J. Physiol. (1966), **183**, *pp.* 37–57
With 9 text-figures
Printed in Great Britain

THE EFFECT OF MEMBRANE FIXED CHARGES ON DIFFUSION POTENTIALS AND STREAMING POTENTIALS

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(*Received* 8 *June* 1965)

SUMMARY

1. Electrical potential differences (p.d.'s) have been measured across an *in vitro* preparation of rabbit gall-bladder.

2. When the gall-bladder separates identical bathing solutions, the p.d. is always zero, regardless of the composition of the bathing solution. Hence the gall-bladder is symmetrical: i.e. the mucosal and serosal cell membranes have the same relative permeability coefficients.

3. Osmotic water flow causes streaming potentials of up to 20 mV, of a sign indicating greater permeability to cations than to anions.

4. At constant osmolarity, streaming potentials increase slightly with NaCl concentration. Streaming potentials decrease considerably with changes in osmolarity resulting from changes in NaCl concentration.

5. Diffusion potentials resulting from electrolyte concentration gradients are fitted well by the constant-field equation with the relative permeability coefficients $P_{Na} = 1{\cdot}00$, $P_{Cl} = 0{\cdot}33$, $P_K = 2{\cdot}3$. These permeability coefficients are independent of osmolarity and of salt concentration.

6. Relative to 0·25 mM-Ca, 5 mM-Ca reduces streaming potentials by 40 %, NaCl diffusion potentials by 62 %, and potassium diffusion potentials by 43 %.

7. The aqueous channels through which water and electrolytes traverse the cell membranes of the gall-bladder contain negative fixed charges, which are blocked by Ca. The physiological significance of the charges may be to reduce chloride permeability and thereby to increase the effectiveness of the gall-bladder in concentrating bile.

8. The effect of pH, and analogy with surface charges of other cells, suggest that the charges are organic acids of low pK_a.

INTRODUCTION

The present series of papers is concerned with the electrical properties of rabbit gall-bladder and their implications for the structure of the cell membrane. This first paper discusses the diffusion potentials and streaming

potentials resulting from concentration gradients across the organ, and the effects of pH, salt concentration, osmolarity, and calcium on these potential differences. The results indicate that the membrane channels through which passive exchanges of water and electrolytes take place contain stable negative fixed charges. These charged groups are probably organic acids with a low pK_a, similar to those responsible for the surface charge of the erythrocyte. The charges may be of physiological significance in reducing the chloride permeability and thereby facilitating the reabsorption of bile by the gall-bladder.

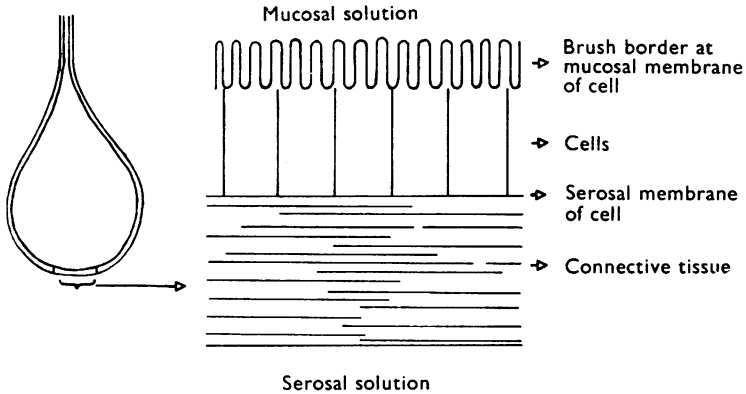

Fig. 1. Diagram of gall-bladder structure (not to scale). *In vivo*, the gall-bladder is a sac (left), in which a single layer of epithelial cells faces the inside and is supported by connective tissue. *In vitro*, the solution facing the cells is called the mucosal solution, and that facing the connective tissue is called the serosal solution. Under magnification (right), the cell membranes facing the mucosal solution are seen to possess a brush border.

In interpreting the electrical characteristics of the gall-bladder, some points about the organ's structure and function must be borne in mind. The gall-bladder (Fig. 1) is a sac consisting of a single layer of epithelial cells (the mucosa), supported on the outside by connective tissue (the serosa) several hundred micra thick, containing a few smooth muscle fibres. The cells abut directly on the inside of the sac (the lumen). Hence the half-time with which a substance added to the mucosal solution diffuses to the cells is a few seconds, but for the serosal solution over a minute (Diamond, 1966*b*). Substances traversing the gall-bladder must cross two sets of cell membranes—a membrane separating the cell contents from the mucosal bathing solution, and another membrane separating the cell contents from the serosal connective tissue. Whereas adjacent cells are cemented together firmly at the mucosal surface by a terminal bar, they frequently separate somewhat at the serosal surface, and the

'functional' serosal membrane may therefore include some of the basal end of the lateral cell membranes. The electrical potential differences (p.d.'s) measured between the mucosal and serosal bathing solutions are therefore the sum of two p.d.'s, one at the luminal and the other at the serosal membranes of the epithelial cells. However, the situation is considerably simplified by the fact, to be demonstrated, that these two cell membranes have the same relative permeability coefficients and behave in some respects as a single membrane.

As regards transport mechanisms, the gall-bladder concentrates bile by transporting sodium chloride and water in isotonic proportions from the mucosal to the serosal surface (Diamond, 1962*a*). With the possible exception of the small intestine (Barry, Dikstein, Matthews, Smyth & Wright, 1964), the mechanism of salt transport in the gall-bladder is unique in that sodium and chloride are transported in a one-to-one ratio by an electrically neutral NaCl pump, which conveys no net charge and hence sets up no p.d. (Diamond, 1962*b*; Wheeler, 1963; Dietschy, 1964). Active salt transport thus makes no contribution to the electrical measurements discussed in these papers, which reflect solely the passive movements of electrolytes. All the experiments described here were carried out at temperatures of 17–21° C, where active salt transport is in any case slow; but the results are in quantitative agreement with those obtained by Dietschy (1964) on transporting preparations at 37° C.

The first section of experimental results describes the p.d. in the absence of concentration gradients; the second concerns diffusion potentials; the third, streaming potentials; and the fourth, the effects of calcium on both these types of p.d..

METHODS

Dissection. White rabbits weighing 3–6 kg were anaesthetized by intra-peritoneal injection of Nembutal (sodium pentobarbitone). The gall-bladder was removed to a dish of Ringer's solution, where it was washed free of bile, and a polyethylene cannula 4 cm long was ligated into the neck of the gall-bladder. The dissection is described in more detail in a previous publication (Diamond, 1964).

Gall-bladders were everted by pushing the organ through a hole in its neck with a blunt glass rod, then cannulating as usual.

Experimental procedure. Electrical measurements were carried out with the cannulated gall-bladder in a 20 ml. beaker of solution. The cannula was held fast in a lucite ring by a screw. The beaker was stirred by a stream of oxygen bubbles saturated with water vapour by passage through Ringer's solution. The luminal bathing solution could be changed by withdrawing it with a polyethylene capillary mounted on a hypodermic needle and syringe, then refilling with fresh solution, and repeating this procedure 4–6 times. The outer bathing solution was changed by lifting out the beaker of solution from below and substituting a fresh beaker, the procedure requiring less than a second.

The p.d. across the gall-bladder was measured with polyethylene bridges filled with 4 % agar in 0·15 M-NaCl (or 0·1 M-Na_2SO_4 in experiments involving sulphate solutions). One bridge went down the cannula into the lumen of the gall-bladder, while the other was

in the outside bathing solution. Each bridge led to a beaker of saturated KCl, connected in turn by a saturated KCl bridge to another beaker of saturated KCl, into which dipped a calomel electrode. The p.d. was amplified by a Keithley 600 A electrometer and recorded on a Varian graphical recorder. All p.d.'s are reported as the potential of the mucosal with respect to the serosal surface. The asymmetry potential of the circuit was measured frequently throughout the experiment as the p.d. with both agar–NaCl bridges dipping into the solution bathing the outside of the gall-bladder. It was generally a few tenths of a millivolt and was subtracted from all experimental p.d.'s. In addition, when the solutions bathing the mucosal and serosal surfaces of the gall-bladder were not identical in electrolyte composition, the junction potentials arising at the agar–NaCl bridges were measured with a saturated KCl bridge, and subtracted from the experimental p.d.'s. All measurements were carried out at ambient room temperatures (17–21° C).

TABLE 1. Composition of experimental solutions (mM)

	NaCl	KCl	$CaCl_2$	Na_2HPO_4	NaH_2PO_4	Sucrose	Na_2SO_4	K_2SO_4	$CaSO_4$
A	148	6	0·25	2·125	0·375	—	—	—	—
B	148	6	0·25	2·125	0·375	600	—	—	—
C	—	154	0·25	2·125	0·375	—	—	—	—
D	—	6	0·25	2·125	0·375	267	—	—	—
E	—	—	—	2·125	0·375	—	118	3	2
F	—	—	—	2·125	0·375	600	118	3	2

If a gall-bladder is carefully dissected and handled, repeated measurements of the same p.d. are highly reproducible. For example, in measurements of the diffusion potential resulting from the same concentration gradient of NaCl, applied 13 times at intervals throughout a 10 hr experiment involving 135 changes of solution, the S.D. was $\pm 5{\cdot}7\,\%$; and for thirteen measurements of a streaming potential, $\pm 5{\cdot}6\,\%$. When the effect of any agent (changed osmolarity, calcium, etc.) on a p.d. was being measured, the p.d. was always measured under normal conditions beforehand and afterwards, to rule out the possibility of irreversible changes.

Solutions. The composition of the experimental solutions is shown in Table 1. Solution *A* is referred to in the text as 'NaCl Ringer's solution'. Hypertonic solutions were made by mixing *B* with *A*, or *F* with *E*. Mixing *D* with *A* (the two solutions have approximately the same osmolarity, as checked with the Fiske osmometer) reduced the NaCl concentration while maintaining constant osmolarity with sucrose. Solutions with high [K] were obtained by mixing *A* and *C*.

RESULTS

The p.d. in the absence of concentration gradients

When the same bathing solution was present simultaneously on the mucosal and serosal surfaces, the p.d. was invariably less than 1 mV and usually less than 0·3 mV, regardless of the composition of the bathing solution. Thus, the average p.d. for seventeen gall-bladders both of whose surfaces were bathed by NaCl Ringer's solution (solution *A*, Table 1) was $-0{\cdot}2 \pm 0{\cdot}2$ mV (i.e. mucosal surface negative; all errors quoted are standard deviations). Of these seventeen gall-bladders, five gave p.d.'s of $-0{\cdot}5$ to $-0{\cdot}3$ mV; four, $-0{\cdot}2$ mV; four, $-0{\cdot}1$ mV; two, 0·0 mV; and two, $+0{\cdot}1$ mV. For nine gall-bladders in Na_2SO_4 Ringer's solution (solution *E*), the average p.d. was $0{\cdot}0 \pm 0{\cdot}2$ mV, with seven of the nine values equal to

or less than 0·1 mV. The other bathing solutions for which this absence of a p.d. (i.e., p.d. under 1 mV) in the absence of concentration gradients has been specifically confirmed are: (1) choline chloride Ringer's solution (all the NaCl in solution *A* replaced by choline chloride); (2) NaCl Ringer's solutions in which [K] had been reduced to 4 mM, or increased to 47 or 65 mM; (3) solutions with raised [Ca] (up to 5 mM in NaCl Ringer's solution, or up to 8 mM in Na_2SO_4 Ringer's solution); (4) solutions with [NaCl] reduced from 200 to 100, 75, 50, 25, or 10 mM, maintaining constant osmolarity with sucrose; (5) solutions with [NaCl] decreased from 148 to 95 or 50 mM, or increased to 210, 250, or 300 mM, without maintaining constant osmolarity; (6) solutions with [Na_2SO_4] reduced from 118 to 59 or 29·5 mM, or increased to 117 mM, without maintaining constant osmolarity; (7) solutions made hypertonic by more than 350%, by addition of up to 600 mM sucrose or mannitol to (*a*) solutions with the normal [NaCl] of 148 mM, or (*b*) solutions with [NaCl] lowered to 90 mM.

The gall-bladder consists essentially of two membranes in series (the mucosal and serosal membranes of the epithelial cells) enclosing a middle compartment (the intracellular contents) and separating two outer compartments (the mucosal and serosal bathing solutions). The experimental finding is that the p.d. across the system is zero when the two outer compartments contain the same solution, regardless of its composition. A necessary and sufficient condition for this finding in such a system of two membranes in series is that the system be symmetrical, i.e. that the two membranes have the same relative permeability coefficients. In other words, the ratio P_{Cl}/P_{Na}, where P is a permeability coefficient, must have the same value at both cell membranes; and similarly for P_K/P_{Na}. The reasoning behind this conclusion is that the concentration gradients across the two membranes will be equal but opposite when the two outer solutions are identical. As the total p.d. is zero, the p.d.'s across the two membranes are also evidently equal but opposite. The p.d. across a membrane is a function of the concentration gradient and the relative permeability coefficients. Since the two membranes give the same p.d. as each other for any concentration gradient, their relative permeability coefficients must be identical. For example, when the osmolarity of the bathing solution is quintupled by addition of sucrose without changes in electrolyte composition, the cells shrink drastically, with consequent changes in the electrolyte concentration gradients and p.d.'s across each face of the cells. Only in a system with the same permeability ratios at both faces will the two p.d.'s at opposite ends of the cell change in parallel and continue to cancel within a fraction of a mV under all circumstances. The conclusion of symmetry by this line of reasoning is supported by the symmetry of diffusion potentials (p. 48) and of streaming potentials (p. 43) in the

gall-bladder. The fact that the two membranes have the same relative permeabilities does not indicate whether they have the same *absolute* permeabilities or electrical resistances.

Streaming potentials

Suppose water is forced through a membrane which separates two solutions of the same electrolyte composition and which is not equally permeable to cations and anions. Then a p.d. will be observed in which the solution into which water is flowing acquires a potential of the same sign as the charge of the more permeant ionic species. These p.d.'s are called streaming potentials, and they arise from the frictional drag exerted on the ions by water during flow through the membrane. Streaming potentials in response to osmotically-induced water flow were demonstrated in fish gall-bladder (Diamond, 1962*c*) and subsequently observed in rabbit gall-bladder (Pidot & Diamond, 1964; Dietschy, 1964), rat small intestine (Smyth & Wright, 1964), and rabbit ileum (S. Schultz, personal communication).

Figure 2 illustrates the effects of adding the impermeant non-electrolyte sucrose to the solution on one side of the gall-bladder to produce osmotic water flow, the two solutions otherwise being identical in composition. The solution containing the sucrose—i.e. towards which water was flowing —went electrically positive. Hence the gall-bladder must be more permeable to cations (chiefly Na) than to anions (sulphate in this experiment). These streaming potentials were not influenced by the nature of the molecule causing the osmotic water flow and could equally well be elicited with other impermeant non-electrolytes, such as raffinose, mannitol, or erythritol. Upon addition of an impermeant molecule to one bathing solution, the streaming potential built up to its steady-state value after a diffusional delay and then remained stable for as long as the osmotic gradient was maintained. The magnitudes of the streaming potentials observed were up to 20 mV.

If the opposite faces of a cell did not have the same relative permeability coefficients, changes in bathing solution osmolarity could cause a change in p.d. because of membrane asymmetry and changes in intracellular concentrations, without a true streaming potential being involved. This artifact is not possible in a symmetrical system such as the gall-bladder. When a p.d. is produced in the gall-bladder by adding an impermeant molecule to one bathing solution, adding the molecule to the opposite bathing solution at the same concentration always brings the p.d. back to zero. This proves that the p.d. is due to water flow and not to changes in osmolarity *per se*. The closely parallel behaviour of p.d. and water flow under conditions where the water flow is undergoing complicated changes

(e.g. Diamond, 1966*a*: Fig. 1 and Fig. 3) is further convincing evidence that true streaming potentials are involved.

Two further points about Fig. 2 deserve notice. One is that the relation between streaming potentials and osmotic gradients is apparently linear for small gradients, and that the proportionality constant is the same whether the water flow is towards the mucosa or the serosa. The average

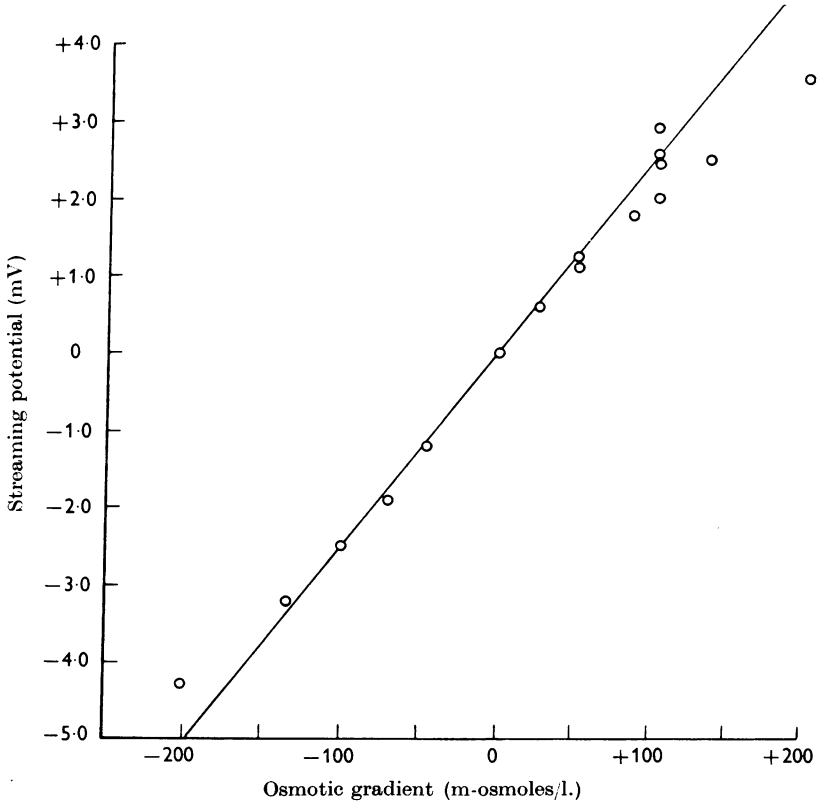

Fig. 2. Streaming potentials in Na_2SO_4 Ringer's solutions (solutions *E* and *F*, Table 1); gall-bladder not everted. Both mucosal and serosal solutions had the same electrolyte composition, but osmotic gradients were created by adding sucrose to one of the solutions. A positive osmotic gradient means mucosal solution hypertonic; a positive p.d. means mucosal solution positive.

value of this constant for ten experiments in NaCl Ringer's solution was $3{\cdot}6 \pm 0{\cdot}9$ mV/100 m-osM. The second point is that the streaming potential shows signs of falling off at the largest concentration of sucrose tested in this experiment, 200 mM. Since streaming potentials are linearly related to rates of water flow, this suggests that the water flow is directly proportional to the osmotic gradient for small but not for large gradients.

The following paper (Diamond, 1966*a*) will show that the relation becomes even more non-linear for larger gradients, and will suggest an explanation.

The effect of anions upon streaming potentials. Since streaming potentials arise from the combined effects of water flow and a greater permeability to cations than to anions, one might expect that replacement of chloride by a more impermeant anion, such as sulphate, should increase the streaming potentials. Contrary to expectation, the proportionality constant for streaming potentials in Na_2SO_4 Ringer's solution was $1{\cdot}7 \pm 0{\cdot}7$ mV/100 m-osM (eleven gall-bladders), one-half of the value in NaCl Ringer's solution. In one experiment where both salts were tested on the same gall-bladder, a 100 m-osM gradient of sucrose gave a streaming potential of 1·9 mV in Na_2SO_4 Ringer's solution and 2·9 mV in NaCl Ringer's solution. Probably the principal explanation is that the water permeability of the gall-bladder is reduced by substitution of sulphate for chloride, as shown by gravimetric measurements of water flow down an osmotic gradient in sulphate and in chloride. Because of the linear relation between water flow and streaming potentials, the streaming potentials in sulphate should also be reduced proportionately. In addition, rabbit gall-bladder is not completely impermeant to sulphate (Dietschy, 1964), so that some shunting effect of anions upon the streaming potential still remains after replacement of chloride by sulphate.

The effect of pH. In two experiments in NaCl Ringer's solution the pH was varied by altering the ratios of HPO_4^{2-} to $H_2PO_4^-$ in the phosphate buffer. The pH was changed simultaneously on both sides of the gall-bladder, so that no hydrogen ion concentration gradient was present. For the same osmotic gradient (100 mM of sucrose) the streaming potential was approximately the same at pH 6·3, 7·15, 7·2, 7·35, and 7·62. Hence pH changes in this range have little or no effect upon the streaming potentials.

The effect of salt concentration. Figure 3 depicts the results of an experiment in which Ringer's solutions with various NaCl concentrations between 50 and 300 mM were used. With a given NaCl concentration on both sides of the gall-bladder, the streaming potential resulting from the addition of 100 mM sucrose to the mucosal surface was measured. As seen in Fig. 3, streaming potentials decreased with increasing salt concentration, the p.d. at 300 mM-NaCl being 26 % of that at 50 mM-NaCl.

In this experiment not only the salt concentration of the Ringer's solution was varying but also the osmolarity, since NaCl accounts for most of the osmolarity of Ringer's solution. An experiment was then devised in which the effect of salt concentration could be studied independently of osmolarity. To solutions with [NaCl] between 10 and 200 mM, sufficient sucrose was added to make each solution isosmolar with the

Ringer's solution containing 200 mM-NaCl (387 m-osM). The effect of salt concentration at constant osmolarity was then studied by measuring the streaming potential resulting from the addition of an extra 100 mM sucrose to the mucosal bathing solution. Figure 4 shows that streaming potentials increased slightly with increasing salt concentration, but the effect is small, since the streaming potential at 10 mM-NaCl was only 35 % below that at 200 mM-NaCl.

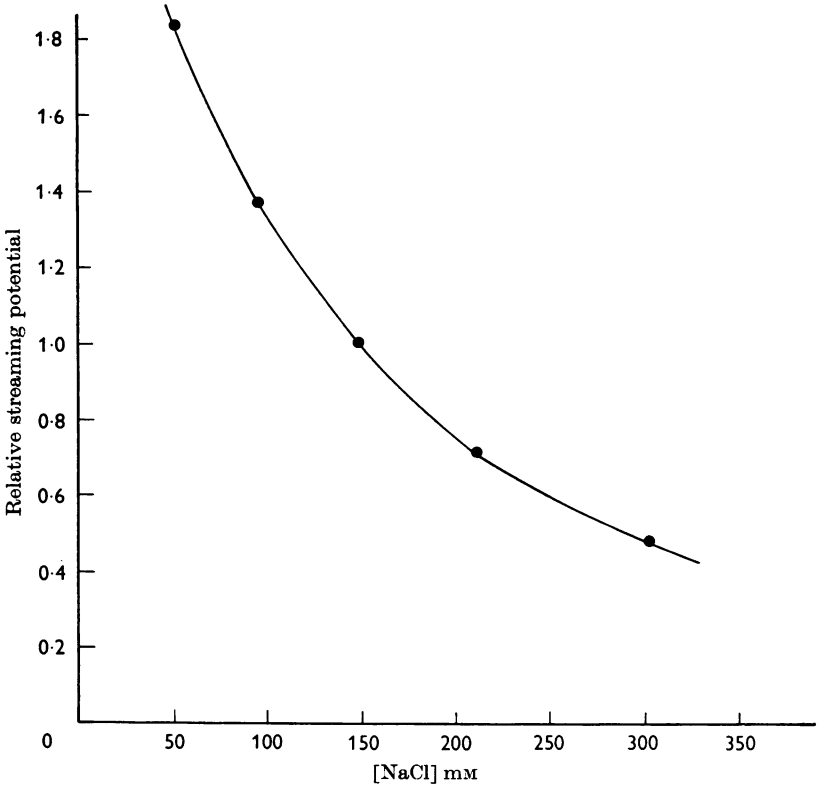

Fig. 3. Streaming potentials as a function of [NaCl] in an everted gall-bladder (osmolarity not maintained constant). Both bathing solutions had the same electrolyte composition (as solution *A*, but with [NaCl] as indicated on the abscissa). At any given [NaCl], the streaming potential resulting from addition of 100 mM sucrose to the mucosal solution was then measured. The ordinate gives the streaming potential at a given [NaCl] relative to the streaming potential at [NaCl] = 148 mM.

Since increases in salt concentration at constant osmolarity fail to decrease the streaming potential, the large decreases in streaming potentials with increasing salt concentration seen in Fig. 3, where the osmolarity was not held constant, must be due to the osmotic effect of the salt. That is, increases in osmolarity *per se* must decrease the streaming

potential. The magnitude of this effect was estimated from Figs. 3 and 4 on the assumption that the effects of osmolarity and of salt at constant osmolarity are multiplicative. For example, from Fig. 3 (osmolarity varying) the streaming potential at [NaCl] = 50 mM is 1·84 times the streaming potential at [NaCl] = 148 mM. From Fig. 4, the effect of this decrease in [Na] at constant osmolarity is to reduce the streaming potential by 20 %, i.e. to multiply it by 0·80. Hence the effect of osmolarity alone is to multiply the streaming potential at [NaCl] = 50 mM by 2·30 (1·84 ÷ 0·80) with respect to the streaming potential at [NaCl] = 148 mM. By this procedure Fig. 5 was constructed to show the effect of osmolarity

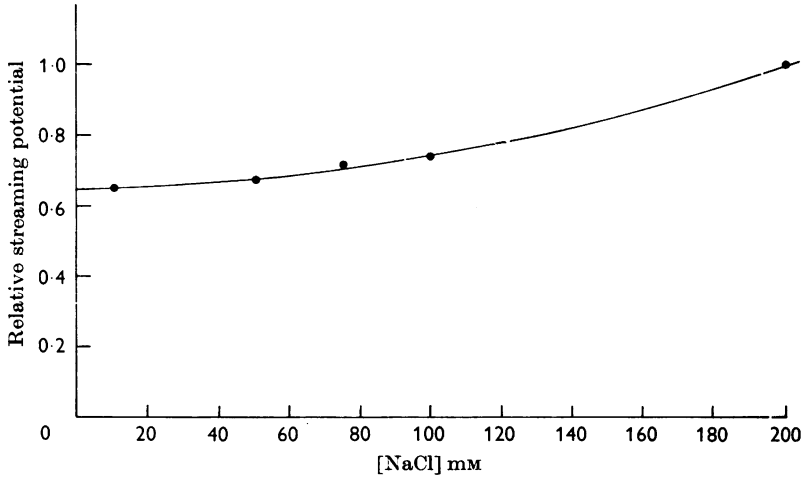

Fig. 4. Streaming potentials as a function of [NaCl] in an everted gall-bladder (osmolarity maintained constant). Both bathing solutions had the same electrolyte composition (as solution *A*, but with [NaCl] as indicated on the abscissa). The experiment differs from that of Fig. 3 in that sufficient sucrose was added to both bathing solutions to maintain a constant osmolarity of 387 m-osM. At any given [NaCl], the streaming potential resulting from addition of a further 100 mM sucrose to the mucosal solution was then measured. The ordinate gives the streaming potential at a given [NaCl] relative to the streaming potential at [NaCl] = 200 mM.

alone upon the streaming potential, and it appears that an increase in osmolarity from 163 to 620 m-osM due to added NaCl reduces the streaming potential by 87 %. Increases in osmolarity due to non-electrolytes have qualitatively and quantitatively similar effects (Diamond, 1966*a*), and the same increase in osmolarity (from 163 to 620 m-osM) due to added sucrose reduces streaming potentials by 90 %.

A priori, the decrease in streaming potentials brought about by higher osmolarities might result either from a decrease in the difference between cation and anion permeabilities, or else from a decrease in water flow. The

former postulate is shown not to hold in this paper (p. 51). The following paper (Diamond 1966*a*) shows the latter explanation to be the correct one.

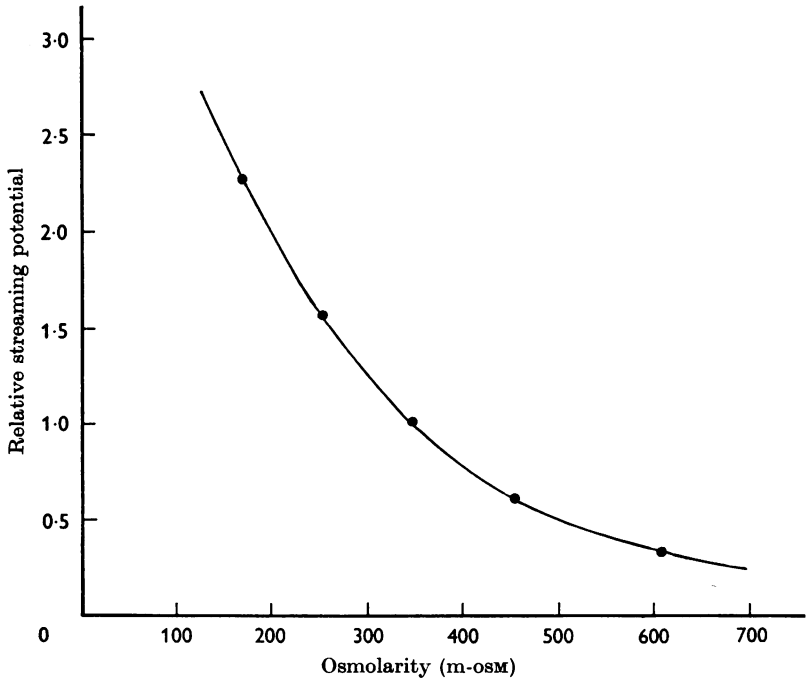

Fig. 5. The effect of osmolarity upon streaming potentials in an everted gall-bladder. Both bathing solutions had the same electrolyte composition (as solution *A*, but with different values of [NaCl] chosen). At any given [NaCl] the streaming potential resulting from addition of 100 mM sucrose to the mucosal solution was then measured. The abscissa gives the average osmolarity of the mucosal and serosal solutions during the measurement of each streaming potential. The ordinate gives the relative streaming potential at each average osmolarity (each [NaCl]), corrected for the effect of [NaCl] at constant osmolarity as explained in the text.

Diffusion potentials

NaCl diffusion potentials. Whereas streaming potentials are the p.d.'s resulting from an osmotic gradient in the absence of an electrolyte concentration gradient, diffusion potentials can result from an electrolyte concentration gradient in the absence of an osmotic gradient. Diffusion potentials were elicited in the gall-bladder by replacing part of the NaCl (or Na_2SO_4) in the bathing solution on one side isosmotically with sucrose. It was found that the side with the lower salt concentration became electrically positive by up to 27 mV, indicating greater permeability to cations than to anions. Figure 6 shows the results of an experiment in which one gall-bladder was exposed to eight different concentrations of

NaCl in the mucosal bathing solution. Reversing the bathing solutions generally gave a p.d. of the same magnitude, though of opposite sign. However, reducing the NaCl concentration in the serosal bathing solution to less than half that in the mucosal solution led to decaying p.d.'s and irreversible damage, whereas p.d.'s resulting from complete replacement of NaCl in the mucosal bathing solution were stable and reversible.

The size of diffusion potentials across a membrane permeable to both cations and anions is not uniquely determined by the concentration gradient and relative permeabilities, but depends also upon the voltage profile through the membrane. Should the voltage gradient through the membrane be linear (i.e. the field constant), then diffusion potentials (E, in mV) may be calculated from the constant-field equation (Goldman, 1943; Hodgkin & Katz, 1949)

$$E = \frac{RT}{F} \log \frac{C^m_{Na}\gamma^m_{Na}P_{Na} + C^m_K\gamma^m_K P_K + C^s_{Cl}\gamma^s_{Cl}P_{Cl}}{C^s_{Na}\gamma^s_{Na}P_{Na} + C^s_K\gamma^s_K P_K + C^m_{Cl}\gamma^m_{Cl}P_{Cl}},$$

where C's are concentrations, γ's single-ion activity coefficients, and P's relative permeability coefficients; and the superscripts m and s refer to the mucosal and serosal bathing solutions respectively. R, T, and F have their usual meanings, and the factor RT/F is 58 mV at room temperature. If P_{Na} is taken arbitrarily as 1·00, then one may calculate the relative chloride and potassium permeabilities (P_{Cl} and P_K) from this equation, the measured p.d. (E), the bathing solution concentrations (C), and γ's taken from Robinson & Stokes (1959) and Moore & Dietschy (1964).

The results given in the next section show P_K in rabbit gall-bladder to be on the average 2·3 (i.e. relative to a P_{Na} of 1). Then the observed NaCl diffusion potentials inserted into the constant-field equation imply that P_{Cl} is $0{\cdot}33 \pm 0{\cdot}05$ (eight gall-bladders). For any one gall-bladder the calculated P_{Cl} was independent of the NaCl concentration gradient. The theoretical curve in Fig. 6 is the p.d. expected for $P_{Cl} = 0{\cdot}31$ (the average value in this particular gall-bladder), and the p.d.'s obtained from this gall-bladder for a range of mucosal NaCl concentrations from 0 to 200 mM all fall close to this theoretical curve. Furthermore, the calculated P_{Cl} was independent of the absolute NaCl concentrations as well as of the concentration gradient. For example, a 2:1 concentration gradient was tested on one gall-bladder at two different absolute concentrations: 200 mM-NaCl serosal, 100 mucosal; and 100 serosal, 50 mucosal. The p.d. in the first case was +7·4 mV, yielding $P_{Cl} = 0{\cdot}31$; and in the second case was +7·0 mV, yielding $P_{Cl} = 0{\cdot}29$.

It is somewhat surprising that the constant-field equation fits the experimental results so well. Prevailing models of the cell membrane picture the voltage gradient as being far from linear, and often assume

a voltage step analogous to a Donnan potential at either boundary between the membrane and bathing solution. In addition, the gall-bladder is not a single membrane but two membranes in series. While it is true that almost any assumed voltage gradient would fit the experimental results if the anion permeability were sufficiently low, this is not true in the present case, where P_{Cl} is high enough to reduce the p.d. for a ten-fold activity ratio to 22 mV from the Nernst value of 58 mV.

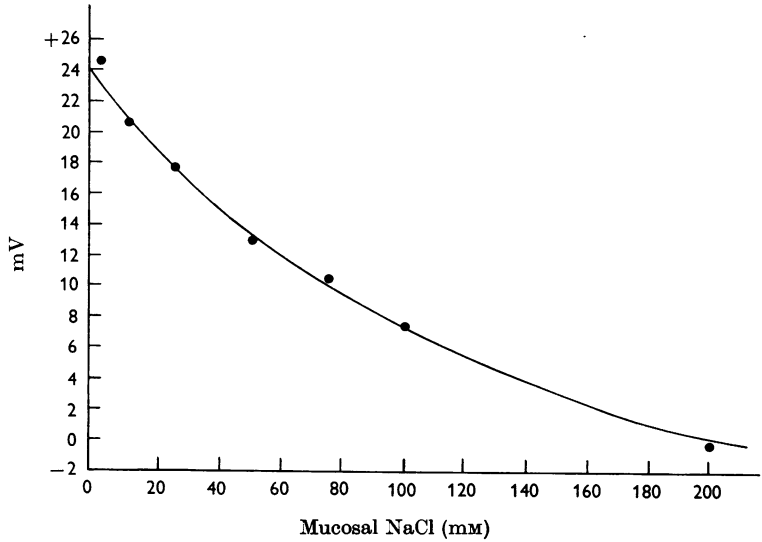

Fig. 6. Diffusion potentials resulting from a NaCl concentration gradient in an everted gall-bladder. Throughout the experiment the serosal solution was as solution *A* but with [NaCl] = 200 mM. Each experimental point is the p.d. (mV) when [NaCl] in the mucosal solution was reduced to the value shown on the abscissa by isosmotic replacement with sucrose. The curve is the p.d. predicted by the constant-field equation for $P_{Cl}/P_{Na} = 0{\cdot}31$, $P_K/P_{Na} = 2{\cdot}3$. A positive p.d. means mucosal solution positive.

Potassium diffusion potentials. In the case of streaming potentials and NaCl diffusion potentials, a change in bathing solution composition was followed by a rise of the p.d. to a new, stable steady-state level after a diffusion delay, and this p.d. decayed back to zero when the former bathing solution was restored. An exception to this pattern is provided by changes of potassium concentration in the mucosal bathing solution. As seen in Fig. 7, a sudden increase in mucosal [K] led to the rapid build-up of a transiently high negative p.d. ($-8{\cdot}3$ mV), which decayed more slowly to a negative steady-state p.d. ($-4{\cdot}0$ mV). Reducing mucosal [K] back to the level of the serosal bathing solution made the p.d. rapidly overshoot zero and go positive to $+3{\cdot}2$ mV, before slowly decaying back to zero. When [K] was changed in the serosal bathing solution, the p.d.

gradually approached a new steady-state value without any overshoot. The steady-state p.d. was the same in magnitude, though opposite in sign, for an increase in mucosal or serosal [K]. The sign of the p.d. indicates that the gall-bladder is more permeable to K than to Na.

It is unlikely that these potassium transients involve changes in potassium permeability with time or with concentration, since the relative potassium permeability (P_K/P_{Na}) deduced from steady-state p.d.'s is independent of concentration. The transient overshoot is apparently related to the fact that the gall-bladder is relatively permeable to K, because similar overshoots appear in streaming potentials resulting from

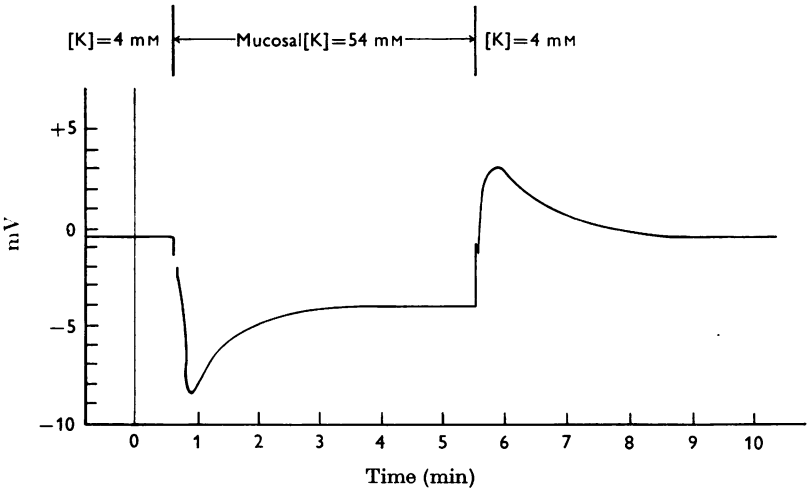

Fig. 7. Transient p.d. responses to changes of mucosal [K] in an everted gall-bladder. The serosal solution was as solution *A* throughout but with [K] = 4 mM. The mucosal solution was identical to the serosal solution, except that between the arrows [K] was increased to 54 mM by mole-for-mole replacement of NaCl with KCl. A positive p.d. means mucosal solution positive.

addition of permeant non-electrolytes to the mucosal solution, whereas streaming potentials caused by gradients of impermeant non-electrolytes, and diffusion potentials from gradients of the relatively impermeant NaCl and Na_2SO_4, involve no such overshoots. Probably K diffuses through the epithelial cells from a high-[K] mucosal solution sufficiently fast for an appreciable concentration of K to build up in the connective tissue just beyond the serosal face of the epithelial cells. The effective concentration gradient of K across the cells thus gradually decreases, causing the p.d. to decrease below its maximal value. Restoring a mucosal solution with low [K] unmasks this locally high [K] at the serosal face, temporarily causing a reversed K diffusion potential and thus making the p.d. overshoot. Since the cells face the mucosal solution directly but are separated from

the serosal bathing solution by connective tissue, K leaking through the cells from the serosal side diffuses immediately into the mucosal bathing solution, hence changes in serosal [K] cause no overshoot in the p.d.

To obtain P_K/P_{Na} from the constant-field equation, the maximal value of the p.d. after an increase in mucosal [K] was inserted, before the effective concentration gradient had begun to decline. The average value of P_K/P_{Na} so calculated for five gall-bladders was $2{\cdot}3 \pm 0{\cdot}3$, but even this may be an underestimate of the true value. It is interesting that fish gall-bladder, unlike rabbit, does not discriminate between K and Na ($P_K/P_{Na} = 1$ in fish gall-bladder; Diamond, 1962*b*).

The effect of osmolarity upon diffusion potentials. The results of this paper (Fig. 5) and the following paper (Diamond, 1966*a*) show that streaming potentials are lower in solutions of high osmolarity. If this effect were due to a decrease in P_{Cl}/P_{Na}, then diffusion potentials should also be smaller at higher osmolarities. To test this hypothesis, diffusion potentials were measured when mucosal [NaCl] was reduced from 200 mM to 100, 50, or 0 mM, serosal [NaCl] always being maintained at 200 mM. The experiment was repeated on the same gall-bladder with the osmolarity of the mucosal and serosal bathing solutions increased equally by addition of 200 mM sucrose, and then by addition of 400 mM sucrose. The results are presented in Fig. 8, where it is obvious that the p.d. resulting from a given concentration gradient of NaCl is independent of the osmolarity. Hence there can be no decrease in P_{Cl}/P_{Na} with osmolarity to explain the decrease of streaming potentials with osmolarity.

Simultaneous diffusion potentials and streaming potentials. For measurement of the NaCl diffusion potentials discussed previously, reductions in [NaCl] in one bathing solution were balanced by isosmotic addition of sucrose so that there could be no osmotic gradient or streaming potential. As mentioned above, the solution with lower [NaCl] went electrically positive by up to 27 mV. However, when a small NaCl concentration gradient (e.g. 210 mM-NaCl serosal, 148 mM mucosal) was present across the gall-bladder without sucrose to maintain osmolarity, the solution with higher [NaCl] went positive by up to 2 mV. Under these circumstances, the salt concentration gradient also represented an osmotic gradient. It is not surprising that the resulting p.d.'s were small, since osmotic water flows and the streaming potential were oriented to make the concentrated solution positive, while the contribution of the diffusion potential would have been to make the concentrated solution negative. Evidently the streaming potential is larger for the small gradients tested, as was to be expected on quantitative grounds.

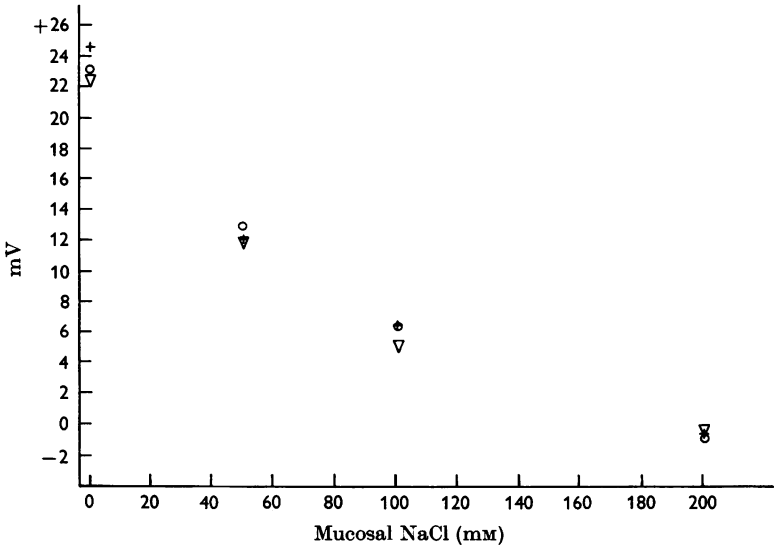

Fig. 8. The effect of osmolarity upon diffusion potentials in an everted gall-bladder. Throughout the experiment the electrolyte composition of the serosal solution was as solution *A* but with [NaCl] = 200 mM. Each experimental point is the p.d. (mV) when [NaCl] in the mucosal solution was reduced to the value shown on the abscissa by isosmotic replacement with sucrose. +, 400 mM; ▽, 200 mM; ○, 0 mM additional sucrose added to both mucosal and serosal solutions.

The effect of calcium

Figure 9 illustrates an experiment in NaCl Ringer's solution, in which both bathing solutions initially contained 0·25 mM-Ca. Reducing mucosal [NaCl] from 150 to 75 mM gave a diffusion potential of 6·35 mV. Increasing mucosal [Ca] from 0·25 to 5 mM then reduced this diffusion potential to 3·8 mV, and the p.d. returned to 6·1 mV when the original low [Ca] was restored. On the average, this increase in mucosal [Ca] reduced NaCl diffusion potentials by $45 \pm 5\%$ (four determinations) and streaming potentials by $31 \pm 5\%$ (three determinations). The recovery of the p.d.'s was often still incomplete 2 min after low [Ca] had been restored, suggesting that some Ca was difficult to wash off the membrane.

When [Ca] was increased from 0·25 to 5 mM in both mucosal and serosal bathing solutions, p.d.'s were reduced still further (Table 2): by $40 \pm 7\%$ (six determinations) for streaming potentials, by $62 \pm 1\%$ (six determinations) for NaCl diffusion potentials, and by $43 \pm 13\%$ (three determinations) for potassium diffusion potentials. The results given in Table 2 are typical of four experiments. In these experiments the effect was completely reversible when 90 min were allowed to wash away high [Ca]. An increase

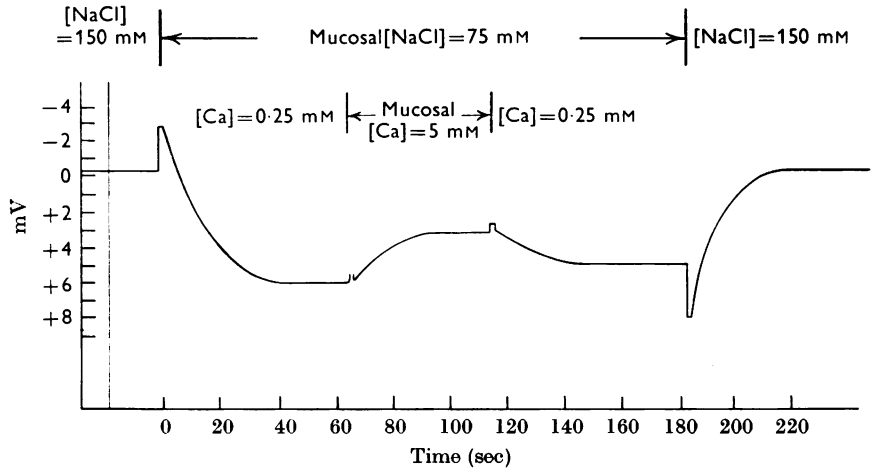

Fig. 9. Effect of calcium upon NaCl diffusion potentials in an everted gall-bladder. The serosal solution was solution *A* throughout. The mucosal solution was identical to the serosal solution, except that between the upper arrows [NaCl] was reduced to 75 mM by isosmotic replacement with sucrose, and between the lower arrows [Ca] was increased to 5 mM by addition of isosmotic $CaCl_2$. The instantaneous deflections of the p.d. trace when mucosal [NaCl] is changed are the transient junction potentials between the new mucosal solution and the former mucosal solution clinging to the gall-bladder.

TABLE 2. Effect of raised [Ca] on p.d.'s

	[Ca] = 0·25 mM (mV)	[Ca] = 5·0 mM (mV)	p.d. at 5·0 mM-Ca / p.d. at 0·25 mM-Ca
NaCl diffusion potentials			
NaCl/50% sucrose	6·95	2·7	0·39
NaCl/75% sucrose	12·5	4·6	0·37
NaCl/100% sucrose	19·4	7·1	0·37
K diffusion potentials			
NaCl/50% KCl	6·3	3·6	0·57
Streaming potentials			
NaCl/NaCl + 50 mM sucrose	2·2	1·15	0·52
NaCl/NaCl + 200 mM sucrose	6·6	3·95	0·60
NaCl/NaCl + 400 mM sucrose	10·6	7·1	0·67

The numbers are the p.d. (mV) when [Ca] on both sides of the gall-bladder was 0·25 mM (second column) or 5·0 mM (third column). The fourth column is the ratio of the third column to the second. The first column gives the concentration gradient. The serosal solution was solution *A* (Table 1) throughout ('NaCl'). Diffusion potentials were produced by replacing 50, 75, or 100% of the NaCl in the mucosal solution with sucrose, or 50% of it with KCl; streaming potentials were produced by adding 50, 200, or 400 mM sucrose to the mucosal solution. The expression 'NaCl/50% sucrose' thus means, for example, that the serosal solution is NaCl Ringer's solution, while the mucosal solution is NaCl Ringer's solution in which 50% of the NaCl has been replaced with sucrose.

in [Ca] from 0·25 to 1·5 mM decreased streaming potentials by almost as much as did an increase from 0·25 to 5 mM.

The percentage of the full inhibition (5 mM-Ca both sides) caused by 5 mM-Ca in the mucosal solution alone was approximately the same for streaming potentials and NaCl diffusion potentials: 77 and 73%, respectively.

DISCUSSION

Comparison with other published results. The values for diffusion potentials and streaming potentials presented in this paper are in good quantitative agreement with results obtained in other studies on rabbit gall-bladder. Dietschy's (1964) value for P_{Cl}/P_{Na} is 0·33, identical with ours. He calculated P_K/P_{Na} from steady-state potassium diffusion potentials rather than from the peak p.d., and obtained a value of 1·5. The same method of calculation would have yielded $1{\cdot}59 \pm 0{\cdot}16$ in our case, but the value obtained from the peak p.d. ($P_K/P_{Na} = 2{\cdot}3$) is probably more meaningful, as already discussed. Wheeler's (1963) flux values for K and Na also imply a P_K/P_{Na} of about this size. Our streaming potentials in NaCl Ringer's solution (3·6 mV/100 m-osM) are higher than those of Dietschy (2·3–2·7 mV/100 m-osM) or of Pidot & Diamond (1964; 2·2 mV/100 m-osM) by about the amount to be expected from our lower calcium concentrations.

Fixed charges as the cause of the p.d.'s. The signs of the diffusion potentials and streaming potentials in NaCl or Na_2SO_4 Ringer's solutions indicate that rabbit gall-bladder is more permeable to Na and K than to either Cl or SO_4, even though the hydrated diameter of Na is 50% larger than that of Cl. Fish gall-bladder is also more permeable to Na and K than to all anions tested (Cl, Br, CH_3SO_4, SO_4), and discriminates even more sharply (P_{Cl}/P_{Na} 0·04).

The only plausible explanation for these large and consistent differences between cation and anion permeabilities is the presence of negative fixed charges in the channels through which electrolytes and water traverse the cell membrane. These negative fixed charges would hinder the diffusion of anions, and reduce the concentration of mobile anions relative to mobile cations in the membrane channels. It may help in picturing the origin of streaming potentials if one thinks of the fluid in the membrane channels as having an excess of mobile cations over mobile anions, sufficient to balance the negative fixed charges of the membrane matrix. Then water flow through these channels would sweep out net positive charges until that streaming potential was built up at which the sum of frictional and electrical forces drove through Na and Cl at the same rates. While the higher permeability to K than to Na might be due in part to the smaller hydrated diameter of K, the fixed charges may also play a role here, as

suggested by the fact that high [Ca] reduces K diffusion potentials as well as streaming potentials and NaCl diffusion potentials. Eisenman (1961) has discussed how negative fixed charges might be responsible for discrimination among cations.

However, an important consequence of these results is that Na does not diffuse across the cell membrane by leaving the aqueous phase and moving along a series of fixed charge sites. The existence of streaming potentials implies a frictional transfer of momentum from water to ions, and this would be impossible if water and ions had quite separate routes of passive permeation through the membrane.

The experiments on diffusion potentials show that these fixed charges are relatively stable entities, unaffected by the salt concentration or the osmolarity of the bathing solution. The only agent found to reduce NaCl diffusion potentials was calcium, which probably acts by binding and masking the fixed charges. Changes in streaming potentials, however, can be caused by changes either in fixed charges or in water permeability. The effect of calcium on streaming potentials must involve the fixed charges, as indicated by its simultaneous effect upon diffusion potentials. However, the changes in streaming potentials with osmolarity are due solely to changes in water permeability (Diamond, 1966*a*), and the same is probably true of the small effects of NaCl at constant osmolarity.

Physiological significance of the fixed charges. Low values of P_{Cl}/P_{Na} suggest that negative fixed charges are a property of gall-bladders from all species examined so far (pike, roach, cat, rabbit). The physiological significance of the charges may be to expedite the main function of the gall-bladder, the selective reabsorption of bile. As synthesized by the liver, bile consists of an isotonic solution of sodium chloride and of the sodium salts of bile acids. In the gall-bladder the bile is concentrated to as little as one-tenth of its original volume by reabsorption of NaCl but not of the sodium bile salts (Diamond, 1962*a*). Hence the activity of sodium in concentrated gall-bladder bile remains near plasma levels, but that of chloride drops far below plasma levels. The limit to the concentrating process occurs when [Cl] in bile drops to that level at which the rate of outward active transport of Cl equals the rate of inward diffusion of Cl down its concentration gradient from plasma. The lower the chloride permeability, the lower the rate of inward chloride diffusion will be, and hence the lower a limiting chloride concentration can be maintained. Vertebrates may thus have evolved negative fixed charges in the gall-bladder because, by reducing the chloride permeability, the charges increase the effectiveness of the gall-bladder in concentrating bile.

Identity of the fixed charges. Finally, it is appropriate to enquire into the molecular basis of the negative fixed charges, which are probably some

ionizable (acidic) group in the membrane. The same problem comes up in connexion with the negative surface charges responsible for the electrophoretic mobility of such isolated cells as erythrocytes (Ponder, 1948), lymphocytes (Bangham, Pethica & Seaman, 1958), HeLa cells (Glaeser, 1963), and TA3 ascites tumour cells (Glaeser, 1963). At neutral pH all these cells bear negative surface charges, whose magnitude decreases at lower pH's and becomes zero at an iso-electric point between pH 1 and 3. It has recently been shown for erythrocytes (Eylar, Madoff, Brody & Oncley, 1962) and ascites tumour cells (Wallach & Eylar, 1961) that the charged group responsible is sialic acid (*N*-acetyl neuraminic acid). Whatever acidic group is responsible for the low permeability of the gall-bladder to anions, it too must have a low pK_a, since pH changes in the range 6·3–7·62 had little or no effect on streaming potentials. It is therefore possible that the same kinds of group are responsible for negative charges in the aqueous channels through cell membranes as for the charges at cell surfaces. Alternatively, the charged groups in the channels could be certain amino-acid side-chains or else membrane lipids (phosphate diesters).

This work was carried out during the tenure of a Junior Fellowship from the Society of Fellows, Harvard, by one of us (Jared M. Diamond), and was supported in part by the Atomic Energy Commission. We are indebted to Dr A. K. Solomon for advice and support, to Dr S. Schultz for a critical reading of the manuscript and to Dr W. Bossert for computer calculations.

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