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Single-File Diffusion Multi-Ion Mechanism of Permeation in Paracellular Epithelial Channels

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Summary. The transepithelial fluxes, conductances and permeabilities of Li^+, Na^+, K^+, Cs^+, NH_4^+ and $H_3CNH_3^+$ were studied under ionic concentrations ranging from 12 to 250 mM in *Bufo arenarum* gallbladders. When these measurements are carefully corrected in order to get only the component due to the paracellular cation channels, the following results are obtained: (1) The permeability ratios (cationic/anionic) are a decreasing function of salt concentration. (2) The partial conductances through paracellular cationic channels show nonlinear saturable concentration kinetics. (3) Moreover, partial conductance kinetics of K^+, Cs^+ and NH_4^+ present a maximum followed, at higher concentratons, by a negative-slope region. (4) The selectivity sequences obtained from biionic potentials do not agree with those obtained from partial conductance measurements. (5) The unidirectional ^{22}Na tracer flux (serosal to mucosal) is inhibited by 63% when the K^+ symmetrical concentration in the bathing solutions is raised from 25 to 200 mM. (6) When the unidirectional ^{42}K fluxes (serosal to mucosal) at 200 mM KCl Na-free solutions are compared with K^+ partial conductance by means of the Hodgkin and Keynes (Hodgkin, A.L., Keynes, R.D. 1955. *J. Physiol London* **128**:61–88) expression, the n' factor is 2.0. These results indicate that cations do not follow the independence principle and behave as in single-file diffusion multi-ion pores when crossing the paracellular cation channels of *Bufo arenarum* gallbladder epithelium.

Key words gallbladder · epithelia · single-file diffusion · paracellular cation channels · ionic permeability

Introduction

The main route for passive ion permeation across gallbladder and other "leaky" epithelia is across channels located at the tight junctions, between the cells (Frömter, 1972; Frömter & Diamond, 1972). This route is usually referred to as "the shunt"; however, it consists of two independent and parallel pathways: (i) The *cation channels*, permeable only to cations; (ii) The *free solution shunt*, a nonspecific leakage pathway permeable to all ions with free solution characteristics, which includes (or perhaps consists of) the damage produced to the *in vitro* preparation (Barry, Diamond & Wright, 1971; Wright, Barry & Diamond, 1971; Moreno & Diamond, 1974; Moreno, 1975*a*, *b*).

A physical model of the cation channels, devised from studies on the passive ion permeation and selectivity in bullfrog and rabbit gallbladders, describes a system of large (about 10 to 20 Å equivalent diameter) hydrated channels, whose ionized acidic groups render them cation selective (Moreno & Diamond, 1974; 1975*a*, *b*; Moreno, 1975*a*).

Protons, 2,4,6-triaminopyrimidinium (TAP) and divalent cations interfere with the permeability of the cation channels to other monovalent cations (Wright & Diamond, 1968; Moreno & Diamond, 1974; Moreno, 1974; 1975*a*). This, plus the fact that there is a net charged ligand interacting with ions crossing through, suggested to us that ions might compete with each other for the sites, i.e., that ions might not obey the independence principle within the channel.

In the present study we show evidence indicating that cations permeating through cation channels do not obey the independence principle, but show competition. The study of this competition indicates that the mechanism of cation permeation corresponds to a single-file diffusion in a multi-ion channel (Hille & Schwarz, 1978).

Materials and Methods

Experimental material consisted of gallbladders from the South American toad *Bufo arenarum*[1] weighing *ca.* 200 g and white

[1] When placed in symmetrical 100 mM NaCl solutions, hybernated toad gallbladders showed a partial sodium conductance of 3.8 ± 1.6 mmho cm^{-2} (22 experiments), while for nonhybernated toads it was 19.1 ± 8.5 mmho cm^{-2} (10 experiments). The difference between the two populations is statistically significant. The experiments reported in this paper are only from hybernated toads.

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rabbits weighing 2.5 to 3.0 kg. Techniques and methods for obtaining *in vitro* preparation of gallbladder and measuring transepithelial potential differences (PD) and conductances (G) were similar to those described previously (Moreno & Diamond, 1974; Moreno, 1975*a*). Briefly, the gallbladder was carefully removed from the animal, washed free of bile, cut open, and a piece of tissue was mounted as a flat sheet in a 19-mm^2 area window between chambers of 2 ml vigorously stirred by magnetic bars.

The transepithelial PD's were measured with Ag-AgCl electrodes and registered with a Keithley 604 differential electrometer connected to a two-channel Cole-Parmer recorder. Transepithelial PD's measured with asymmetrical bathing solutions were corrected for the difference in electrode potentials:

$$E=(RT/F)\ln(a'_{Cl}/a''_{Cl})$$

where a'_{Cl}/a''_{Cl} is the chloride activity ratio as calculated from activity coefficients in Robinson and Stokes (1959). The experimental temperature was 21 ± 3 °C.

Solutions

The bathing solution has the following composition (in millimolal, mM): 0.25, $CaCl_2$; 1.63, K_2HPO_4; 0.73, KH_2PO_4; and 300, 200, 100, 50, 25, 12.5 or 6.25 XCl (X^+ being Li^+, Na^+, K^+, Cs^+, NH_4^+ or $H_3CNH_3^+$). The solutions were made isosmotic with the 300 mM salt solution by adding sucrose[2]. The pH was 6.75. In the experiments where XCl concentration ([XCl]) was less than 13 mM, the amount of phosphate buffer was reduced, so that total [K^+] was 1 mM.

The ratio P_X/P_{Cl} (permeability of X^+/permeability of Cl^-) was obtained from PD arising from changing the mucosal solution by an isosmotic solution containing diluted or concentrated XCl, resulting in a "dilution" or "concentration" potential.

Since P_X/P_{Cl} is dependent on salt concentration (*see* p. 105) its value for a given concentration was obtained by linear interpolation between a "dilution" and a "concentration" potential (for instance, the P_X/P_{Cl} for 200 mM XCl was the mean of the P_X/P_{Cl} obtained from a 300/200 and a 100/200 diffusion potential; mucosal concentraton/serosal concentration). The ratio P_X/P_{Cl} was the direct value obtained when the diffusion potential for concentrations of 18.25, 37.5, 75, 150 and 250 mM was measured (i.e., the P_X/P_{Cl} for 75 mM was obtained measuring 50/100 or 100/50 diffusion potentials).

The biionic potentials were obtained by replacing the mucosal solution of a salt with the same concentration of another one as previously described (Moreno & Diamond, 1974).

Analysis of the Data

The calculation of the ionic permeabilities (P) and conductances (G) have been extensively described and discussed elsewhere (Moreno & Diamond, 1974; 1975*a*, *b*). Briefly, P ratios were extracted from measured diffusion potentials by means of the Goldman-Hodgkin-Katz (GHK) equation. The partial conductance of the ion X^+ across the cation channels (G_X), across the free solution shunt (G_X^{sh}), and the partial conductance of chloride (G_{Cl}) were related as follows (from Moreno & Diamond, 1974; 1975*b*; Moreno, 1975*a*):

$$G_{Cl}=G^t/(1+P_X/P_{Cl}) \tag{1}$$

$$G_X^{sh}/G_{Cl}=\mu_X/\mu_{Cl} \tag{2}$$

$$G_X=G^t-(1+\mu_X/\mu_{Cl})\,G_{Cl} \tag{3}$$

where G^t is the measured epithelial conductance, μ_X and μ_{Cl} the free solution mobilities as obtained from Robinson and Stokes (1959); MacInnes (1961), and Moreno and Diamond (1975*b*, *Appendix*).

The main assumptions of Eqs. (1)–(3) are that there is a free solution shunt in parallel with cation channels and that Cl^- is permeable only through it. Extensive evidence showing that this is the case has been presented previously (Barry et al., 1971; a summary in Moreno, 1975*a*, pp. 99–100), and further evidence is presented here.

The GHK equation was originally derived from the Nernst-Planck equations and involves assumptions that are not expected to hold for permeation through cation channels (*see* Hille, 1975). In this work we used the GHK equation to determine the ratio of G due to cations and anions, and since cations can migrate through the cation channels and the free solution shunt:

$$P_X/P_{Cl}\equiv G_X^t/G_{Cl}=G_X/G_{Cl}+G_X^{sh}/G_{Cl} \tag{4}$$

where G_X^{sh}/G_{Cl} is the ratio of G's at the free solution shunt and it is well defined by the GHK equation. There is much empirical evidence supporting this (*see* Barry et al., 1971; Moreno & Diamond, 1974; Moreno, 1975*a*): when one measures permeabilities of cations known to be impermeant through cation channels ($G_X=0$); when the epithelium is severely damaged ($G_X^{sh}\gg G_X$); or when blockers of the cation channels are used ($G_X\to0$) the value P_X/P_{Cl} becomes or closely approaches $\mu_X/\mu_{Cl}\,(=G_X^{sh}/G_{Cl})$.

The ratio G_X/G_{Cl} of Eq. (4) is the ratio of G's for X^+ crossing through the cation channels and Cl^- crossing through the free solution shunt. There can be no coupling between G_X and G_{Cl} since they migrate through separate channels. The following experimental facts further support the use of the GHK equation for obtaining empirical P_X/P_{Cl} ratios: (a) When different ions X^+ (with different values of P_X) are tested in the same membrane, the value of G_{Cl} obtained through the GHK equation and Eqs. (1) and (4) remains constant (*see* Fig. 4). (b) When the value of G_{Cl} changes spontaneously in a preparation (because of damage, for instance) the value G_X does not change. (c) At the start some exceptionally good preparations show $P_X/P_{Cl}>100$ and after an hour the ratio approaches the usual value (near 4, the change is probably due to different degrees of damage), while the value of G_X remains constant. (d) When G_X is selectively blocked, the value of G_{Cl} does not change.

Measurement of Tracer Fluxes

The method for measuring tracer fluxes was described previously (Moreno, 1975*b*). The radioactive isotope (0.3 to 0.5 μCi/ml of carrier-free ^{22}Na from New England Nuclear, Boston, Mass.; or 2 to 2.5 μCi/ml of carrier-free ^{42}K from the Comisión Nacional de Energía Atómica of Argentina) was added to the serosal half chamber, and the flux was calculated from the rate of appearance of isotope in aliquots extracted from the mucosal side. Before and after the flux measurement, the membrane G^t and P_X/P_{Cl} were electrically measured.

The paracellular unidirectional flux of the cation X^+ from serosal to mucosal, J_X^t, is:

[2] Diamond and Harrison (1966) did not find differences in dilution potentials when measured in isosmotic solutions with total osmolarity of 400 or 800 mOsm. We confirmed this and did not find differences among the electrical properties of membranes when placed in 100 mM NaCl bathing solution with total osmolarity of 370 mOsm ($G_{Na}=4.2\pm0.5$; $G_{Cl}=1.2\pm0.2$ mmho cm^{-2}; $n=10$) or 736 mOsm ($G_{Na}=4.7\pm0.5$; $G_{Cl}=1.4\pm0.4$ mmho cm^{-2}; $n=4$).

$$J_X^t = J_X + J_X^{sh} \quad (5)$$

where J_X is the flux of X^+ through cation channels and J_X^{sh} is the flux across the free solution shunt. Since the free solution shunt has free solution characteristics:

$$J_X^{sh} = (RT/F^2)(\mu_X/\mu_{Cl})\, G_{Cl}. \quad (6)$$

The tracer permeability of the ion X^+ through the cation channels (P_X) is:

$$P_X \equiv J_X/a_X \quad (7)$$

where a_X is the activity of X^+ in the solution with the radioactive tracer. The actual value of a_X was calculated as in Smulders and Wright (1971, Eq. (2)) as:

$$a_X = a'_X - J_X^t\, d/D \quad (8)$$

where a'_X is the activity in the bulk solution, d is the thickness of the unstirred layer, and D is the free solution diffusion coefficient of XCl. The difference $a'_X - a_X$ resulted in less than 5% of a'_X in all cases.

Statistics and Calculations

Calculations were carried out on an IBM/360 computer, DOS system, using FORTRAN IV language. Unless otherwise stated, results are expressed as mean ± SE (number of independent experiments). The statistical significance of the mean differences was tested through Student's t distribution. In such cases the values in brackets ($P<\ldots$) mean the probability of a random difference.

The values of G's are normalized to the average of G_{Na} at 100 mM (22 experiments) except in the experiments with radioactive tracer where G's are not normalized.

Results

General Features of the Bufo arenarum Gallbladder

The physiological characteristics of *Bufo arenarum* gallbladder are very similar to those previously reported for bullfrog and rabbit gallbladders (Diamond, 1968; Moreno & Diamond, 1975*a*). In symmetrical 100 mM NaCl solution the spontaneous transepithelial PD was 1.7 ± 0.7 mV (serosal positive), the membrane resistance was $183 \pm 18\ \Omega\ cm^2$, the ratio P_{Na}/P_{Cl} extracted from diffusion potentials was 4.1 ± 0.3 (data from 22 membranes). The thickness of the unstirred layers measured from dilution and streaming potentials as in Diamond (1966) was 124 ± 18 μm at the mucosal side and 359 ± 23 μm at the serosal side (25 experiments). When the appropriate solutions (described above) are used, these characteristics persist for hours.

The anatomical structure of *Bufo arenarum* gallbladder does not significantly differ from bullfrog or rabbit gallbladder when observed under the light and electron microscope (P.J.I. Salas & J.H. Moreno, *unpublished observations*).

Table 1. Diffusion potentials (V in mV) and P_X/P_{Cl} ratios at different salt concentrations

Ion X	[XCl] (mM)	m/s	V	P_X/P_{Cl}	n
Li^+	25	12.5/25	-7.8 ± 1.0	4.7 ± 0.7	5
		50/25	7.7 ± 1.0		
	100	50/100	-9.0 ± 0.8	3.8 ± 0.3	11
		200/100	5.7 ± 1.3		
	200	100/200	-5.5 ± 1.6	2.1 ± 0.3	6
		300/200	2.2 ± 0.5		
Na^+	25	12.5/25	-9.7 ± 0.9	5.5 ± 0.9	5
		50/25	9.3 ± 1.3		
	100	50/100	-9.4 ± 0.5	4.1 ± 0.3	22
		200/100	7.5 ± 0.6		
	200	100/200	-9.5 ± 0.6	4.0 ± 0.6	5
		300/200	4.6 ± 0.7		
K^+	25	12.5/25	-9.1 ± 0.6	4.4 ± 0.7	9
		50/25	9.1 ± 0.8		
	100	50/100	-9.1 ± 0.5	3.7 ± 0.2	24
		200/100	7.0 ± 0.6		
	200	100/200	-6.8 ± 1.0	3.1 ± 0.6	9
		300/200	3.4 ± 0.7		
Cs^+	25	12.5/25	-8.7 ± 0.7	3.5 ± 0.6	5
		50/25	6.4 ± 0.9		
	100	50/100	-5.9 ± 1.0	2.7 ± 0.4	13
		200/100	3.4 ± 0.7		
	200	100/200	-3.4 ± 1.2	1.9 ± 0.4	5
		300/200	1.7 ± 0.7		
NH_4^+	25	12.5/25	-11.7 ± 0.7	8.3 ± 0.6	9
		50/25	10.8 ± 0.8		
	100	50/100	-10.8 ± 0.4	5.1 ± 0.5	16
		200/100	7.4 ± 0.6		
	200	100/200	-6.3 ± 1.4	3.4 ± 0.6	9
		300/200	3.1 ± 0.7		
$H_3CNH_3^+$	25	12.5/25	-8.2 ± 0.3	3.7 ± 0.4	5
		50/25	7.6 ± 1.4		
	100	50/100	-6.7 ± 0.6	2.4 ± 0.3	15
		200/100	3.0 ± 0.5		
	200	100/200	-4.7 ± 1.3	1.5 ± 0.4	5
		300/200	2.2 ± 0.5		

V is the diffusion potential under the m/s concentrations (mucosal/serosal). Dilutions and concentrations were done in a random order. The P_X/P_{Cl} ratios were obtained by linear interpolation between the values from dilutions and concentrations through the Goldman-Hodgkin-Katz equation. Notice that P_X/P_{Cl} decreases when salt concentration [XCl] increases.

The Relation P_X/P_{Cl} as a Function of Salt Concentration

Table 1 shows the experimental values of diffusion potentials and P_X/P_{Cl} at 25, 100 and 200 mM for the cation X^+ being Li^+, Na^+, K^+, Cs^+, NH_4^+ or $H_3CNH_3^+$. In all cases the value P_X/P_{Cl} decreases as concentration increases. The difference between P_X/P_{Cl} at 25 mM *vs.* 200 mM is statistically significant for Li^+, K^+, Cs^+, NH_4^+ and $H_3CNH_3^+$ ($P<0.05$).

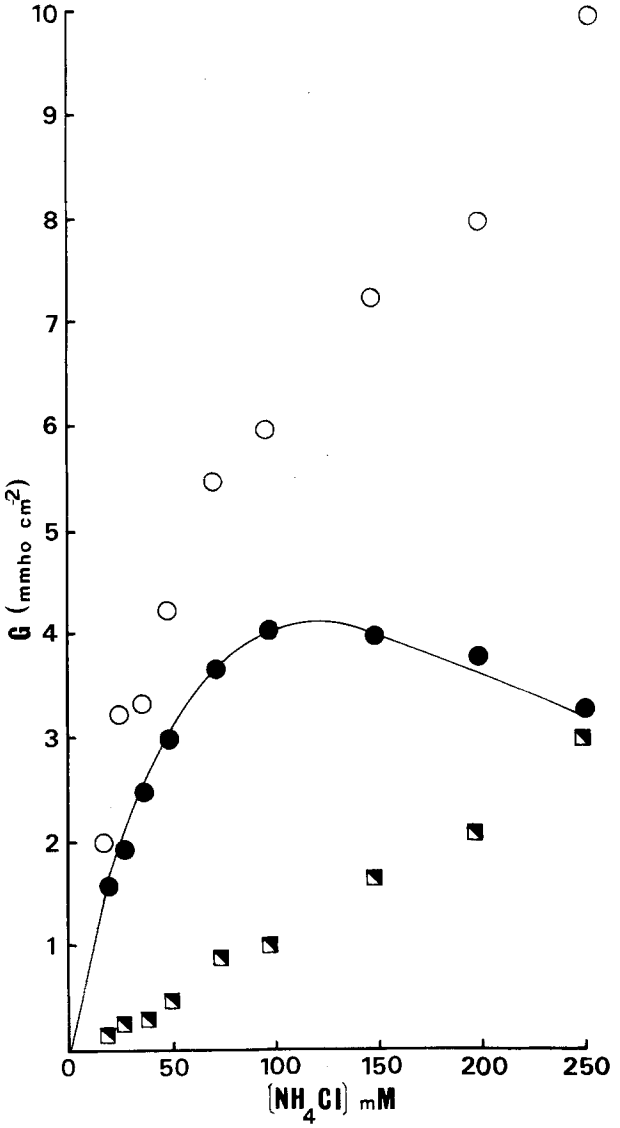

Fig. 1. Total membrane conductance, G^t (○); NH_4^+ conductance, G_{NH_4} (●), and Cl^- conductance G_{Cl} (◪) are plotted against $[NH_4Cl]$. Each value is the average of 9 experiments. Notice that as concentration increases G^t increases nonlinearly, G_{Cl} increases linearly while G_{NH_4} increases up to a maximum and then decreases. The line is drawn by eye

Table 2. Partial conductances (in mmho cm^{-2}) at different concentrations

Concentration (mM)	K^+	Cs^+	NH_4^+
100	3.5 ± 0.1	1.8 ± 0.08	4.1 ± 0.1
250	2.1 ± 0.6	1.0 ± 0.3	3.1 ± 0.3
	(5)	(4)	(9)

Each value is the mean (± SE) of G_X measured (number of experiments in brackets). The differences are statistically significant ($P<0.05$, paired data). Notice that G_X decreases when concentration increases.

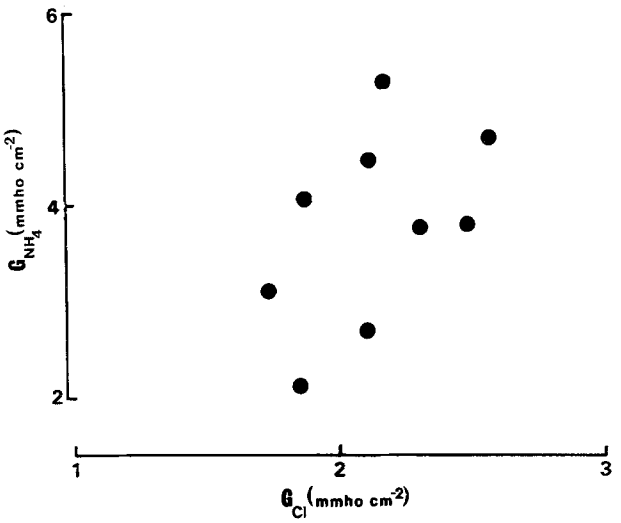

Fig. 2. G_{NH_4} is plotted against G_{Cl}. Each dot represents the value of a single experiment at $[NH_4Cl]=200$ mM. Notice that there is no negative dependence of G_{NH_4} on G_{Cl}. The slight positive dependence is probably due to different folding in different membranes (i.e., different effective areas)

Conductance Measurements

Figure 1 shows the values of the total membrane conductance (G^t, open circles), the ammonium conductance through cation channels (G_{NH_4}, filled circles) and the Cl^- conductance (G_{Cl}, squares) as a function of $[NH_4Cl]$. As concentration increases G^t increases nonlinearly, G_{Cl} increases linearly with concentration, interpolating at $[NH_4Cl]=0$ to $G_{Cl}=0$ (which is consistent with the fact that Cl^- migrates through the free solution shunt). G_{NH_4} increases sharply as concentration increases up to a maximum near $[NH_4Cl]=100$ mM and $G_{NH_4}=4.1$ mmho cm^{-2}, but beyond this concentration G_{NH_4} decreases as concentration increases. The paired data of 9 gallbladders indicates that G_{NH_4} is significantly larger at 100 mM (4.1 mmho cm^{-2}) than at 250 mM (3.1 mmho cm^{-2}) ($P<0.05$) (*see* Table 2). Since the correction due to the leakage current calculated from G_{Cl} (Eq. (3)) is larger for 250 mM than for 100 mM, one should be particularly careful in checking if this correction is responsible for the negativity of the slope seen in the negative-slope region of G_{NH_4} kinetics. This is tested in Fig. 2, which depicts the correlation of G_{Cl} and G_{NH_4} for different experiments at $[NH_4Cl]=200$ mM. As shown in the figure there is not negative dependence between G_{Cl} and G_{NH_4}, but a slight positive one. This positive correlation is probably due to the variations in the folding of the different epithelia which, in turn, varies the effective area of the membrane and thus affects G_{NH_4} and G_{Cl} by the same factor. Therefore,

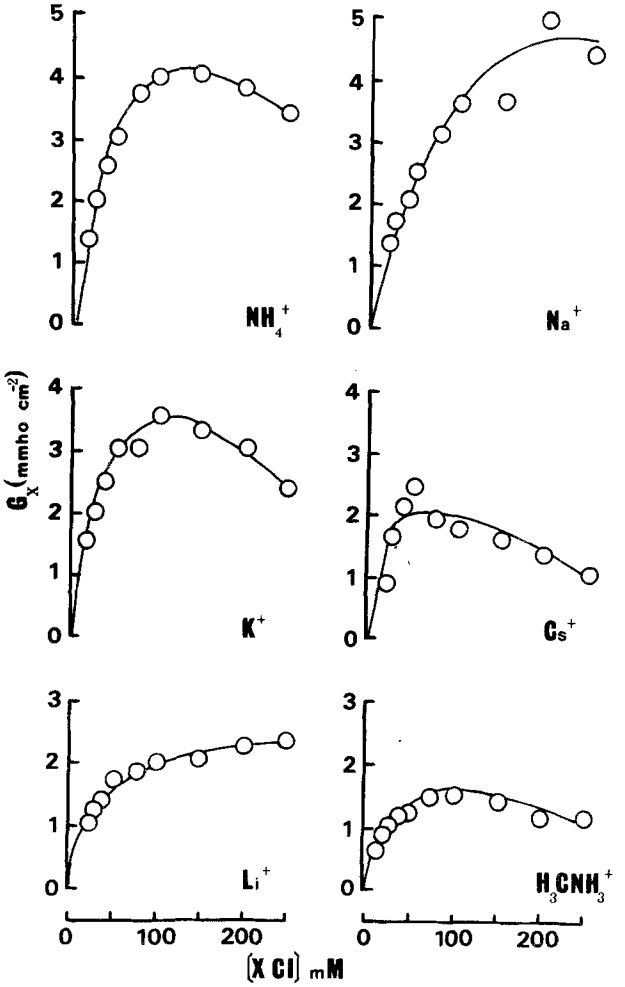

Fig. 3. The conductance of the cation X^+ through the cation channels is plotted against salt concentration; each value is the average of 3(Li^+), 22(Na^+), 5(K^+), 4(Cs^+), 9(NH_4^+), and 7($H_3CNH_3^+$) individual experiments. The lines are drawn by eye

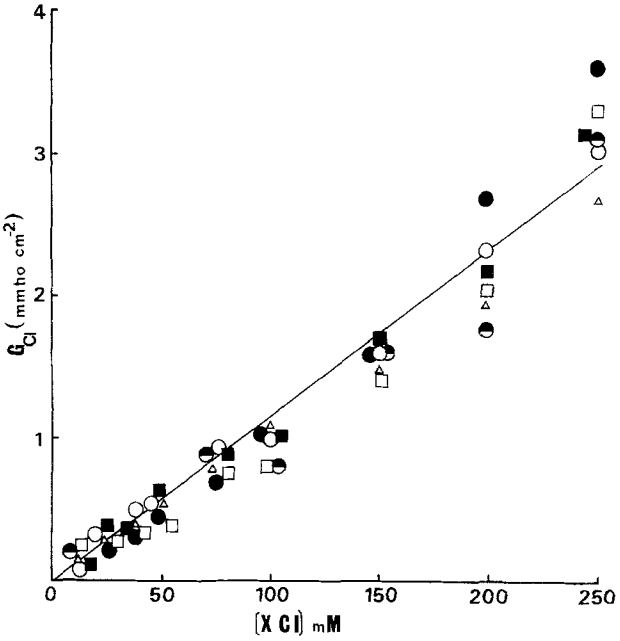

Fig. 4. Cl^- conductance is plotted against XCl for $X^+ = Li^+$(△), Na^+(●), K^+(○), Cs^+(□), NH_4^+(■), and $H_3CNH_3^+$(◓). Each value is the average of at least 3 individual gallbladders. Notice: (i) that there is a linear relation between G_{Cl} and XCl; (ii) that it interpolates at $G_{Cl}=0$, $[X\,Cl]=0$; (iii) that the values of G_{Cl} do not depend on the cation present (in spite of their different behavior depicted in Fig. 3). Statements (i), (ii) and (iii) are in accordance with the fact that Cl^- migrates through a free solution shunt

the data demonstrate that G_{NH_4} saturates and has a maximum near $[NH_4Cl]=100$ mM and a region of negative slope beyond the maximum.

Figure 3 shows the values of G_X as a function of $[X\,Cl]$ for the cations studied. All the kinetics of G_X show saturation. For K^+, Cs^+ and $H_3CNH_3^+$ the kinetics are similar to the one shown above for NH_4^+: G_X increases, reaches a maximum and then decreases as concentration increases. Table 2 shows that the values of NH_4^+, K^+ and Cs^+ conductances are significantly larger at 100 mM than at 250 mM concentration. The mean value of $G_{H_3CNH_3}$ at 100 mM is larger than at 250 mM, but the difference is not statistically significant. Na^+ and Li^+ exhibit saturation but do not show a maximum up to 250 mM.

Figure 4 shows the values of G_{Cl} as a function of $[X\,Cl]$ for all the studied cations. G_{Cl} increase linearly with $[X\,Cl]$, interpolates at $[X\,Cl]=0$ to $G_{Cl}=0$, and is independent of the cation species. This is consistent with an independent free solution pathway for Cl^- as demonstrated previously (Barry et al., 1971; Moreno & Diamond, 1974; Moreno, 1975*a*; *b*).

The data on G_X and P_X/P_{Cl} indicate that there is no validity of the independence principle within the cation channels. Rather, the interference between permeating ions is such that beyond a certain concentration the cation conductance for NH_4^+, Cs^+ and K^+ *decreases* with increasing concentration. This is the behavior predicted for single-file multi-ion channels (Hille & Schwarz, 1978).

Selectivity Sequences at Different Ionic Concentration

The ratios P_X/P_{Cl} calculated from biionic potentials through the GHK equation were obtained at 25, 100 and 200 mM salt concentrations. Table 3 shows the values for $X^+ = Li^+$, Na^+, Cs^+, NH_4^+ and $H_3CNH_3^+$. The biionic "selectivity" (obtained by biionic potentials) is not coincident with the conductance "selectivity," neither are the changes which occur in the biionic sequences comparable with those of the con-

Table 3. P_X/P_K ratios obtained from biionic potentials

Ion	Concentration (mM)		
	25	100	200
Li^+	0.8±0.1 (5)	1.2±0.1 (11)	1.3±0.2 (6)
Na^+	1.1±0.09 (8)	1.2±0.08 (19)	1.2±0.2 (6)
Cs^+	0.7±0.08 (8)	0.6±0.09 (17)	0.7±0.1 (5)
NH_4^+	1.7±0.2 (11)	1.4±0.06 (21)	1.6±0.2 (10)
$H_3CNH_3^+$	0.5±0.04 (8)	0.3±0.03 (19)	0.3±0.03 (8)

The values of P_X/P_K (mean ±SE (number of experiments in brackets)) were obtained from biionic potentials through the Goldman-Hodgkin-Katz equation and corrected for the leakage current through Eq. (3) of Moreno and Diamond (1975*b*).

ductance sequences when the concentration is altered.

At 25 mM salt concentrations, the sequence of P's obtained through biionic potentials (P_X/P_K values in brackets) is: $NH_4(1.7) > Na(1.1) \geqq K(1) > Li(0.8) \geqq Cs(0.7) > H_3CNH_3(0.5)$. The sequence for G_X at the same concentration (G_X/G_K values in brackets) is: $K(1) \cong NH_4(1) > Na(0.85) \geqq Cs(0.82) > Li(0.65) > H_3CNH_3(0.49)$.

At 200 mM, the "biionic" sequence of permeabilities (P_X/P_K) is: $NH_4(1.6) > Li(1.3) \geqq Na(1.2) > K(1) > Cs(0.7) > H_3CNH_3(0.3)$. While the "conductance" sequence (G_X/G_K) is: $Na(1.75) > NH_4(1.31) > K(1) > Li(0.81) > Cs(0.46) \geqq H_3CNH_3(0.42)$.

The Unidirectional Na^+ Flux is Inhibited by K^+

In these series of experiments the unidirectional passive serosal to mucosal ^{22}Na flux (J_{Na}) was measured in the presence of symmetrical isosmotic 25 and 200 mM KCl solutions in the same gallbladder (transepithelial PD was clamped to 0). The serosal Na^+ concentration was 150 μM (the carrier of the ^{22}Na tracer). The choice of these K^+ concentrations was based on the G_K kinetics depicted in Fig. 3 which shows that G_K is not saturated at 25 mM but it is already saturated and shows negative slope at 200 mM. Thus the two conditions are expected to reflect two different states of "vacancies" in the channel ligands.

Figure 5 shows two individual experiments and Table 4 the results of seven experiments. The measured uncorrected tracer permeability (P_{Na}^{uc}) was calculated through Eq. (7) but using J_{Na}^t instead of J_{Na}. P_{Na}^{uc} is in all cases smaller at 200 than at 25 mM KCl (22.6 *vs.* 10.6 cm sec^{-1} 10^{-6}, $P<0.01$). The value of the shunt Na^+ permeability (P_{Na}^{sh}) calculated from Eqs. (6) and (7) varies largely from experiment to experiment but is not significantly affected by K^+ concentration (3.4 *vs.* 3.9 cm sec^{-1} 10^{-6}), while the cation channel or corrected Na tracer permeability (P_{Na}^{*}) is inhibited by 63% (19.2 *vs.* 7.1 cm sec^{-1} 10^{-6}) when K^+ concentration is increased from 25 to 200 mM. There is no relation between the percentage inhibition of Na^+ permeability and the difference between Na^+ shunt permeabilities at 25 and 200 mM ($P_{Na}^{sh}(200) - P_{Na}^{sh}(25)$), indicating that the correction for the free solution shunt does not influence the value of the inhibition.

The Relation between Passive K^+ Fluxes and Conductances

According to Hodgkin and Keynes (1955), when the membrane potential equals the ionic equilibrium potential the passive tracer flux of an ion X (J_X) and its conductance (G_X) across a channel are related through the expression:

$$G_X = n' J_X F^2/RT. \quad (9)$$

The value of n' provides valuable information on the permeation mechanism across a channel (*see* Discus-

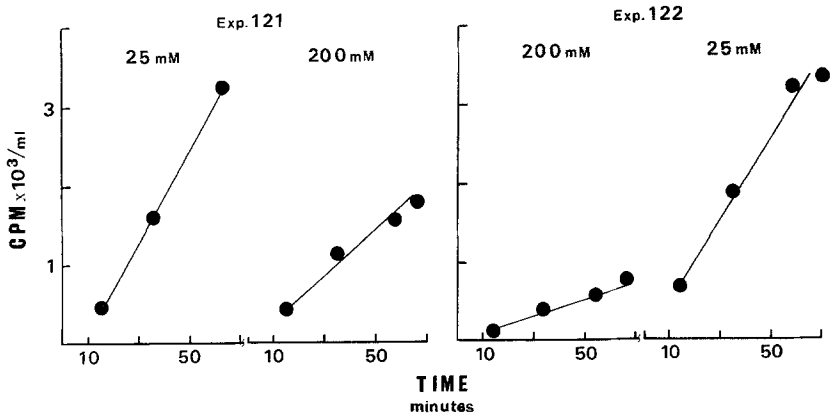

Fig. 5. Each point corresponds to a measurement of tracer activity in the mucosal side. The activity (in cpm/ml) in the serosal side was the same during the whole experiment; therefore, the slope is proportional to the isotope permeability. Notice that the permeability to the isotope decreases when symmetrical salt concentration increases

Table 4. Effect of K^+ concentration on Na^+ tracer permeability (in cm sec^{-1} 10^{-6})

Exp.	KCl (mM)	P^{uc}_{Na}	P^{sh}_{Na}	P^{*}_{Na}	Inhibition (%)	Order of measurement
121	25	17.3	6.8	10.5		
	200	8.9	5.1	3.8	63.5	25–200
122	25	25.3	2.9	22.4		
	200	6.6	2.2	4.4	80.2	200– 25
123	25	31.3	1.8	29.5		
	200	12.7	5.4	7.3	75.3	25–200
125	25	19.3	1.9	17.4		
	200	12.4	1.8	10.6	38.9	200– 25
136	25	31.2	3.7	27.5		
	200	17.4	1.6	15.8	45.1	200– 25
137	25	20.1	2.1	18.0		
	200	7.0	4.9	5.1	71.0	25–200
138	25	14.1	4.7	9.4		
	200	9.5	6.7	2.7	70.5	25–200
$\bar{X}\pm$SE	25	22.6 ±2.6	3.4 ±0.7	19.2 ±2.9		
	200	10.6 ±1.4	3.9 ±0.8	7.1 ±1.7	63.5 ±5.9	

Each pair of values represents the measurements of permeabilities in a single gallbladder in the presence of 25 or 200 mM KCl on both sides. The Na^+ fluxes were measured from serosal to mucosal (serosal Na^+ concentration 150 μM). P^{uc}_{Na} is the total permeability, P^{sh}_{Na} is the leakage permeability calculated from Eq. (6) and P^{*}_{Na} is the cation channel permeability. Notice the inhibitory effect of K^+ on P^{uc}_{Na} and P^{*}_{Na}. Notice also that the inhibitory effect does not depend on P^{sh}_{Na} nor on the order of measurement.

Table 5. Comparison of K^+ tracer fluxes (in μmol hr^{-1} cm^{-2}) and conductances (in mmho cm^{-2})

Exp.	J^t	G_K	G^{sh}_K	J_K	n' uncorrected	n' corrected
118	3.4	4.3	1.5	2.0	1.6	2.0
120	2.5	2.4	1.8	0.8	1.6	3.0
126	2.5	0.9	2.4	0.2	1.2	3.9
127	2.7	2.3	1.5	1.2	1.4	1.8
128	7.7	7.0	5.2	2.8	1.5	2.4
129	4.8	4.4	2.3	2.6	1.3	1.6
130	4.7	1.5	4.2	0.8	1.1	1.9
131	4.5	3.5	2.3	2.4	1.2	1.4
132	3.5	2.0	2.9	0.7	1.3	2.7
133	8.5	3.9	6.4	2.4	1.2	1.6
181	8.2	5.4	2.9	5.4	1.1	1.1
182	5.0	4.0	2.6	2.5	1.4	1.6
183	7.2	6.4	2.2	5.1	1.2	1.3
184	10.5	10.6	3.0	7.6	1.3	1.5
185	5.3	4.2	2.7	2.7	1.4	1.6
$\bar{X}\pm$SE	5.4 ±0.6	4.2 ±0.6	2.9 ±0.4	2.6 ±0.5	1.3 ±0.04	2.0 ±0.2

The measurements were made in symmetrical 200 mM KCl solutions (PD = 0). The values of J and G on the same line were obtained from the same experiment. The fluxes were measured with ^{42}K added to the serosal side. J^t_K is the measurement of the total K^+ flux; J_K is the calculated flux through the cation channels (i.e., corrected by means of Eqs. (5) and (6)). G^{sh}_K is the shunt potassium conductance and G_K the cation channel potassium conductance; n' values are obtained through Eq. (9) with J^t_K and G^t_K ($G^t_K = G_K + G^{sh}_K$) for uncorrected n', and J_K and G_K for corrected n'. Notice that n' is always greater than 1 and that for cation channels it reaches the value of 2.0.

sion and Hille & Schwarz, 1978). In order to evaluate n', K^+ tracer fluxes and conductances were measured simultaneously at 200 mM KCl symmetrical solutions (PD clamped to zero). Table 5 shows the results of 15 individual experiments. J^t_K is the measured K^+ flux while J_K is the flux corrected by the shunt (Eqs. (5) and (6)). When n' is calculated from uncorrected values (J^t_K and G^t_K, $G^t_K = G_K + G^{sh}_K$) its value is 1.3 ± 0.04 (statistically larger than 1, $P < 0.01$ for paired data). When the free solution shunt flux and conductances are substracted (i.e., J_K and G_K are inserted in Eq. (9)), the value of n' becomes 2.0 ± 0.2.

Equation (9) also implies that within a channel at a given concentration G_X and $J_X F^2/RT$ should be linearly related with a slope equal to n'. Figure 6 shows that the relation between G_X and $J_X F^2/RT$ is linear with slope 2.0 and that intercepts near $G_K = J_K F^2/RT = 0$.

If one considers the transcellular route of permeability, the measured flux of K^+ should be:

$$J^t_K = J_K + J^{sh}_K + J^{cell}_K \qquad (10)$$

where J^{cell}_K is the flux of K^+ through the cells. In a leaky epithelium the J^{cell}_K carrying current is much smaller than J_K (in *Necturus* gallbladder exposed to standard amphibian Ringer solution, transcellular resistance averages 7500 Ω while paracellular resistance averages 230 Ω; data from Reuss, 1979). It has also been demonstrated that active J^{cell}_K (serosal to mucosal) is either very small or null (Diamond, 1962; Wheeler, 1963; Frizzell & Schultz, 1972). However, we do not know if there exists an electrically silent transcellular flux of K^+ (for instance arising from a K^+ exchange diffusion). The fact that in Fig. 6 J_K is linearly related to G_K and that it interpolates near $J_K = G_K = 0$ indicates that any hypothetical electrically silent J^{cell}_K must be much smaller than J_K. Anyway, if any active or passive electrically silent flux of K^+ had existed, the n' value of gallbladder cation channels would have been actually *larger* than 2.0.

A value $n' > 1$ means that the independence principle does not hold within the channel and is found in single file diffusion multi-ion pores. In single-file

multi-ion pores the n' coefficient is expected to be a nonmonotonic function of salt concentration (Hille & Schwarz, 1978). We measured the n' value in 100 mM NaCl and KCl symmetric solutions (isosmotic with a 370 mOsm solution). The results were in NaCl $n'=1.7\pm0.3$ (5 experiments) and in KCl $n'=1.5\pm0.2$ (8 experiments). These results agree with the theoretic predictions for multi-ion pores and shown that $n'>1$ also holds under more physiological salt concentrations.

Discussion

The Ions Do Not Follow the Independence Principle within the Channel

Fluxes have independence within a channel if the chance for one ion to cross channel remains unmodified by the presence of other ions. The results presented here show that cations compete with each other to cross the channels (i.e., they show saturation and competition), and this is in agreement with previous findings in this epithelium: (a) In order to permeate, cations have to interact with a net charged acidic group within the channel; (b) Other ions are known to compete with sodium permeation: protons, TAP and divalent cations (Wright & Diamond, 1968; Moreno & Diamond, 1974; Moreno, 1974).

Barry et al. (1971) and Wright et al. (1971) proposed an electrically neutral thick membrane model for the cation channels, where ions behaved near the independence principle within the concentrations they used (20 to 400 mM). They proposed that the *total* conductance increased linearly with concentration and that P_{Rb}/P_{Cl} obtained from dilution potentials was only slightly dependent on salt concentration (*see* Wright et al., 1971, Figs. 4 and 8). These two findings seem to disagree with the results presented here (Figs. 1 and 3 and Table 1). As a matter of fact, the slight deviation from the linear behavior reported by Wright et al. is in agreement with our findings. We think that the reasons for the apparent disagreement are the following: (a) Wright et al. (1971) and Barry et al. (1971) were in fact the first to realize that there is a free solution shunt in parallel with cation channels, but, unfortunately, they did not correct their permeabilities and conductances for this leakage current so that their results are in terms of what we call here G^t, P_X^{uc} and J^t; and (b) they did not use phosphate in their solutions (but the buffer TRIS), and it has been demonstrated (Moreno & Diamond, 1974, p. 280) that the lack of phosphate results in rapid damages of the preparations (*see*

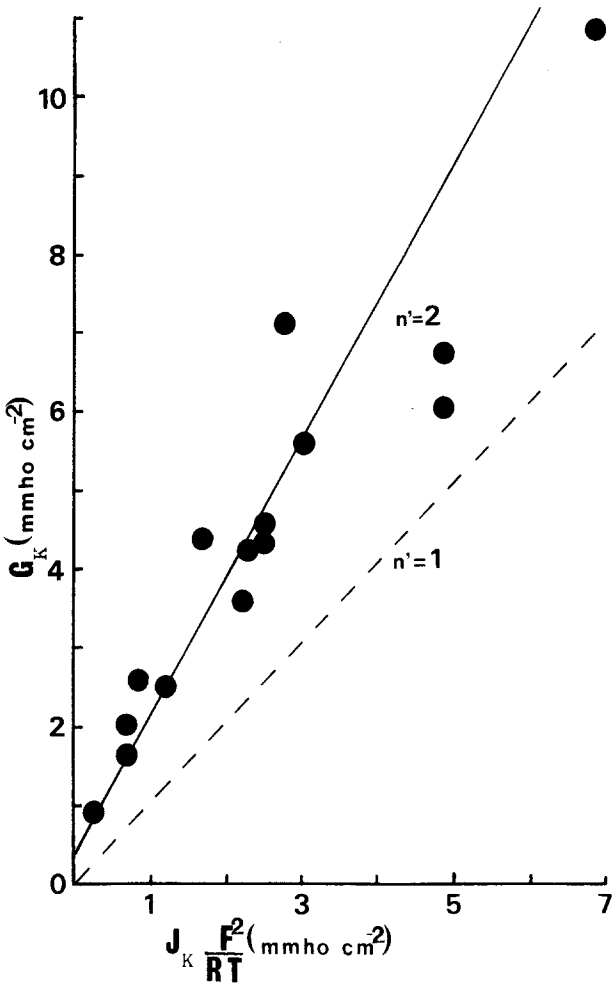

Fig. 6. The transepithelial K^+ conductance is plotted *vs.* the transepithelial tracer flux. The measurements were done in 200 mM KCl solutions and corrected for the solution shunt. Each value of G_K and $J_K F^2/RT$ was obtained in a single gallbladder. Notice: (i) that the values are linearly related, as expressed in Eq. (8); (ii) that the slope is larger than 1, suggesting a single-file diffusion in a multi-ion channel; and (iii) that the relation interpolates near $G_K=J_K=0$, indicating that there is not any significant additive component as, for instance, would an electrically silent flux be due to exchange diffusion or a neutral pump

Figs. 3 and 9 of Wright et al., 1971). These authors were working with relatively large free solution shunt conductances which probably masqueraded the G_X saturation kinetics and the negative dependence of P_X/P_{Cl} on concentration (i.e., they were adding a large component of fluxes that behave in agreement with the independence principle: the free solution shunt, which in some cases was larger than nonindependence cationic flux).

Since Barry et al. (1971) and Wright et al. (1971) worked with rabbit gallbladders, an alternative explanation to the apparent discrepancies might be found in a species difference. Figure 7 shows the results of experiments performed on two white rab-

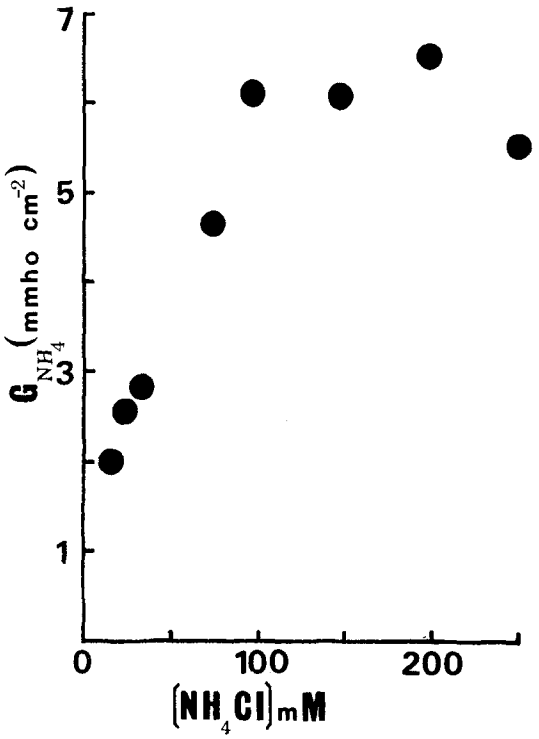

Fig. 7. Ammonium conductance G_{NH_4} is plotted against $[NH_4Cl]$. Each dot represents the average from two experiments in rabbit gallbladder

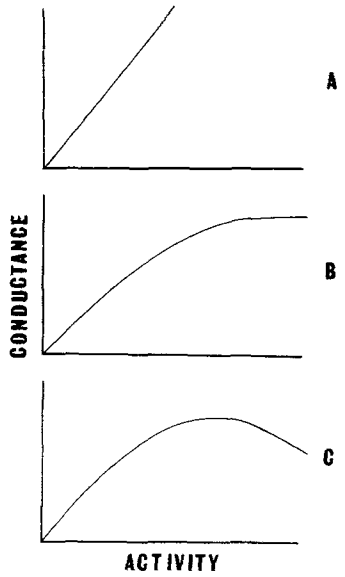

Fig. 8. The theoretical kinetics conductance against ionic activity are shown for (*A*) a free diffusion system, (*B*) single-ion channels, and (*C*) a case of single-file diffusion multi-ion channels (*see* Hille & Schwarz, 1978)

bit gallbladders measuring G_{NH_4} as a function of salt concentration. They suggest that the saturable G_{NH_4} kinetics demonstrated here for *Bufo* gallbladder is also present in rabbit gallbladder.

The Mechanism of Permeation

Thus, ions interact with each other within cation channels. The mechanism of permeation through a pore which better describes these interactions is single-file diffusion. Single-file diffusion models of permeation have been discussed by Hodgkin and Keynes (1955), Heckmann (1965, 1972), Läuger (1973), Köhler (1977), Hille and Schwarz (1978), Köhler and Heckmann (1979), and Hille (1979), among others.

From these works, important distinctions can be made between the behavior of single-ion channels (i.e., those that accept only one ion at a time) and multi-ion channels (i.e., those that admit more than one ion within the channel at a time):

a) Permeability as a Function of Ionic Concentration. Both single-ion and multi-ion channel permeabilities are negatively dependent on concentration. Table 1 shows the relative permeability of all the studied cations decreasing when concentration increases, while P_{Cl} (from conductances, *see* Fig. 4) remains constant.

b) Competitive Effects. In single file diffusion pores the flux of an ion X depends on the concentration of other permeating ions Y in the medium. This is because the chance of finding a vacant site is dependent on the average number of ions at the ligand surroundings, so that the increment of the number of Y ions decreases the migrating probability of X ions. We found that the unidirectional flux of tracer Na^+ is decreased by 63% when K^+ concentration rises from 25 to 200 mM. The effect of TAP (Moreno, 1974, 1975*a*) should be also understood as a competitive effect of this type. TAP has stronger affinity to the sites than the ions studied here, and therefore its blocking properties are more evident. This is probably also the case in blockage by protons (Wright & Diamond, 1968; Moreno & Diamond, 1974).

c) The G_X Kinetics. When ions of one species do not compete with each other while crossing a pore (i.e., the independence principle does hold), the conductance-concentration kinetics is linear (Fig. 8*A*). Single-ion channels show simple saturation (Heckmann, 1972; Läuger, 1973) as in Fig. 8*B*, while multi-ion channels present a maximum and a region of negative slope (Hille & Schwarz, 1978) as in Fig. 8*C*. Our results demonstrate that as concentration increases G_X saturates for all the ions shown in Fig. 3. NH_4^+, K^+ and Cs^+ kinetics show a maximum followed by a region of negative slope. The same seems

to happen with $H_3CNH_3^+$, but the negative slope could not be statistically demonstrated. G_{Na} and G_{Li} show saturation but not negative slope up to 250 mM. This could be either because they do not have negative-slope region or because they do have it, but beyond 250 mM.

d) The Factor n'. The factor n' at $V_m = 0$ (membrane potential) and for symmetrical salt solutions can be written (from Hille & Schwarz, 1978):

$$n' = P_{net}/P^* = (a/J)(\partial J/\partial a) \quad (11)$$

where P^* is the value of P for equilibrium unidirectional fluxes (tracer fluxes) and P_{net} the net permeability $(\partial J/\partial a)$; n' can also be expressed as in Hodgkin and Keynes' (1955) expression (Eq. (9) of this paper).

A values of $n' = 1$ indicates either that ions do not interact significantly with channel ligands or that the channel admits only one ion at a time. A value of $n' > 1$ suggests that the diffusing particle is a multimer of the single ion and can be obtained in single-file pores containing more than one ion at a time; if it is the case, the maximum value of n' for a given concentration is *not larger* than the maximum number of ions permitted in the channel at a time (Heckmann, 1972; Hille & Schwarz, 1978). Our results at 200 mM KCl show a value of $n' = 2.0$. This indicates that the cation channel is a single-file pore with at least two sites which can be simultaneously occupied.

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References

Barry, P.H., Diamond, J.M. 1971. A theory of ion permeation through membranes with fixed neutral sites. *J. Membrane Biol.* **4**:295-330

Barry, P.H., Diamond, J.M., Wright, E.M. 1971. The mechanism of cation permeation in rabbit gallbladder - Dilution potentials and biionic potentials. *J. Membrane Biol.* **4**:358-394

Diamond, J.M. 1962. The mechanism of solute transport by the gallbladder. *J. Physiol. (London)* **161**:474-502

Diamond, J.M. 1966. A rapid method for determining voltage-concentration relations across membranes. *J. Physiol. (London)* **183**:83-100

Diamond, J.M. 1968. Transport mechanisms in the gall-bladder. *In:* Handbook of Physiology: Alimentary Canal. C.F. Cole, editor. Vol. 5, pp. 2451-2482. American Physiological Soc. Washington

Diamond, J.M., Harrison, S.C. 1966. The effect of fixed charges upon diffusion potentials and streaming potentials. *J. Physiol. (London)* **183**:37-57

Frizzell, R.A., Schultz, S.G. 1972. Ionic conductances of extracellular shunt pathways in rabbit ileum. *J. Gen. Physiol.* **59**:318-346

Frömter, E. 1972. The route of passive ion movement through the epithelium of *Necturus* gallbladder. *J. Membrane Biol.* **8**:259-301

Frömter, E., Diamond, J.M. 1972. Route of passive ion permeation in epithelia. *Nature New Biol.* **235**:9-12

Heckmann, K. 1965. Zur Theorie der "Single File"-Diffusion, I. *Z. Phys. Chem.* **44**:184-203

Heckmann, K. 1972. Single file diffusion. *Biomembranes* **3**:127-153

Hille, B. 1975. Ionic selectivity of Na and K channels of nerve membranes. *In:* Membranes. A Series of Advances. G. Eisenman, editor. Vol. 3, pp. 255-323. Marcel Dekker, New York

Hille, B. 1979. Rate theory models for ion flow in ionic channels of nerve and muscle. *In:* Membrane Transport Processes. C.F. Stevens and R.W. Tsien, editors. Vol. 3, pp. 5-16. Raven Press, New York

Hille, B., Schwarz, W. 1978. Potassium channels as multi-ion single file pores. *J. Gen. Physiol.* **72**:409-442

Hodgkin, A.L., Keynes, R.D. 1955. The potassium permeability of a giant nerve fibre. *J. Physiol. (London)* **128**:61-88

Köhler, H.H. 1977. A single-file model for potassium transport in squid giant axon. *Biophys. J.* **19**:125-140

Köhler, H.H., Heckmann, K. 1979. Unidirectional fluxes in saturated single-file pores of biological and artificial membranes. I. Pores containing no more than one vacancy. *J. Theoret. Biol.* **79**:381-401

Läuger, P. 1973. Ion transport through pores: A rate theory analysis. *Biochim. Biophys. Acta* **311**:423-441

MacInnes, D.A. 1961. The Principles of Electrochemistry. Dover, New York

Moreno, J.H. 1974. Blockage of cation permeability across the tight junctions of gallbladder and other leaky epithelia. *Nature (London)* **251**:150-151

Moreno, J.H. 1975*a*. Blockage of gallbladder tight junction cation selective channels by 2,4,6-triaminopyrimidinium (TAP). *J. Gen. Pjysiol.* **66**:97-115

Moreno, J.H. 1975*b*. Routes of non-electrolyte permeability in gallbladder: Effects of 2,4,6-triaminopyrimidinium (TAP). *J. Gen. Physiol.* **66**:117-128

Moreno, J.H., Diamond, J.M. 1974. Discrimination of monovalent inorganic cations by "Tight" junctions of gallbladder epithelium. *J. Membrane Biol.* **15**:277-318

Moreno, J.M., Diamond, J.M. 1975*a*. Cation permeation mechanisms and cation selectivity in "Tight-Junctions" of gallbladder epithelium. *In:* Membranes: A series of Advances. G. Eisenman, editor. Vol. 3, pp. 383-497. Marcel Dekker, New York

Moreno, J.H., Diamond, J.M. 1975*b*. Nitrogenous cations as probes of permeation channels. *J. Membrane Biol.* **21**:197-259

Reuss, L. 1979. Mechanisms of sodium and chloride transport by gallbladder epithelium. *Fed. Proc.* **38**:2733-2738

Robinson, R.A., Stokes, R.H. 1959. Electrolyte solutions. Butterworth, London

Smulders, A.P., Wright, E.M. 1971. The magnitude of nonelectrolyte selectivity in the gallbladder epithelium. *J Membrane Biol.* **5**:297-318

Wheeler, H.O. 1963. Transport of electrolytes and water across wall of rabbit gallbladder. *Am. J. Physiol.* **205**:427-438

Wright, E.M., Barry, P.H., Diamond, J.M. 1971. The mechanism of cation permeation in rabbit gallbladder. *J. Membrane Biol.* **4**:331-357

Wright, E.M., Diamond, J.M. 1968. Effects of pH and polyvalent cations on the selective permeability of gallbladder epithelium to monovalent ions. *Biochim. Biophys. Acta* **163**:57-74

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