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The Role of the Lateral Intercellular Spaces and Solute Polarization Effects in the Passive Flow of Water across the Rabbit Gallbladder

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Summary. Osmotic water flows were measured across *in vitro* preparations of the rabbit gallbladder by a gravimetric technique. The bladders exhibited asymmetrical osmotic behavior, in which the L_p (hydraulic conductivity) for water flow from mucosa to serosa was up to four times greater than the L_p for water flow in the opposite direction. This result is similar to the effects of osmotic gradients on ion and nonelectrolyte permeability reported in the first paper. As in the case of solute permeability, these changes in L_p are accounted for by changes in the dimensions of the lateral intercellular spaces of the epithelium. These spaces are thus a final common pathway for the movement of both solutes and water across the epithelium. We also observed osmotic flow transients in which the initial L_p was about an order of magnitude greater than the steady state L_p. These transients are largely explained by solute polarization in the unstirred layers adjacent to the epithelial membranes. A comparison between streaming potentials and water flows showed that streaming potentials are directly proportional to the rate of flow only over a limited range. These observations are readily explained on the basis of structural changes and solute polarization effects. Finally, the routes of water flow across epithelia are discussed in the light of our observations.

In the previous paper (Smulders, Tormey & Wright, 1972, hereafter referred to as Paper 1) we found that osmotic gradients modified both the permeability and structure of the rabbit gallbladder. Increasing the osmolarity of the mucosal fluid reduced ion and nonelectrolyte permeation to about one-third of the control value and reduced the width of the lateral intercellular spaces. On the other hand, increasing the osmolarity of the serosal fluid had a much smaller effect on permeation, although the lateral spaces dilated widely. Our interpretation of these results is that the lateral

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intercellular spaces are a common pathway shared by all solutes crossing the epithelium and that diffusion along these spaces becomes rate limiting when they are collapsed.

Since there is reason to suspect that passive water flows across the epithelium also proceed via the lateral spaces, the question now arises whether osmotic gradients produce asymmetrical effects on water flows. To answer this question we decided to investigate the effect of osmotic gradients on the hydraulic conductivity (L_p) of the rabbit gallbladder. Our results show that there is indeed an asymmetry of flow across the tissue that is largely accounted for by the changes in dimensions of the lateral spaces. In addition, these experiments show that solute polarization effects (unstirred layer effects) greatly alter the magnitude of the observed L_p's. These phenomena have widespread implications regarding the mechanisms of water flow across epithelial membranes.

Methods

The hydraulic water permeability (L_p) of the rabbit gallbladder was determined at room temperature by the gravimetric procedure described in detail by Diamond (1962, 1964, 1966*a*). Briefly, the gallbladder (everted or non-everted) was cannulated, and the lumen filled with bicarbonate-free saline before suspending the preparation in a beaker of saline stirred with a stream of oxygen bubbles. Water flow was measured by weighing the sac and its contents at 5- or 15-min intervals on an analytical balance. L_p's were obtained from the change in water flow when either the luminal or external solution was replaced with saline made hypertonic with sucrose. In the present experiments we may neglect any possible effects of osmotic gradients on solute linked water transport, since at room temperature in bicarbonate-free saline the rate of fluid transfer across the gallbladder is very low, i.e., about 5 mg/hr/cm^2. The serosal area of the sac was estimated at the end of the experiment by slitting it open and measuring the area of the flat sheet.

All other experimental methods and procedures were carried out at 22 to 24 °C with chamber preparations as described in previous papers (Smulders & Wright, 1971, and Paper 1). Statistical errors are quoted as the standard error of the mean.

Results

L_p Measurements

a) Osmotic Water Flows from Mucosa to Serosa. These flows were generated by replacing the serosal saline solution (the external solution in non-everted gallbladders and the luminal solution in everted sacs) with saline made hypertonic by the addition of sucrose. Within 10 min of replacing the serosal solution the flow reached a steady state. Table 1 lists the L_p's obtained with 50 and 300 m-molal (mM) sucrose. Although there was

Table 1. Gallbladder L_p's

Sucrose (mM)	L_p (cm/sec × 10^3)		
	$J_{s\to m}$	$J_{m\to s}$	P
50	2.4±0.4 (7)	3.3±0.6 (10)	>0.20
300	1.4±0.1 (5)	4.7±0.6 (8)	<0.001

These L_p's were obtained from gravimetric water flow experiments on everted and non-everted gallbladders where osmotic flows were generated by the addition of sucrose to either the mucosal or serosal solutions to give final concentration of either 50 or 300 mM. All values are steady state L_p's and these are expressed on the basis of the serosal area of the sac. Included are the P values for the differences between the L_p's measured in opposite directions at the same gradient. In addition, it should be pointed out that there is no significant difference between the two L_p's obtained by the addition of 50 and 300 mM sucrose to the serosal fluid. ($P>0.1$), but that there is a significant difference between the two values obtained by the addition of sucrose to the mucosal fluid ($P<0.05$). Finally, it should be noted that a L_p of 1 cm/sec is equivalent to 7.5 cm/sec, atmos, and 0.02 cm/sec, osmol.

no statistical difference between the average L_p's obtained with the two gradients, in the three gallbladders where we measured the flows at both 50 and 300 mM sucrose the L_p at the higher concentration was on the average only about 80% of that obtained at the lower gradient.

This deviation from linearity in flow is close to the 31% deviation predicted for the sweeping away effect (Dainty, 1963), i.e., with 300 mM sucrose in the serosal solution the sucrose concentration at the serosal face of the epithelial cells should be reduced to about 210 mM by the flow of water across the tissue. [$C_m = C_s e^{(-V\delta_s/D)} \simeq 210$ mM; where $C_s = 300$ mM; V, the velocity of water flow in the unstirred layer, is 5.2×10^{-6} cm/sec (calculated from the Table 1 assuming that about half the volume of the subepithelial tissue is available for flow); $D_{\text{sucrose}} = 5.2\times10^{-6}$ cm^2/sec; and δ_s, the thickness of the serosal unstirred layer in sac preparations is 375 μ (calculated from streaming potential half times obtained by Diamond, 1966*b*)[1].]

b) Osmotic Water Flows from the Serosa to the Mucosa. These flows were produced by replacing the mucosal solution with saline-made hypertonic with sucrose. In everted sac preparations, upon addition of sucrose to the mucosal fluid there was a high rate of flow which declined rapidly to a steady state value of about one-tenth of the initial rate of flow. This is illustrated in Fig. 1 for one experiment: In the absence of an osmotic gradient the rate of change in weight was constant at a few mg/hr, but on addition of sucrose (50 mM) to the mucosal solution there was a high initial rate of flow of about 300 mg/hr, which declined to a steady state flow of about 30 mg/hr some 30 min later. Owing to the limitations of the gravi-

1 *See* pages 209 through 215 for further details of unstirred layer effects.

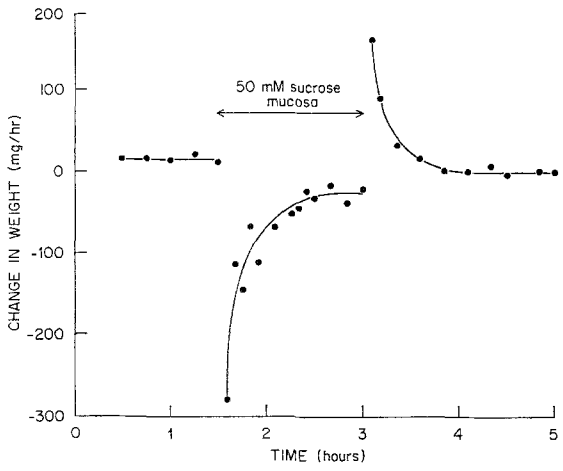


Fig. 1. The rate of water flow across rabbit everted gallbladder as a function of time. Water flows were measured gravimetrically every 5 min and the rate of change in weight (in mg/hr) is plotted on the ordinate against time on the abscissa; positive changes in weight mean that water is flowing from mucosa to serosa and negative changes mean that water is flowing from serosa to muscosa. The serosal solution (luminal) was regular Ringer's solution throughout and the external solution (mucosal) was also Ringer's solution except during the time indicated, where it was replaced with a solution identical in ionic composition but containing in addition 50 mM sucrose

metric technique it was not possible to obtain accurate measurements before a time lapse of 5 min. Thus the initial rate may be grossly underestimated and the time course somewhat distorted. On returning a sucrose-free solution to the mucosal surface of the gallbladder there was a rebound in the weight of the sac and its contents, i.e., water flowed from the mucosa to serosa at a high initial rate which declined rapidly towards the rate initially obtained in the absence of osmotic gradients.

These transients did not arise in non-everted gallbladders probably because the gravimetric procedure does not record transient water movements between the submucosal and luminal compartments. The presence of the thick serosal unstirred layers (Diamond, 1966*b*; Smulders & Wright, 1971) probably explains why similar transients were not seen when sucrose was added to the serosal fluid in non-everted preparations (*see also* p. 202).

The L_p's obtained from steady state flows from serosa to mucosa in everted and non-everted gallbladders are included in Table 1. The first point to note is that there was no statistically significant difference between the L_p's for the flows produced by 50 mM gradients in either direction. The second point is that the L_p for the $S \rightarrow M$ flow is about three times less than the L_p for the $M \rightarrow S$ flow when 300 mM sucrose was used to generate

the osmotic flows. (In three gallbladders, one everted and two non-everted, the mucosa to serosa flow with a gradient of 300 mM was on the average four times greater than the reverse flow.) Thus, there is an asymmetry of osmotic flow across the gallbladder at the higher osmotic gradients. This asymmetry may be described as a decrease in the hydraulic conductivity as the sucrose concentration in the mucosal fluid is increased. The magnitude of this decrease in L_p is comparable to the decrease in ion and nonelectrolyte permeability obtained with the same increase in the mucosal sucrose concentration (Paper 1).

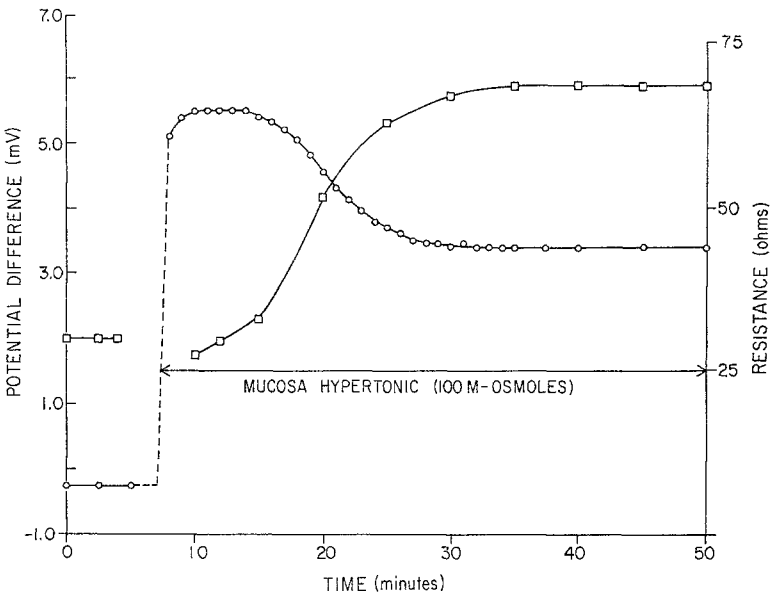
In summary, these steady state measurements show that there is an asymmetry of osmotic flow across the gallbladder and that this may be ascribed to a decrease in water conductivity as the osmolarity of the mucosal solution is increased. There is little change in water conductivity as the osmolarity of the serosal fluid is increased.

Streaming Potentials

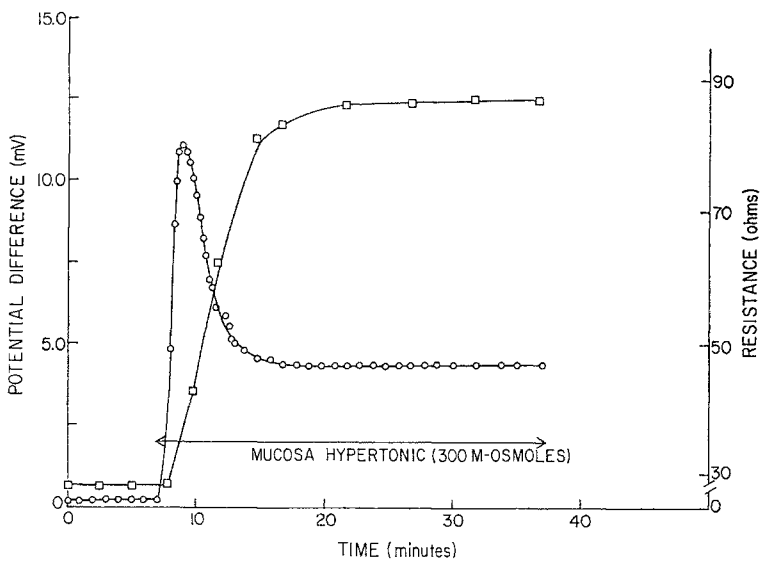
In some biological membranes osmotic water flows generate electrical potential differences which have been called streaming potentials; for example, in the gallbladder (Diamond & Harrison, 1966), in the intestine (Smyth & Wright, 1966), in nerve (Vargas, 1968) and in the choroid plexus (Wright & Prather, 1970).

In the present series of experiments we studied streaming potentials produced by the addition of sucrose to either the mucosal or serosal solutions. Streaming potentials and the resistance changes produced by addition of sucrose to the mucosal bathing solution are shown in Fig. 2. On adding 100 mM sucrose (Fig. 2*a*), a streaming potential of 5.5 mV built up with a half time of 12 sec. The potential difference (p.d.) remained stable for about 5 min and then it slowly declined to a new value some 2 mV lower; the half time for the decay was about 9 min. Addition of 300 mM sucrose (Fig. 2*b*) generated a streaming potential of about 11 mV, but in this case the potential did not remain stable and decayed rapidly to about 4 mV. In both experiments it will be noted that both the onset and the half time for the decay in streaming potentials coincided closely with the resistance changes. This behavior of the streaming potentials is in direct contrast to that observed in sac preparations of the gallbladder. For example, Dietschy (1964) showed an experiment where the streaming potentials, produced by addition of 300 mM sucrose to the mucosal solution, remained stable for 30 min.

Sucrose added to the serosal solution produced stable streaming potentials. This was probably because the diffusion of sucrose up to the serosal



a



b

Fig. 2. Osmotically induced potentials and resistances as a function of time. The electrical potential difference across the gallbladder (in mV) and the gallbladder resistance (in Ω) on the ordinate are plotted against time (in minutes) on the abscissa. Initially, in both experiments, the mucosal and serosal compartments contained identical Ringer's solution, but at the times indicated the mucosal solutions were replaced with Ringer's solution identical in ionic composition but containing in addition either 100 (Fig. 2*a*) or 300 (Fig. 2*b*) mM sucrose. In both graphs the open circles (○) represent the electrical potential and the open squares (□) represent the resistance

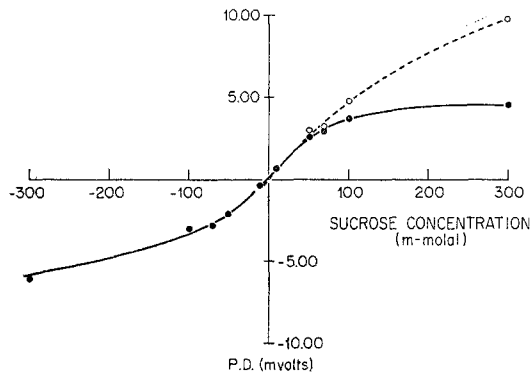


Fig. 3. Streaming potentials across the gallbladder as a function of the sucrose concentration gradient. The ordinate is the potential difference, mucosa with respect to serosa, in mV. The abscissa is the sucrose concentration gradient across the gallbladder in mosm; the osmotic gradient is taken as positive when sucrose was added to the mucosal solution and negative when sucrose was added to the serosal solution. The open circles (○) represent the maximum potentials observed while the closed circles (●) represent steady state values. All potentials plotted are average values obtained from at least two gallbladders

face of the epithelium is retarded by a rather thick unstirred layer and this will tend to damp out any transients that might otherwise occur.

Fig. 3 shows streaming potentials plotted against sucrose concentration gradients. Where sucrose was added to the mucosal solution, both the maximum p.d. and the final, steady state p.d. are indicated: The figure shows that the initial streaming potentials increased linearly with increasing sucrose concentration up to about 100 mM and thereafter became non-linear. The steady state streaming potentials did not appear to increase above 100 mM (*see also* Fig. 2*a* and *b*). Potentials produced by the addition of sucrose to the serosal fluid increased in a linear manner with concentration up to about 75 mM and above this concentration were non-linear. The non-linearity was such that the potentials obtained with 300 mM sucrose were only about three times greater than those obtained with 50 mM sucrose.

Discussion

These results confirm the earlier observation by Diamond (1966*a*) that there is non-linear osmosis across the rabbit gallbladder; i.e., there is an apparent decrease in the hydraulic conductivity as the osmolarity of the mucosal fluid is increased (compare the results in Table 1 with Diamond, 1966*a*, Fig. 3). We have, however, uncovered four new phenomena: (1) asymmetry of water flow; (2) osmotic flow transients; (3) an imperfect

correlation between streaming potentials and water flows; and (4) streaming potential transients. It will become apparent in the ensuing discussion that these phenomena and non-linear osmosis are all interrelated, that the asymmetry of flow is largely due to changes in the dimensions of the lateral intercellular spaces, and that water flow transients are largely due to polarization effects in the serosal unstirred layer. The implications of these phenomena for the route of water flow across epithelial membranes will be explored.

Asymmetrical Water Flows

The steady state water flow measurements showed that, although there was no significant difference between the osmotic flows directed towards the mucosa and serosa at low osmotic gradients (50 mM sucrose), there was a considerable degree of asymmetry at higher gradients. With gradients of 300 mM sucrose the flow from mucosa to serosa was on the average of three times greater than the flow in the opposite direction. A similar conclusion can be drawn from previous experiments on the gallbladder which showed that hypertonic mucosal solutions generate less osmotic flow than hypotonic mucosal solutions (Diamond, 1966*a*, Fig. 1). There have been reports of asymmetry of osmotic flow across other epithelial membranes; for example, intestine (Loeschke, Bentzel & Csáky, 1970), proximal tubule (Bentzel, Parsa & Hare, 1969) and toad urinary bladder (Bentley, 1961). In all cases the flow to the serosa was greater than the flow in the opposite direction. For instance, in the intestine the flow to the serosa was found to be 6 to 9 times greater than the osmotic flow in the opposite direction.

In the rabbit gallbladder the results are similar to the effects of osmotic gradients on ion and nonelectrolyte permeability; e.g., with 300 mM sucrose in the mucosal solution the permeability was about one-third of that obtained with 300 mM sucrose in the serosal solution (*see* Paper 1). We concluded in the case of solute permeation that the permeability differences were largely due to changes in morphology of the tissue. Osmotic flows towards the mucosal solution caused the extracellular spaces of both the epithelium and the other parts of the gallbladder wall to collapse while flow in the opposite direction caused these spaces to dilate. Calculations showed that diffusion through these spaces became rate limiting as they collapsed, the dominant effect being in the lateral intercellular spaces. The qualitative and quantitative similarities between the effects of osmotic gradients on the permeability of the gallbladder to water and solutes suggest that the changes in the dimensions of the lateral spaces also account for the asymmetry of water flow.

The asymmetrical effects of osmotic gradients on water flow cannot be satisfactorily explained on the basis of unstirred layer effects. The line of reasoning is similar to that used in Paper 1 to eliminate these as a possible explanation for the effect of osmotic gradients on solute permeation. (In the present case we have to take into account the effect of water flow on both the sucrose and sodium chloride concentrations; *see* p. 209 for further details.)

One way to establish whether or not the changes in the dimensions of the lateral spaces are sufficient to explain the asymmetry of water flow is to calculate the L_p of the lateral spaces under various osmotic gradients. The L_p of the spaces was calculated from the equation describing laminar flow through a narrow slit where the relation between pressure and flow is given by

$$\text{Flow} = \frac{2\Delta P B^3 W}{3\eta l}$$

(Bird, Steward & Lightfoot, 1960), where B is the half distance between the lateral membranes, W and l are the width (i.e., half the cell circumference/cm^2, *see* Paper 1, Table 2) and length of the lateral spaces, respectively, ΔP is the pressure difference between the ends of the channel (in dynes/cm^2), and η is the viscosity of the fluid in the lateral spaces. Since flow also equals $L_p\ \Delta P$ (where L_p is the hydraulic conductivity), it follows that the L_p of the channels is given by the equation

$$L_p = \frac{2B^3 W}{3\eta l}.$$

Using the dimensions of the lateral channels given in Table 2, Paper 1, the channel L_p was calculated for three conditions: namely, (1) in the absence of flow; (2) with 50 mM sucrose in the mucosal solution; and (3) with 300 mM sucrose in the mucosal solution. In making these calculations it is assumed that the viscosity of the fluid in the spaces is identical to that in the bulk phase solutions, i.e., $\eta = 0.01$ poise.

In the absence of water flow, and in the presence of flow to the mucosa generated by a 50 mM sucrose gradient, the calculations show that the L_p of the lateral spaces ($> 3.0 \times 10^{-1}$ cm/sec) was at least 100 times the measured steady state L_p. Even if the steady state L_p's are underestimated by a factor of 10, the major barrier to osmotic flow of water across the gallbladder under these conditions must be somewhere in the epithelium other than in the lateral spaces. This is corroborated by the observation that there was no significant difference between the steady state L_p's obtained

when sucrose (50 mM) was added to either the mucosal or serosal solutions; if the lateral spaces were rate limiting with sucrose (50 mM) in the mucosal solution then we should obtain a significantly higher L_p when sucrose is added to the serosal solution, since in this case the spaces would be much wider (Paper 1).

On the other hand, with 300 mM sucrose in the mucosal fluid the L_p of the lateral spaces is computed to be about 2.6×10^{-2} cm/sec. To assess the significance of this value we shall have to consider the route by which water flows across the epithelium. Water, like solutes, may flow across the epithelium by two separate pathways; namely, via the tight junctions and via the epithelial cells. (The line of reasoning is similar to that taken for solute permeation in Paper 1.) In the former case, water would flow between the mucosal solution and the lateral intercellular spaces through the tight junctions. The L_p of the epithelium ($L_p(e)$) is related to the L_p of the tight junctions ($L_p(tj)$) and lateral spaces ($L_p(ls)$) by the expression

$$1/L_p(e) = 1/L_p(tj) + 1/L_p(ls).$$

In the latter case, water would flow between the lateral spaces and the mucosal solution through the epithelial cell, traversing in turn the lateral and apical membranes. Then $L_p(e)$ is given by the expression

$$1/L_p(e) = 1/L_p(am) + 1/L_p(lm, ls),$$

where $L_p(am)$ and $L_p(lm, ls)$ are the hydraulic conductivities of the apical membrane and the lateral membrane/lateral space complex. As discussed for solute flow, $L_p(lm, ls)$ is given by the equation

$$L_p(lm, ls) = \frac{1}{\sqrt{\frac{1}{L'_p(ls)} \times \frac{1}{L'_p(lm)}} \coth \sqrt{\frac{L'_p(lm)}{L'_p(ls)}}\, l}$$

where $L'_p(ls)$ and $L'_p(lm)$ are the L_p's per unit length of the lateral space and lateral membranes, respectively, and l is the length of the lateral spaces.

We have estimated the contribution of the lateral spaces to the overall L_p when the flows are generated by 300 mM sucrose in the mucosal solution. First, we take 2.9×10^{-3} cm/sec as the value for the L_p of either the tight junctions or the combined cell membranes. [This is the average L_p obtained when sucrose (50 mM) is added to the mucosal or serosal solutions (*see* Table 1). This is a reasonable starting point, as we have already shown that the L_p of the spaces does not contribute significantly to the overall L_p under these conditions.] The results show that the closed lateral channels would

drop the L_p to 2.6×10^{-3} cm/sec, if flow is across the tight junctions, or to 2.8×10^{-3} cm/sec, if flow is through the epithelial cells, i.e., a reduction of 10% and 5%, respectively.

Apart from the uncertainties about this type of calculation that were raised in the previous paper, the significance of these particular calculations is subject to an additional uncertainty; namely, the reliability of the L_p measurements. There is good reason to believe that the gallbladder L_p's are underestimated by at least a factor of 10 (*see* Fig. 1 and below). If so, we are underestimating the importance of the lateral channels in regulating osmotic flow. Taking an L_p (of the tight junctions or cell membranes) a factor of 10 higher (i.e., 2.9×10^{-2} cm/sec), we find that the change in dimensions of the lateral channels now drops the L_p to 1.1×10^{-2} cm/sec, if flow is via the tight junctions, or to 2.1×10^{-2} cm/sec, if flow is via the cell, i.e., a reduction of about 60% and 25%, respectively. Similar calculations of the change in L_p when the sucrose concentration in the serosal solution is increased from 50 to 300 mM show that the resistance to flow along the lateral channels is infinitesimally small, i.e., increasing the sucrose concentration in the serosal fluid from 50 to 300 mM should have no effect on the hydraulic conductivity. These calculations therefore predict that the flow to the mucosal solution should be less than the flow to the serosal solution with 300 mM sucrose gradients. In other words, the changes in the dimensions of the lateral spaces appear to be sufficient to account for the observed asymmetry of flow.

Osmotic Flow Transients

It will be recalled that in everted gallbladders when sucrose was added to the mucosal solution there was a high initial rate of osmotic flow which decayed rapidly to about one-tenth of the initial rate (Fig. 1). A rebound phenomenon was also observed when the sucrose gradient was removed. Comparison of the time course of the decay in the rate of water flow and the increase in gallbladder resistance (with 50 mM sucrose in the mucosal fluid) showed that there was no close correlation between the two phenomena. In fact, the resistance had not significantly increased before the rate of flow had dropped by one-fifth. This suggests that the water flow transient is not basically related to the osmotically induced changes in solute permeability or changes in geometry. This view is supported by theoretical and experimental considerations which show that the lateral spaces are too wide to be a significant barrier to osmotic flow when 50 mM sucrose is present in the mucosal fluid (*see* above).

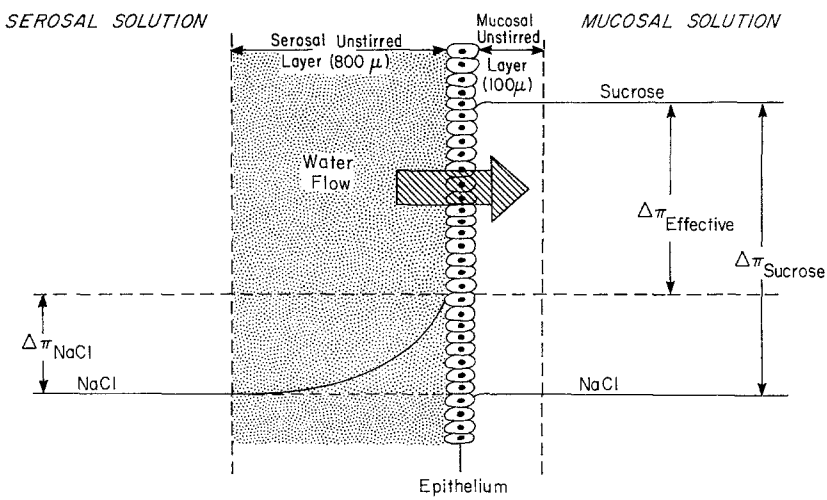


Fig. 4. A diagrammatic representation of the unstirred layer effect which gives rise to time dependent L_p's, the rebound phenomenon and streaming potentials in rabbit gallbladder. This shows the gallbladder wall separating the mucosal and serosal solutions, and the sucrose and NaCl concentration profiles across the tissue. The bulk phase mucosal and serosal solutions contain identical concentrations of NaCl. The mucosal solution also contains 50 mM sucrose which generates an osmotic flow ($\sigma_{suc} L_p \Delta\pi_{suc}$) from serosa to mucosa. This flow of water has no significant effect on the sucrose and NaCl concentrations in the mucosal unstirred layer, but in the serosal unstirred layer the flow produces an enhancement of the NaCl concentration adjacent to the serosal face of the epithelium [$C_m = C_s e^{(V\,\delta_s/D)}$, where C_m and C_s are the NaCl concentrations adjacent to the epithelium and in the bulk phase serosal solution, respectively, V the velocity of water flow, δ_s the thickness of the serosal unstirred layer, and D the NaCl-free solution diffusion coefficient]. The local salt gradient across the epithelium creates a diffusion potential which represents most, if not all, of the so-called streaming potential; the magnitude of the p.d. being given by the constant field equation. (*See* Barry, Diamond & Wright, 1971 for a discussion of diffusion potentials across the gallbladder.) It should be noted that the local salt gradient between the serosal face of the epithelium and the serosal solution also gives rise to a liquid junction potential, which increases the size of the apparent streaming potential. The salt gradient produces a local osmotic gradient across the epithelium which is opposite to the sucrose gradient, i.e., the effective osmotic pressure across the epithelium is $\Delta\pi_{effective} = \Delta\pi_{sucrose} - \Delta\pi_{NaCl}$. Therefore, the flow of water across the gallbladder in the steady state is given by $(\sigma_{suc} L_p \Delta\pi_{suc}) - (\sigma_{NaCl} L_p \Delta\pi_{NaCl})$

Similar osmotic flow transients have been observed in measurements of transcellular flow in giant algal cells (Tazawa & Nishizaki, 1956; Barry & Hope, 1969). The probable explanation for the transients in both the gallbladder and the algal cells lies in unstirred layer effects.

The unstirred layer effect that concerns us is the so-called sweeping away effect (Dainty, 1963). Water flow across a membrane tends to concentrate the solution on one side of the membrane, and to dilute the solution

on the other side, by a factor $e^{(V\delta/D)}$, where V is the velocity of water flow, δ the thickness of the unstirred layer on each side of the membrane, and D is the diffusion coefficient of the solute in the unstirred layers. Referring to Fig. 4 we can construct the effects of water flow on the sucrose and NaCl concentrations adjacent to both sides of the gallbladder epithelium in an experiment such as that shown in Fig. 1. First, since the serosal unstirred layer is much thicker than the mucosal unstirred layer (Diamond, 1966*a*; Smulders & Wright, 1971) we expect that the largest effects will occur at the serosal face of the epithelium. Addition of 50 mM sucrose to the mucosal fluid will generate an osmotic flow towards the mucosal solution that will be proportional to the osmotic pressure exerted by sucrose ($\sigma_{\text{sucrose}} L_p \Delta\pi$). This flow across the epithelium will tend to reduce both the sucrose and NaCl concentrations at the mucosal face of the epithelial cell by the factor $e^{(-V\delta/D)}$, but even if the initial rate of flow is 10 times higher than the steady state flow, the changes in concentration are less than 5% [$e^{(-V\delta_m/D)} > 0.95$ where $V = 2.5 \times 10^{-5}$ cm/sec, $\delta_m = 100$ μ, $D_{\text{sucrose}} = 5.2 \times 10^{-6}$ cm^2/sec, and $D_{\text{NaCl}} = 1.48 \times 10^{-5}$ cm^2/sec]. Likewise, the water flow tends to enhance the solute concentration at the serosal face of the cell by a factor $e^{(V\delta_s/D)}$. In this case, however, owing to the thick unstirred layer (800 μ in chamber preparations) and the larger velocity of flow in the extracellular spaces of the gallbladder wall, there will be significant changes in concentration. For example, even if we assume that the velocity of flow is 2.5×10^{-5} cm/sec, $\delta_s = 800$ μ and $D_{\text{NaCl}} = 1.48 \times 10^{-5}$ cm^2/sec, the NaCl concentration at the serosal face of the epithelium will be increased by a factor of 1.15, i.e., the concentration at the cell would be 172 mM[2]. This local salt concentration gradient across the epithelium will tend to produce a back flow of water across the epithelium which is proportional to the osmotic pressure exerted by the salt ($\sigma_{\text{NaCl}} L_p \Delta\pi_{\text{NaCl}}$). Thus, in the steady state, the effective osmotic pressure across the gallbladder is not $\Delta\Pi_{\text{sucrose}}$,

2 Owing to the difficulty of estimating the velocity of flow in the various compartments of the gallbladder wall we are unable to arrive at an accurate estimate of sweeping effects in the serosal unstirred layer by this procedure. The serosal unstirred layer is a heterogeneous structure consisting of the lateral spaces, sub-mucosa, muscularis, etc. Thus in any given experiment, the velocity of water flow varies in a complex fashion from the lateral spaces to the serosal surface of the tissue. Furthermore, the velocity profile varies according to the experimental conditions owing to the changes in the geometry within the epithelium and within the rest of the wall (*see* Paper 1). It is possible, however, to evaluate the importance of sweeping effects in the lateral channels by using the dimensions listed in Table 2, Paper 1. In all cases, except with 300 mM sucrose in the mucosal fluid, there are relatively small changes in concentration between the open and closed ends of the channels; i.e., under most conditions the sweeping effects in the sub-epithelial tissue are relatively large compared with the effects in the lateral channels.

but rather $\Delta\Pi_{\text{sucrose}} - \Delta\Pi_{\text{NaCl}}$. Such an unstirred layer effect could explain the transient osmotic flow shown in Fig. 1.

A similar argument could be used to explain the rebound in flow when the sucrose gradient is taken off, since it would require a finite time for the local salt concentration gradients to dissipate. Thus the initial rate of back flow would be proportional to $\Delta\Pi_{\text{NaCl}}$.

The recent finding that osmotically induced changes in electrical potential across the gallbladder (so-called streaming potentials) are mostly, if not entirely, boundary diffusion potentials (Wedner & Diamond, 1969), provides substantial evidence for this explanation of osmotic flow transients. In other words, the diffusion potential set up by the local salt gradients across the epithelium (Fig. 4) can account for most, if not all, of the observed streaming potentials; therefore, we can estimate the salt concentration at the serosal face of the epithelium from the magnitude of the streaming potential using the constant field equation; i.e.,

$$E = \frac{RT}{F} \ln \frac{P_{\text{Na}}\gamma[\text{Na}]_s + P_{\text{K}}\gamma[\text{K}]_s + P_{\text{Cl}}\gamma[\text{Cl}]_m}{P_{\text{Na}}\gamma[\text{Na}]_m + P_{\text{K}}\gamma[\text{K}]_m + P_{\text{Cl}}\gamma[\text{Cl}]_s}$$

where E is the p.d. (mucosa with respect to serosa), T the absolute temperature, R the gas constant, F the Faraday, P a permeability coefficient, γ an activity coefficient, and the subscripts s and m refer to the serosal and mucosal solutions, respectively. Taking 3 mV as the p.d. induced by 50 mM sucrose in the mucosal fluid (Fig. 3), $P_{\text{Cl}}/P_{\text{Na}} = 0.2$ and $P_{\text{K}}/P_{\text{Na}} = 2.2$ (Wright & Diamond, 1968); and correcting for the liquid junction p.d. that arises between the serosal face of the epithelium and the serosal solution (*see* Machen & Diamond, 1969), the NaCl concentration at the serosal face of the epithelium increased from 148 to 170 mM. This amounts to an osmotic gradient of about 40 mosm after correction for the osmotic coefficient. Thus, the effective osmotic gradient across the tissue, ~ 10 mosm, is about one-fifth of the apparent osmotic gradient; i.e., the polarization effect alone could account for about a fivefold difference between the initial and steady state rates of flow.

Since the half time for the build up of streaming potentials is about 12 sec when sucrose is added to the mucosal solution, the polarization effect will be expected to drop the rate of flow to one-fifth of the initial rate within the first 5 min. Thus, for a more detailed correlation between solute polarization and osmotic transients it will be necessary to develop accurate methods for the rapid measurement of water flows. We note that the polarization effect does not account for the presence of the long tail on the

water flow transients. This suggests additional effects, which could be polarization effects within the luminal fluid of these sac preparations.

Streaming Potentials, Water Flows and Non-Linear Osmosis

Let us inquire further into the relationship between the so-called streaming potentials and the osmotic flow of water across the gallbladder in the light of the finding that these p.d.'s are mostly boundary diffusion p.d.'s. As outlined above, these originate from changes in salt concentration at the serosal face of the epithelium, the magnitude of the p.d. being given by the constant field equation. Since the size of the polarization effect is related to the velocity of water flow across the unstirred layer [$V = D/\delta_s(\ln C_m/C_s)$], it can be seen that the magnitude of the streaming potential is proportional to the velocity of water flow, if, and only if, the thickness of the serosal unstirred layer, the salt diffusion coefficient in the unstirred layer, the dimensions of the extracellular spaces of the gallbladder wall, P_{Cl}/P_{Na}, and P_K/P_{Na} all remain constant.

In view of the observed changes in the geometry of the gallbladder wall in the presence of osmotic gradients (Paper 1), the question now arises whether there is any rational relationship between streaming potentials and the rates of flow. Comparison of the results in Table 1 and Fig. 3 shows, on one hand, that there is a good correlation between the p.d.'s and L_p's when sucrose is added to the mucosal solution: both the streaming potentials and the L_p's indicate that the flow increased in a non-linear fashion with concentration, i.e., as the sucrose concentration was raised from 50 to 300 mM the steady state L_p's and the initial streaming potentials per mosm/liter both decreased by about 50%. This confirms an earlier observation by Diamond (1966*a*, Fig. 3). On the other hand, there is a poor correlation when sucrose is added to the serosal fluid: as in the pervious case, streaming potentials increased in a non-linear manner, but this time there was no significant difference between the L_p's obtained at the two gradients.

We now have to ask, what is the basis for: (1) the non-linear increase in flow when sucrose is added to the mucosal fluid, and (2) the discrepancy between streaming potentials and flows when sucrose is added to the serosal fluid? Both polarization effects and changes in tissue geometry have to be considered.

First, let us consider polarization effects. As discussed in detail in the previous section, water flows change both the salt and sucrose concentrations adjacent to the serosal face of the epithelium, and the effective osmotic gradient can be estimated by considering, in turn, the effects of water flow

on sucrose and salt concentrations. The effect on sucrose can be estimated using the relation $C_m = C_b e^{(V\delta/D)}$, and the effect on the salt can be estimated from the magnitude of the streaming potentials. For example, when 50 mM sucrose was added to the mucosal solution the effective osmotic gradient was shown to be about 10 mosm/liter. The effective steady state gradient can also be shown to be about 150 mosm/liter when 300 mM sucrose is added to the mucosal solution. Therefore, on the basis of polarization effects we would expect the steady state flows to increase by a factor of about 15 when the sucrose concentration is increased from 50 to 300 mM in the mucosal solution; i.e., the apparent L_p should actually increase as the mucosal sucrose concentration is increased. This predicted change is in the opposite direction to that observed, and so we conclude that polarization effects do not account for non-linear osmosis. When sucrose is added to the serosal solution, similar consideration of the polarization effects shows that we should expect osmotic flow to increase in a close to linear manner with concentration. Experimentally it was found that the flow increased by a factor of about 5 when the sucrose concentration was increased from 50 to 300 mM in the serosal fluid. These conclusions also entail asymmetry of flow with 300 mM gradients, but the asymmetry is opposite in direction to that observed experimentally.

These considerations show that polarization effects do not explain either non-linear osmosis or the asymmetry of flow at the higher osmotic gradients. However, additional calculations do indicate that polarization effects can explain the observation by Diamond (1966*b*, Fig. 2) that the apparent L_p of the gallbladder decreased with increasing absolute osmolarity. Thus, it is no longer necessary to postulate that gallbladder membranes behave as osmometers, since all the evidence can now be explained on other, more secure, grounds.

Next, let us consider changes in tissue geometry. We have already shown that when the mucosal fluid is made hypertonic the lateral spaces collapse and exert a non-linear throttling effect on the volume of water flow. We have also seen that when the serosal fluid is made hypertonic the spaces dilate but have no significant influence on volume flow. This appears to explain satisfactorily both the symmetry of flow at low gradients, the asymmetry at high gradients, and non-linear osmosis.

These changes in the lateral spaces of the epithelium, taken together with associated changes in the extracellular spaces in the rest of the wall, also provide an explanation for the non-linear relationship between osmotic gradients and streaming potentials. With flow towards the serosal solution, the cross-sectional areas of the extracellular spaces increase progressively

with increasing rates of flow. As we have seen earlier, this increase in area does not affect the L_p. However, there is a sub-linear increase in the velocity of flow with increasing gradient because of the increase in the area of the channels; i.e., the velocity of flow within the widened channels will be much less than it would have been had the geometry not been altered. This change in velocity produces a non-linear increase in the streaming potential, even though there is a linear increase in the volume of flow. Only when flow is directed towards the mucosal solution do *both* the streaming potentials (velocity of flow) and the volume of flow become functions of the channel area. Thus, when the mucosal solution is hypertonic both the streaming potential and the volume of water flow will be sublinearly related to the gradient.

What, finally, is the explanation for the streaming potential transients which occur when sucrose is added to the mucosal solution (Fig. 2)? A clue comes from the observation that the thickness of the gallbladder wall depends on the direction of osmotic flow across the tissue (Paper 1, Table 1). These anatomical variations correlate well with changes in the thickness of the serosal unstirred layer estimated from the half times for the build up of sodium chloride diffusion potentials (*see* Smulders & Wright, 1971): In the absence of flow the mucosal and serosal unstirred layers were about 100 and 800 μ, respectively. The mucosal unstirred layer was unaffected by osmotic gradients, but upon addition of sucrose (300 mM) to the serosal and mucosal fluids, the thickness of the serosal unstirred layer increased by 150 μ and decreased by 400 μ, respectively. Can these variations in the serosal unstirred layer explain the difference between the initial and steady state streaming potentials shown in Figs. 2 and 3? If the velocity of water flow is assumed to be identical under the two conditions, the decrease in the serosal unstirred layer thickness which is necessary to explain the fall in streaming potential may be calculated from the expression

$$\frac{\delta_s'}{\delta_s} = \ln\left(\frac{C_m'}{C_b}\right) - \ln\left(\frac{C_m}{C_b}\right)$$

where C_m' and C_m are, respectively, the initial and steady state salt concentrations adjacent to the serosal face of the cell, C_b is the salt concentration in the bulk phase serosal solution, and δ_s'/δ_s is the ratio of the thickness of the initial and steady state serosal unstirred layers. From the values of the initial and steady streaming potentials it is calculated that C_m' and C_m are 226 and 182 mM, respectively. Substitution of these values into the equation yields an unstirred layer thickness ratio of 2, which is indistinguishable from the ratios obtained experimentally from the diffusion potential half times.

Thus, it would appear that the discrepancy between the initial and steady state streaming potentials is due to a decrease in the thickness of the serosal unstirred layer. The close correlation between the time course of the decay in the streaming potential and the increase in the resistance (Fig. 2) provides additional evidence to support this suggestion. Apparently the velocity of flow remains constant as the channels close and the volume of flow decreases. In the sac preparations neither the decay in the streaming potentials nor the changes in width of the gallbladder wall are observed.

The Route of Water Flow

We have seen that changes in lateral intercellular space dimensions explain asymmetry of osmotic water flow. A necessary corollary of this is that all or most of the water crosses the epithelium by way of the lateral spaces. This conclusion is reached in a manner essentially different from previous studies on routes of water flow. Dilation of lateral intercellular spaces has been shown to be associated with water flow in a number of epithelia, and from this it has been concluded that the spaces dilate because water flows through them. However, there are more subtle reasons why spaces might dilate in the presence of water flow (hydrostatic pressures outside the epithelia, fixation artifacts, changes in underlying structures, etc.; *cf.* Tormey & Diamond, 1967; DiBona & Civan, 1970; Grantham, Cuppage & Fanestil, 1971). But this does not affect the outcome of our argument, because we have shown that, aside from the fact that water flow affects space dimensions, the space dimensions affect water flow. Therefore the conclusion that the lateral spaces are the route of water flow is now on a much more secure footing.

There has been some question whether active (solute-linked) and passive water flows across epithelia follow the same route. Alternate routes have been proposed in order to explain two observations made in rabbit gallbladders (Diamond, 1964; Pidot & Diamond, 1964): First, that active fluid transport produced no streaming potentials, whereas osmotically-generated, passive flows did. Second, that the L_p estimated from osmotic water flows was much too small to account for isotonic fluid transport. More recent work showed that the potentials generated by osmotic water flows are not true streaming potentials but are mostly boundary diffusion potentials (Wedner & Diamond, 1969), and potentials of a similar nature can be set up by active fluid transport (Machen & Diamond, 1969). Isotonic water transport in the face of a low L_p can be explained on the basis of the standing-gradient osmotic-flow model (Diamond & Bossert, 1967), and in any case

the L_p's obtained previously are probably an order of magnitude too low (this paper). Consequently, there is now no obstacle to concluding that active and passive water flows follow identical routes. Both presumably arise as the result of osmotic gradients between the lateral intercellular space and the mucosal bathing solution, and it should make no difference whether this local osmotic gradient is the result of active solute transport into the space, or an externally applied concentration gradient. This interpretation is, of course, further suggested by our present observation that lateral space changes with passive water flow parallel those reported during active flows (Tormey & Diamond, 1967).

As discussed above, water can reach the lateral spaces from the mucosal bathing solution by either of two routes: either directly through the tight junctions or through the cell.

To evaluate the importance of the tight junction route, we will estimate the L_p of the junctions. Since virtually all the electrical current crosses the epithelium through the junctions (Frömter & Diamond, 1972) the area of the pores in the tight junctions calculated from the resistance is $\sim 4 \times 10^{-5}$ cm^2. ($A = l/kR$, where A is the total channel area; k, the specific electrical conductance of NaCl, is 16×10^{-3} mho/cm^{-1}; l, the length of a tight junction, is 2×10^{-5} cm; and R, the electrical resistance of the gallbladder is 28 Ω.) If the pores in the tight junctions have a radius of 12 Å (*see* Smulders & Wright, 1971) it follows that the tight junctions cannot be thought of as continuous narrow slits. They must be heterogeneous structures with pores at intervals of approximately 1,000 Å. Parenthetically, one could not expect to visualize such channels by conventional electron microscope techniques, for reasons of contrast rather than resolution.

The L_p of a single cylindrical pore is obtained from Poiseuille's law, i.e.,

$$L_p = \frac{\pi r^4}{8 \eta l}$$

where r is the pore radius, and η is the viscosity of bulk water = 0.01 poise. The total number of pores is found by dividing the total pore area/cm^2 bladder wall by the area of a single pore, i.e.,

$$\frac{A}{\pi r^2} = \frac{l}{kR} \times \frac{1}{\pi r^2}.$$

(Here we use the resistance, R, to give an estimate of total pore area, but the permeability of various compounds such as sodium, sucrose or inulin could be used to give the same result.) Combining these two expressions,

we find that the L_p per cm^2 of bladder wall is

$$L_p = \frac{r^2}{\eta k R}.$$

(Note that we can now calculate L_p *independently* of any question of the actual length or tortuosity of the channels, and that, although η and k might deviate from free solution values in a narrow pore, these deviations will be in opposite directions and tend to cancel.)

We thus estimate the L_p of the tight junction route to be $\sim 5 \times 10^{-4}$ cm/sec, assuming a radius of 12 Å. If we take the actual overall L_p to be $\sim 5 \times 10^{-3}$ cm/sec (*see* p. 208), it is obvious that only 10% of the water flow into the lateral channels would be through the junctions. In the more likely event that the actual L_p is even larger, the contribution of the tight junction route would be negligible. Even if the channel radius were as large as 25 Å, the L_p for the junctions would be insufficient to explain observed rates of flow. Therefore, it appears the tight junctions are not the main route of water flow across the epithelium.

Another consideration makes it even more unlikely that the tight junctions could be the route of water flow. At the pore sizes we are considering ($\sim$12 to 25 Å), the reflection coefficient of sucrose will be less than 0.5, and there will be appreciable variation in the reflection coefficients of larger and smaller molecules (*cf.* Durbin, 1960). But in fact the reflection coefficients for all sugars with more than three carbons have been shown to be one in the rabbit gallbladder (Wright & Diamond, 1969). This observation can be reconciled with a pore radius of 12 Å only if almost all the water passes through an entirely different set of much smaller pores. But if the pores for water flow are only, say, 3 Å in radius, it can be calculated that the tight junctions are probably not sufficiently extensive to accommodate enough of them to explain the measured L_p. This suggests that the water pathway is through the cell. Finally, it should be noted that a transcellular route is *a priori* reasonable, since it implies water flows across membranes with L_p's similar to those reported for artificial membranes and red cells (*cf.* Dick, 1970, for review).

Finally, a word about our finding (Paper 1) that the subserosa is a major hydraulic barrier to flow across the bladder wall and that the submucosa swells and shrinks in accord with the magnitude and direction of water flow. This suggests that the submucosa could be the middle compartment of Curran's three-compartment model for fluid transport, and that the subserosa could be the second membrane (Curran & MacIntosh, 1962; Patlak, Goldstein & Hoffman, 1963). But it should be clear that, *if* the

fluid emerging from the lateral intercellular spaces is already isotonic, these structures can play no role in the attainment of isotonicity. Indeed, the subserosa almost certainly does not play a role in the gallbladder *in vivo*, where fluid leaves by way of the capillaries rather than across the serosal surface. Thus our results do not appear to bear on the relative merits of the standing-gradient and three-compartment models of fluid transport.

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