or hypoosmotic by the addition of KCl they do not behave as perfect osmometers. This would be the case if the membrane had a low reflection coefficient for K^+. Theories for transepithelial transport of water involving low reflection coefficients for K^+ have been suggested (House, 1964; Schilb & Brodsky, 1970). If the reflection coefficient for K^+ was lower for the serosal than for the mucosal membrane, transepithelial water transport would be aided.

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[2] It is relevant to consider the case where L_p is increased with decreased bath osmolarity: The water permeability of epithelial cell membranes has been reported both to be an increasing function of osmolarity (toad bladder: Ripoche, Bourget & Parisi, 1973; Hardy, 1979) and a decreasing one (frog skin: House, 1964; Franz & Van Brüggen, 1967; Lau et al., 1982).

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