The Mechanism of Isotonic Water Transport

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ABSTRACT The mechanism by which active solute transport causes water transport in isotonic proportions across epithelial membranes has been investigated. The principle of the experiments was to measure the osmolarity of the transported fluid when the osmolarity of the bathing solution was varied over an eightfold range by varying the NaCl concentration or by adding impermeant non-electrolytes. An *in vitro* preparation of rabbit gall bladder was suspended in moist oxygen without an outer bathing solution, and the pure transported fluid was collected as it dripped off the serosal surface. Under all conditions the transported fluid was found to approximate an NaCl solution isotonic to whatever bathing solution used. This finding means that the mechanism of isotonic water transport in the gall bladder is neither the double membrane effect nor codiffusion but rather local osmosis. In other words, active NaCl transport maintains a locally high concentration of solute in some restricted space in the vicinity of the cell membrane, and water follows NaCl in response to this local osmotic gradient. An equation has been derived enabling one to calculate whether the passive water permeability of an organ is high enough to account for complete osmotic equilibration of actively transported solute. By application of this equation, water transport associated with active NaCl transport in the gall bladder cannot go through the channels for water flow under passive conditions, since these channels are grossly too impermeable. Furthermore, solute-linked water transport fails to produce the streaming potentials expected for water flow through these passive channels. Hence solute-linked water transport does not occur in the passive channels but instead involves special structures in the cell membrane, which remain to be identified.

The present paper is concerned with the mechanism of isotonic water transport across epithelial membranes. Familiar examples of this process are: the secretion of gastric juice by the stomach, of bile by the liver, of pancreatic juice by the pancreas, and of cerebrospinal fluid by the choroid plexus; and the absorption of the digesta by the small and large intestines, of bile by the gall bladder, and of the glomerular filtrate by the proximal tubule of the kidney. The qualitative properties of the secretion or absorbate vary widely in different organs

(*e.g.*, bile *vs.* gastric juice), depending upon what solute is actively transported, and in the past decade much information has accumulated about the specificity and mechanism of various solute "pumps." However, all these fluids are quantitatively mostly water, and there are four constant features suggesting that the mechanism of water transport, which is still poorly understood, may be the same among these various organs, regardless of the identity of the solute transported: (*a*) whenever tested, the ability to do osmotic work on water by transferring it against osmotic gradients; (*b*) active transport of some solute, be it hydrochloric acid, bile salts, NaCl, $NaHCO_3$, or glucose; (*c*) the ability to transfer water in the absence of external driving forces, when the membrane separates identical bathing solutions; and (*d*) under these conditions (membrane separating identical bathing solutions), no water transport in the absence of net solute transport, and a constant ratio between the amounts of water and solute transported, equal (isotonic) to the ratio in plasma (anisotonic transport presents separate problems which are not the subject of this paper). It has been shown by irreversible thermodynamics (Diamond, 1961, 1962 *c*) that the movement of water against osmotic gradients in the presence of active solute transport may be a passive consequence of the latter and is not *per se* evidence for active water transport. The problem of isotonic water transport should therefore be rephrased in the following terms: what is the coupling mechanism by which the active transport of one molecule of solute causes the transport of several hundred molecules of water?

Of the many theories advanced to explain water transport, it was previously found in the fish gall bladder that this coupling mechanism is neither classical osmosis, filtration, electroosmosis, or pinocytosis (Diamond, 1962 *c*, 1964). Recently attention has been concentrated on two other theories which are *a priori* capable of explaining water-to-solute coupling and have been indistinguishable experimentally from the existing evidence. These are the theories to be tested here and may be summarized as follows:—

1. *Local Osmosis* Active solute transport might set up a high concentration of solute in a restricted space in or just beyond the cell membrane, and water would then cross the membrane in response to this local osmotic gradient. Water transport could then be maintained against an opposing osmotic gradient in the external bathing solutions, until this external gradient balanced the local osmotic gradient caused by active solute transport.

2. *The Double Membrane Effect* Suppose active transport of solute takes place into an area between two membranes, the first of them with narrow channels relatively impermeable to solute, the second with wider channels relatively permeable to solute. Since the solute will exert a greater effective osmotic pressure difference across the first membrane than across the leaky second membrane, water will be drawn across the first membrane by osmosis,

build up hydrostatic pressure in the intervening space, and be forced out of the leaky second membrane under this pressure. In such a system active solute transport into the middle space would cause a net movement of water even if the two external bathing solutions were identical, or against an external osmotic gradient. The theory of double membrane effect has been worked out by Patlak, Goldstein, and Hoffman (1963), and the effect has been placed on a firm experimental basis in model systems of artificial membranes (Durbin, 1960; Curran and McIntosh, 1962; Ogilvie, McIntosh, and Curran, 1963). Quantitatively, the general effect is considerably more complicated than as summarized above, since the rates of water and solute movement depend upon the permeability coefficients, reflection coefficients, and filtration constants of both membranes and upon the active transport rate. The essential feature is that the two membranes must have differing reflection coefficients to obtain any water movement between identical external solutions. If the first membrane is completely impermeable to solute (*i.e.*, if the reflection coefficient σ is 1) and the second is freely permeable ($\sigma = 0$), then the double membrane model yields the same predictions as local osmosis. However, the two theories are distinct because under all other circumstances the double membrane effect leads to different predictions, and local osmosis may be visualized in ways other than this limiting case of the double membrane model. Either an epithelial cell with its two limiting membranes might correspond to a double membrane system, or else each of the two cell membranes might have double membrane properties.

Both of these theories could in principle account for how active solute transport carries water between identical bathing solutions and up osmotic gradients. Both could also explain production of secretions or absorbates which are isotonic to plasma. Local osmosis automatically would give isotonic water-to-solute ratios if the actively transported solute could equilibrate with water completely. The double membrane effect can in theory give almost any ratio, since the transported osmolarity would be a function of several independent parameters (the hydraulic conductivities, solute permeabilities, and reflection coefficients of both membranes), and in particular could yield isotonicity if the membrane had evolved the right structure and parameters.

The principle devised to discriminate between these theories was to measure the osmolarity of the transported fluid when the osmolarity of the bathing solution is varied, since the theories now yield differing predictions. That is, is the transported fluid still isotonic when the osmolarity of the bathing solution differs from that of plasma? According to local osmosis, which depends upon osmotic equilibration within the membrane, the transported fluid should be isotonic to the bathing solution, whatever the latter's osmolarity. However, by the double membrane effect the transported fluid should be isotonic only at one bathing solution osmolarity, since the transported osmolarity depends

upon six parameters describing inherent properties of the membranes (Patlak, Goldstein, and Hoffman, 1963). At lower bathing osmolarities the transported fluid should be hypertonic to the bathing solution, and at higher bathing osmolarities the transported fluid should be hypotonic.

The experimental material chosen for this study was the gall bladder of the rabbit because this organ has a simple and convenient anatomy, transports well *in vitro* for many hours, tolerates at least a tenfold range in osmolarities, and maintains the highest rate of water transport yet described. At plasma osmolarities it transports NaCl and water in isotonic proportions by means of coupled active transport of Na and Cl (Diamond, 1962 *b*, 1964; Wheeler, 1963; Dietschy, 1964). The success of the experiment depended upon measuring the transported osmolarity as accurately as possible, and the conventional indirect method of obtaining by differences the amounts of solute and water absorbed and taking the ratio was insufficiently precise for this purpose. Accordingly, a new gall bladder preparation was devised on the lines of Smyth and Taylor's (1957) preparation of the small intestine, in which the organ is suspended in moist air without a serosal bathing solution and the pure transported fluid is collected directly. The actual experiment consisted of measuring the osmolarity of the transported fluid when the osmolarity of the luminal bathing solution was varied (*a*) by varying the concentration of NaCl itself, using three slightly different types of Ringer's solutions; (*b*) by adding varying concentrations of an impermeant non-electrolyte, sucrose or raffinose.

METHODS

Methods for dissecting out and cannulating rabbit gall bladders have already been described (Diamond, 1964). In general, the cannulated gall bladder was first allowed to equilibrate for 15 to 20 minutes at 37°C in an oxygenated beaker of the experimental solution, with which the lumen was also filled. The preparation was weighed at 5 minute intervals to ±1 mg on a Mettler balance to obtain the rate of fluid transport. At the end of this period the lumen was refilled with fresh experimental solution, to which the dye phenol red had been added. After a further 5 minutes in a beaker of experimental solution, the gall bladder was transferred for 10 minutes to a pyrex Goetz tube (a pear-shaped tube with stem, made by Corning Glass Works, Corning, New York) with a total volume of 100 ml and a 1 ml stem graduated in units of 0.05 ml. The Goetz tube was submerged almost up to the top of its joint in a water bath at 37.0 ± 0.3°C, ensuring that there were no temperature gradients in the tube down which the gall bladder could lose water by thermal distillation. The tube had previously been filled with 95 per cent O_2–5 per cent CO_2 (or with 100 per cent O_2 if the experimental solution did not contain bicarbonate) saturated with water vapor by having been bubbled through experimental solution at 37°C. In a few early experiments the Goetz tube was gassed continuously throughout the experiment, but this procedure proved inconvenient and unnecessary and was abandoned, since the expected oxygen uptake of a gall bladder was negligible compared to the

100 ml volume of the tube. The fine thread connecting the weighing hook of the gall bladder to the cannula plug lay in the ground-glass joint of the Goetz tube, and a ground-glass stopper was then inserted into the joint, thus sealing off the Goetz tube and also leaving the gall bladder hanging in the center of the tube (Fig. 1). After 10 minutes the gall bladder was removed from the Goetz tube, weighed on a Mettler balance (weighing *a*), and transferred to another Goetz tube, which had also been filled with 95 per cent O_2–5 per cent CO_2, saturated with water vapor, and then weighed (weighing *b*). The Goetz tube containing the gall bladder was quickly weighed (weighing *c*) and resubmerged in the water bath.

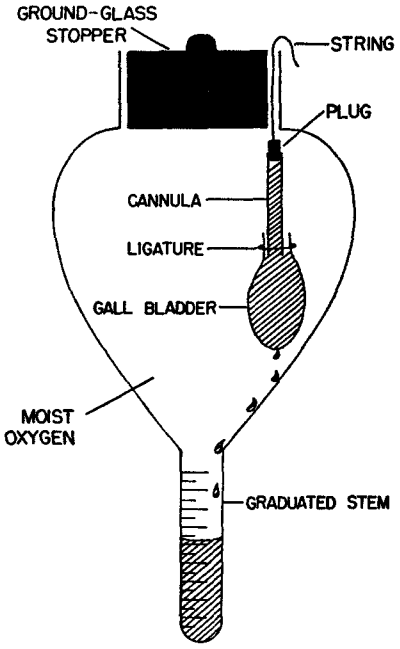

FIGURE 1. Gall bladder preparation with no outer bathing solution. A rabbit gall bladder is suspended in a Goetz tube by a string held in place by the ground-glass stopper. Fluid transported out of the lumen of the gall bladder drips off into the graduated stem. The tube is filled with oxygen (or 95 per cent O_2-5 per cent CO_2) saturated with water vapor, and is submerged almost up to the top of the stopper in a constant-temperature bath.

Droplets of fluid began to appear on the outside of the gall bladder and fall off into the graduated stem. When between 10 and 50 per cent of the fluid initially in the lumen of the gall bladder had been secreted into the Goetz tube (requiring from 1 to 4 hours), the experiment was terminated. The Goetz tube with the gall bladder was weighed (weighing *d*) and then unstoppered, the gall bladder lifted out, and the Goetz tube restoppered. Weighings *c* and *d* were virtually identical, showing that no fluid could evaporate from or leak into the Goetz tube. The gall bladder was suspended from the weighing hook of the balance, and its weight was noted three times at 15 second intervals. Since the gall bladder lost weight by 1 to 3 mg/15 second by evaporation after removal from the Goetz tube, its weight at the moment when the Goetz tube was unstoppered was obtained by extrapolation (weighing *e*). The gall bladder was cut, and the residual luminal fluid was allowed to run out into a tared centrifuge tube, which was promptly stoppered and weighed. Finally, the Goetz tube containing the secreted fluid was weighed (weighing *f*). The secreted fluid and

residual luminal fluid were diluted for chemical analysis, their volumes before dilution being calculated from their respective weights and densities. The weight of fluid lost by the gall bladder (weighings *a* minus *e*) was on the average 2.8 per cent greater than the weight of fluid gained by the Goetz tube (weighings *f* minus *b*) because of evaporation of fluid at the beginning and end of the experiment while the gall bladder was being transferred into and out of the Goetz tube (*a* plus *b* less than *c*, *e* plus *f* less than *d*). The weight loss of the gall bladder (*a* minus *e*) was taken as the more correct approximation to the true weight of secreted fluid.

The chemical composition of the secreted fluid and residual luminal fluid was calculated directly from the dilution factor and the chemical analyses after dilution. The average composition of the luminal fluid during the experiment might have been obtained most simply by averaging the concentrations in Ringer's solution and in the residual luminal fluid. However, it was considered slightly more accurate to take account of the fact that the gall bladder had last been refilled with fresh Ringer's solution 15 minutes before entering the second Goetz tube to collect secreted fluid, and

TABLE I

COMPOSITION OF EXPERIMENTAL SOLUTIONS

	NaCl	$NaHCO_3$	KCl	$CaCl_2$	$MgSO_4$	Glucose	NaH_2PO_4	KH_2PO_4	K_2HPO_4	Sucrose or raffinose
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
A	Variable	25.0	7.0	2.0	1.2	11.1	1.2	—	—	—
B	Variable	25.0	7.0	2.0	1.2	—	1.2	—	—	—
C	Variable	25.0	7.0	2.0	1.2	—	1.2	—	—	Variable
D	Variable	—	—	1.5	—	—	—	0.375	2.125	—

modification of the luminal fluid would already have commenced in those 15 minutes. Accordingly, the composition of the luminal fluid when the collection of secreted fluid began was calculated by linear interpolation, based on weight loss by the gall bladder, between concentrations in Ringer's solutions and in the residual luminal fluid. This calculated initial fluid and the known residual fluid were then averaged to obtain the average composition of the luminal fluid during an experiment. The corrections involved were more complicated than important, since, as will be seen, the final and average luminal fluid had much the same concentrations as Ringer's solution except in the experiments involving sucrose or raffinose. In the text the symbols $[Na]_{lumen}$, $[Cl]_{lumen}$, etc., will always refer to these time-average luminal concentrations.

The composition of the experimental solutions is given in Table I. In any one experiment the same solution was used to carry out the dissection, to bathe both sides of the gall bladder during the preliminary 15 or 20 minute equilibration period, and to fill the lumen when the gall bladder was secreting in the Goetz tube. An exception to this procedure was made if the lumen was to be filled with a solution containing sucrose or raffinose (solution C). In such cases the dissecting fluid and the outer

bathing solution during the preliminary equilibration period differed from the experimental solution in containing no sucrose or raffinose.

Sodium and potassium were analyzed by flame photometry, and chloride by potentiometric titration, as already described (Diamond, 1964). Phenol red was determined colorimetrically in the alkaline form at 560 mμ with the Beckman model B spectrophotometer after addition of NaOH. Glucose was estimated by a colorimetric method (glucostat, Worthington Biochemicals Corporation) after reaction with glucose oxidase. Raffinose and sucrose were determined by a colorimetric resorcinol method (Schreiner, 1950). C^{14}-inulin and C^{14}-sucrose were counted for 30 minutes or to one million counts with the Packard tri-carb liquid scintillator and were corrected for quenching by phenol red. The concentrations of phenol red present in the experimental solution were found not to interfere with Na, K, or glucose determinations but did raise the blank value in the colorimetric determination of sucrose and raffinose, and this blank was, of course, subtracted. Results of colorimetric and radioactive sucrose determinations were averaged, as the two methods agreed consistently to within 4 per cent.

RESULTS

Transport in the "Unilateral" Preparation When a gall bladder filled with a solution containing the dye Evans Blue or phenol red was suspended in a Goetz tube in an atmosphere of water-saturated 95 per cent O_2–5 per cent CO_2, colorless droplets of fluid appeared on the outside of the gall bladder and fell into the Goetz tube. For example, at the beginning of one experiment a gall bladder contained 2.418 ml of solution with the dye Evans Blue at an optical density ($O.D._{610 m\mu}$) of 5.20. After 5 hours 33 minutes, 2.157 ml of fluid colorless to the eye, or 89.2 per cent of the original luminal volume, had been secreted into the Goetz tube, and the measured $O.D._{610 m\mu}$ of this secreted fluid was 0.006. The $O.D._{610 m\mu}$ of the small amount of residual fluid in the lumen of the gall bladder was now 45.1, and this 8.68-fold (45.1 ÷ 5.2) rise in concentration of Evans Blue implied that the luminal volume was now only 11.5 per cent (1 ÷ 8.68) of its original value, or that 88.5 per cent of the original luminal fluid had been secreted. The absence of Evans Blue in the secreted fluid and the excellent agreement (89.2 per cent *vs.* 88.5 per cent) between the gravimetric and colorimetric measurements of secretion mean that there was no bulk leakage of fluid out of the lumen of the gall bladder.

Besides Evans Blue several other compounds of intermediate or high molecular weight were found not to appear in the secreted fluid when present in the lumen. For example, C^{14}-inulin was lost from the lumen into the secreted fluid at only 0.2 per cent/hour, and corresponding figures are 0.2 to 0.4 per cent/hour for phenol red, raffinose, or C^{14}-sucrose. As routine it was found convenient to add a few milligrams of phenol red to the luminal solution, and if the secreted fluid was colored, as happened in a few cases at very low osmolarities,

the preparation was rejected. From the rise in concentration of phenol red in the residual luminal fluid at the end of the experiment, the volume of fluid secreted was calculated, to serve as a check of the gravimetric measure of fluid secretion.

When the luminal solution contained no sucrose or raffinose, it was noticed that the gall bladder secreted fluid as a unilateral preparation in the Goetz tube, with only a luminal bathing solution, at about the same rate as it had transported fluid in the preliminary 15 or 20 minute equilibration period with bathing solutions on both sides. This point was specifically examined in six experiments by measuring the rate of fluid transport gravimetrically (Diamond, 1964) with bathing solutions on both sides for up to an hour, then observing the rate of fluid secretion as a unilateral preparation. The latter rate was 95 ± 12 per cent of the former, so that the rate of transport is the same within experimental error in the unilateral preparation and in the conventional preparation with outer bathing solution. However, when the luminal solution contained sucrose or raffinose, an outer bathing solution without sucrose or raffinose was used during the preliminary equilibration period, and there was thus an osmotic gradient across the gall bladder opposing water movement out of the lumen. Such gall bladders were found to secrete fluid more rapidly as a unilateral preparation, when manufacturing their own outer fluid, than as a conventional preparation, when opposed by a known osmotic gradient. The reason for this will be discussed later.

While the secretion rate varied considerably from preparation to preparation, there was also a clear dependence upon the osmolarity or NaCl concentration of the luminal solution (Fig. 2). Optimal secretion rates were encountered in the range 125 to 250 mOsM, somewhat below the level of rabbit serum (*ca.* 300 mOsM), while very high and very low osmolarities (or NaCl concentrations) gave low secretion rates because of inhibition of NaCl transport at these extreme osmolarities. At any given NaCl concentration the rate was about the same whether or not glucose was present, but was significantly lower in the absence than in the presence of bicarbonate. Thus, the optimal conditions are the same for the unilateral preparation and the conventional preparation with an outer bathing solution, where bicarbonate was found to stimulate and glucose not to affect the rate of water transport (Diamond, 1964). Secretion rates with sucrose or raffinose added to the lumen fell in the same range as for the experiments without sucrose or raffinose shown in Fig. 2.

Estimated Error Since the two theories of water transport mentioned in the introduction made quantitatively different predictions for solute concentrations in the secreted fluid, it was essential to determine these concentrations as accurately as possible and to have an estimate of their error. The standard deviation for Na determinations by flame photometry, based on twelve sepa-

rate dilutions of one sample, was ±0.6 per cent. However, one would like a combined estimate of all errors, analytical and systematic. This may be obtained from the extent of agreement between the amount of solutes put into the system at the beginning of the experiment and the amounts recovered at the end, since the same errors (in measuring volumes and solute concentrations) that are responsible for imperfect agreement between these two quantities are also the sources of error in the quantity of chief experimental interest (the relative concentration of the secreted and luminal fluids). The input of

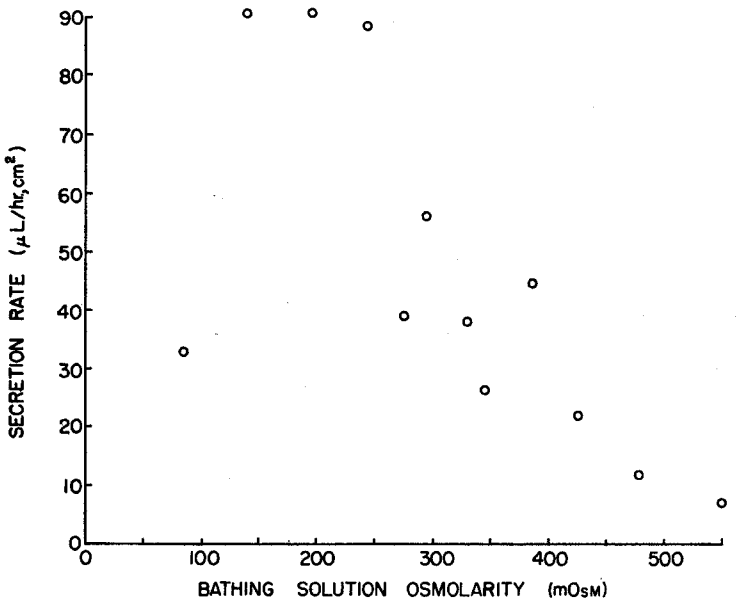

FIGURE 2. Fluid transport rate by preparation of rabbit gall bladder without an outer bathing solution (ordinate). Abscissa, osmolarity of the luminal bathing solution, varied by varying the concentration of NaCl, the principal solute (solutions A, B, or D, Table I). Each point is an average value from three gall bladders.

solutes was calculated from the volume and concentrations of the luminal fluid at the time when the gall bladder was transferred into the second Goetz tube for collection of secreted fluid. The recovery was the sum of the solutes in the secreted fluid and in the residual luminal fluid at the end of an experiment, as obtained from the measured volumes and concentrations of these two fluids. It was found that the Cl recovered (sum of Cl in the secreted fluid and in the residual luminal fluid) was greater than the amount of Cl in the initial luminal fluid in twenty-four cases, less in twenty-eight, and the same (within 0.1 per cent) in two, and the average absolute magnitude of the difference (irrespective of sign) was 2.0 per cent. Corresponding figures are: for Na,

greater in twenty-six cases, less in twenty-eight, average difference 1.7 per cent; for K, greater in thirty-seven cases, less in fourteen, the same in three, average difference 5.9 per cent; for sucrose or raffinose, greater in seven cases, less in five, the same in one, average difference 4.2 per cent; for glucose, greater in one case, less in eleven, average difference 22.2 per cent. Thus, on the whole more K and less glucose were finally recovered from the fluids than were initially present, and these shifts imply that not unexpectedly there is some net uptake of glucose by the cells of the gall bladder wall and a slight leakage of K. However, the small changes in amounts of Na, Cl, and sucrose or raffinose, which account for most of the osmolarity of the fluids, are random, and these solutes are conserved within 1.7, 2.0, and 4.2 per cent respectively. Hence, 1.7 to 4.2 per cent is also the approximate uncertainty introduced into the determination of the relative concentration of the secreted and luminal fluids by all experimental measurements. Expressed in another way, the observed conservation of solutes means that the direct measurement of concentrations in the secreted fluid would be confirmed by an indirect measurement based on relative disappearance of solutes and water from the luminal fluid.

Results of Experiments without Sucrose or Raffinose Three series of experiments (forty-one gall bladders) involved solutions in which NaCl accounted for most of the osmolarity. One Ringer's solution (solution D, Table I) contained only small amounts of other ions; a second (solution B) also contained $NaHCO_3$ at 25 mM; and the third (solution A) contained in addition glucose at 11.1 mM. In each experiment a certain concentration of NaCl was added to one of these Ringer's solutions, and the range of sodium concentrations tested extended from 26.6 to 294.5 mM. The results were on the whole similar for all three series of experiments and are presented in Figs. 3 to 5.

During each experiment the luminal Na concentration was found to remain approximately constant in the absence of glucose and to decline slightly in the presence of glucose, whether or not bicarbonate was present. The luminal Cl concentration remained constant in the absence of glucose and bicarbonate, decreased somewhat in the presence of bicarbonate, and decreased more in the combined presence of bicarbonate and glucose. The luminal K concentration increased slightly.

$[Na]_{secreted}$ and $[Cl]_{secreted}$ increased close to linearly with $[Na]_{lumen}$ and $[Cl]_{lumen}$, while $[K]_{secreted}$ was always less than $[K]_{lumen}$. As seen in Fig. 3, $([Na] + [K])_{secreted}$ was virtually the same as $([Na] + [K])_{lumen}$ in the absence of glucose whether or not bicarbonate was present in the lumen. $[Cl]_{secreted}$ was virtually the same as $[Cl]_{lumen}$ in the absence of glucose or bicarbonate, slightly greater in the presence of bicarbonate, and somewhat greater again in the presence of bicarbonate plus glucose (Fig. 4). Fig. 5 shows the ratio $[K]_{secreted}/[K]_{lumen}$, which was less than one in all these experiments without

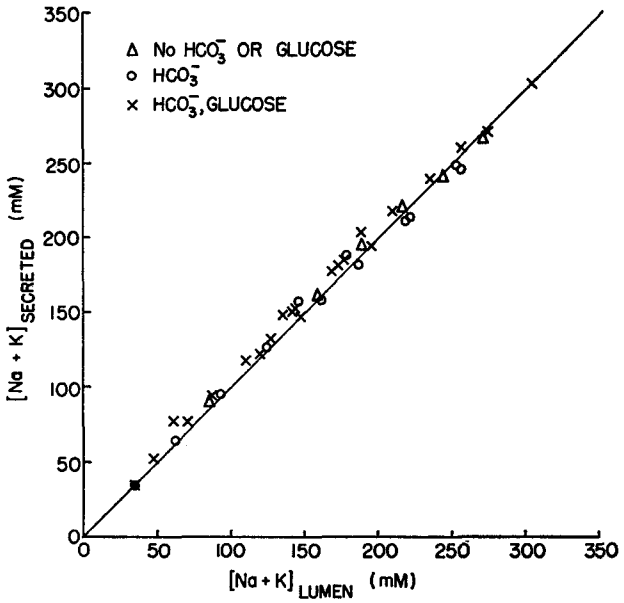

FIGURE 3. [Na] + [K] in the fluid secreted by gall bladders without an outer bathing solution (ordinate), when the luminal [NaCl] was varied. Abscissa, [Na] + [K] in the luminal bathing solution, which was mostly NaCl (Δ, solution D, Table I; O, solution B; x, solution A). [Na] was always considerably larger than [K] in both the secreted and luminal fluids. The straight line is the line of 45° slope.

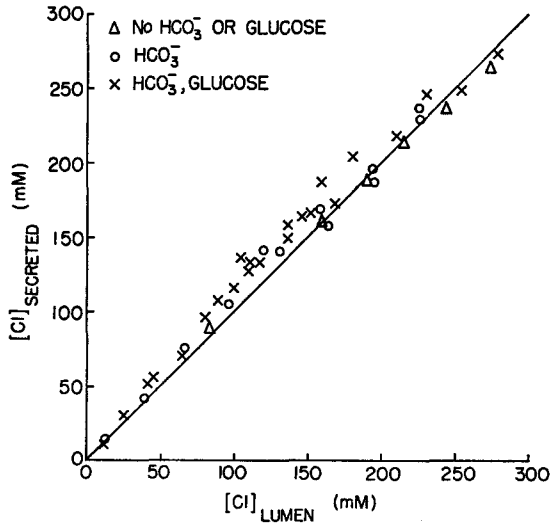

FIGURE 4. [Cl] in the fluid secreted by gall bladders without an outer bathing solution (ordinate), when the luminal [NaCl] was varied. Abscissa, [Cl] in the luminal bathing solution (for composition, see Fig. 3 and Table I). The straight line is the line of 45° slope.

sucrose or raffinose. The ratio tended to decrease with total luminal osmolarity, but the correlation need not imply a direct dependence of the ratio on osmolarity, as some low values of the ratio occurred at low osmolarities. The average value of $[glucose]_{secreted}/[glucose]_{lumen}$ was 0.17, without any clear dependence on the total luminal osmolarity.

$[HCO_3]$ could be computed approximately as ($[Na] + [K] - [Cl]$) in bicarbonate-containing Ringer's solutions, since other ions were present in much smaller concentrations. From this calculation it turned out that $[HCO_3]_{secreted}/[HCO_3]_{lumen}$ was less than 1.0 in thirty-one out of thirty-four

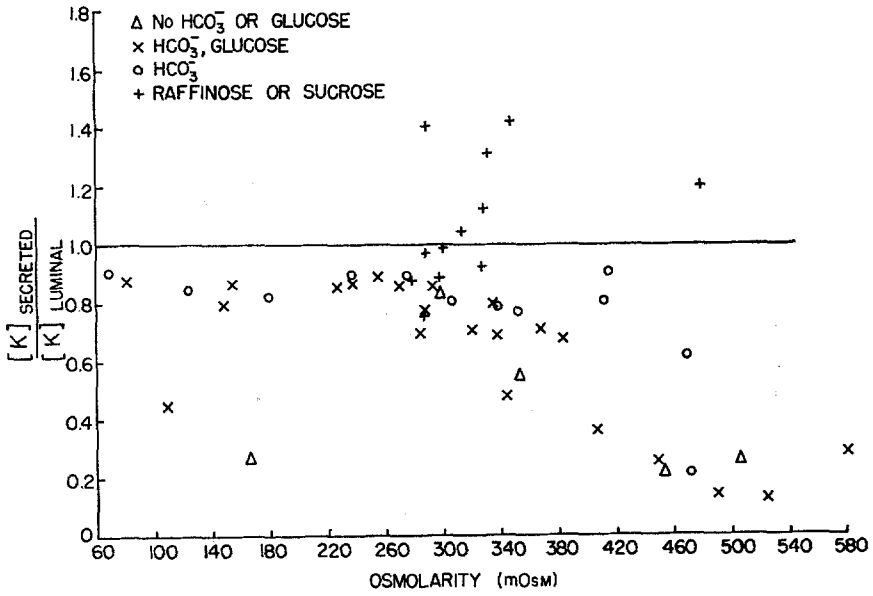

FIGURE 5. $[K]_{secreted}/[K]_{lumen}$ as a function of the osmolarity of the luminal bathing solution. $[K]_{lumen}$ was on the average 8.2 mM. Δ, solution D, Table I; x, solution A; o, solution B; +, solution C.

cases and had an average value of 0.73. The lower values of this ratio appeared to come at intermediate osmolarities and the higher values at the lowest and highest osmolarities, but because of the scatter of the results this conclusion is not certain. Thus, with $[HCO_3]$ at 25 mM in the lumen, it appeared in the secreted fluid at only 18 mM. Conversely, by direct measurement $[Cl]_{secreted}$ was greater than $[Cl]_{lumen}$ in thirty-one out of thirty-four cases in the presence of bicarbonate, hence the gall bladder preferentially transports chloride from the chloride-bicarbonate mixtures. That is, bicarbonate has a lower affinity than chloride for the anion-binding site of the neutral pump, which conveys one anion for each sodium ion. Since, however, higher rates of water and NaCl

transport are observed in the presence than in the absence of bicarbonate, bicarbonate must directly or indirectly stimulate NaCl transport, and this, rather than bicarbonate transport *per se*, is the reason why the gall bladder transports water better in bicarbonate-containing Ringer's solutions. Preferential absorption of chloride over bicarbonate in rabbit gall bladder was previously noted by Wheeler (1963), and the stimulatory effect of bicarbonate was demonstrated reversibly in the conventional gall bladder preparation with an external bathing solution (Diamond, 1964).

Results of Experiments with Sucrose and Raffinose The other series of experiments (thirteen gall baldders) involved solutions in which NaCl and either sucrose or raffinose accounted for most of the osmolarity but which also contained 25 mM $NaHCO_3$ (to get higher secretion rates) and small amounts of other ions (solution C). The luminal Na and Cl concentrations were found to decrease more or less predictably during these experiments, the greater decreases being associated with higher luminal sucrose or raffinose concentrations. Hence an appropriate initial NaCl concentration in the range 100 to 125 mM was chosen for each experiment, depending upon the luminal sugar concentration (the higher the luminal sucrose or raffinose concentration, the higher the initial NaCl concentration chosen), such that the average value of $[NaCl]_{lumen}$ would be about the same for all experiments. Thus, in any one experiment the Ringer's solution contained 100 to 125 mM NaCl plus a certain amount of sucrose or raffinose, and the range of sucrose and raffinose concentrations tested extended up to 182.5 mM. The results were the same for sucrose as for raffinose and are presented in Figs. 5 to 7.

During each experiment the luminal concentration of sucrose or raffinose was found to rise, while those of Na and Cl fell. The secreted fluid contained only small or barely detectable quantities of sucrose or raffinose. $[K]_{secreted}$ was never much less than $[K]_{lumen}$ and was greater than $[K]_{lumen}$ in six out of thirteen cases, although no such case was encountered in the experiments without sucrose or raffinose (Fig. 5). The highest values of $[K]_{secreted}$ occurred in experiments with the highest luminal concentrations of sucrose or raffinose, when $[K]_{secreted}$ might be up to 42 per cent greater than $[K]_{lumen}$. $[Na]_{secreted}$ or $[Cl]_{secreted}$ was always higher than $[Na]_{lumen}$ or $[Cl]_{lumen}$, and the differences between their secreted and luminal concentrations increased with [sucrose or raffinose]$_{lumen}$. In Fig. 6 the difference $([Na] + [K])_{secreted} - ([Na] + [K])_{lumen}$ is plotted against $[sugar]_{lumen} - [sugar]_{secreted}$ (almost the same as just $[sugar]_{lumen}$, since $[sugar]_{secreted}$ was small or negligible). The intercept on the y-axis is virtually 0 (actually, -0.3 mM)—that is $([Na] + [K])_{secreted}$ is the same as $([Na] + [K])_{lumen}$ in the absence of sucrose and raffinose, as described in the preceding section. The slope is 0.55. Fig. 7 depicts the difference $[Cl]_{secreted} - [Cl]_{lumen}$ in the same way, and the slope is 0.49. However, the y-axis intercept

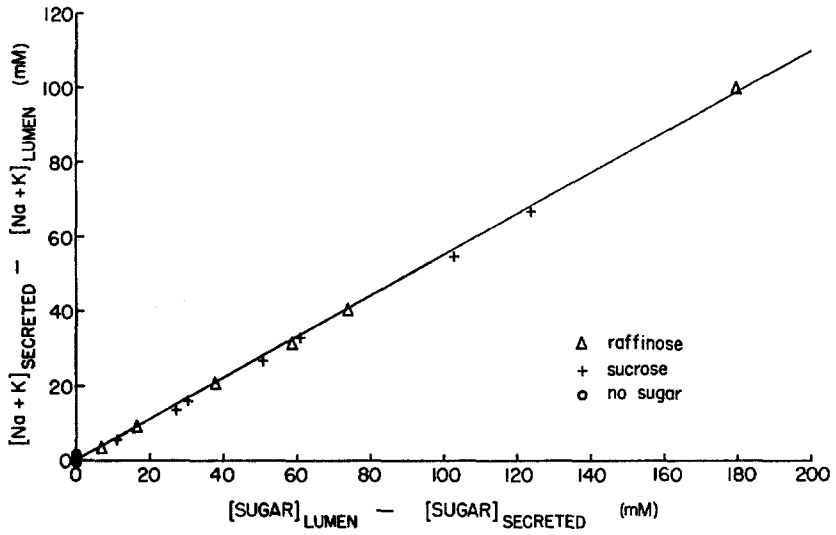

FIGURE 6. Effect of addition of sucrose or raffinose to the luminal bathing solution (solution C, Table I) upon the difference between secreted and luminal cation concentrations. Luminal [NaCl] was always 100 to 125 mM, $[sugar]_{secreted}$ was very small, and [Na] was much larger than [K] in both luminal and secreted fluids. Thus, the main variation is in $[Na]_{secreted}$ as a function of $[sugar]_{lumen}$. The straight line, fitted by least mean squares, has a slope of 0.55 and a y-axis intercept of −0.3 mM.

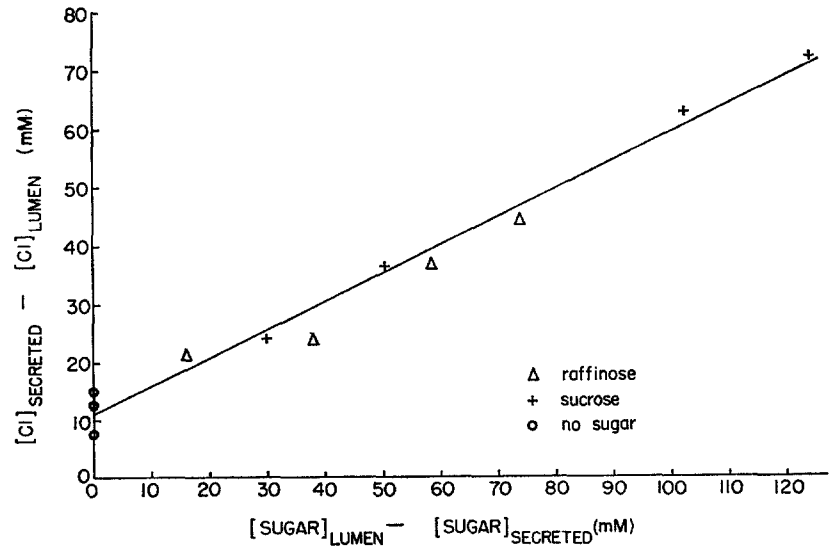

FIGURE 7. Effect of addition of sucrose or raffinose to the luminal bathing solution (solution C, Table I) upon the difference between secreted and luminal [Cl]. Luminal [NaCl] was always 100 to 125 mM, and $[sugar]_{secreted}$ was very small. Thus, the main variation is in $[Cl]_{secreted}$ as a function of $[sugar]_{lumen}$. The straight line, fitted by least mean squares, has a slope of 0.49 and a y-axis intercept of 11 mM.

is 11 mM, meaning that $[Cl]_{secreted}$ is greater than $[Cl]_{lumen}$ even in the absence of sucrose or raffinose. This point was discussed in the preceding section and attributed to preferential transport of chloride over bicarbonate. The slopes of 0.55 and 0.49 in Figs. 6 and 7 mean that for about every mM of sucrose or raffinose in the lumen, an extra 0.5 mM of NaCl appears in the secreted fluid.

The Osmolarity of the Secreted Fluid From the preceding sections it is qualitatively apparent that the transported fluid is approximately isotonic, because: (*a*) when the lumen contained only salt, the salt concentrations of the secreted and luminal fluids were the same; (*b*) when the lumen contained salt plus low concentrations of glucose, the secreted concentration of glucose was lower than the luminal concentration, but this was balanced by the secreted concentration of salt being slightly higher than the luminal concentration; (*c*) when the lumen contained salt plus large concentrations of sucrose or raffinose, the secreted salt concentration increased 1 mM (yielding two osmotically active particles) for every 2 mM of luminal sucrose or raffinose. The following more refined calculation confirms quantitatively the conclusion that the transported fluid is isotonic.

To calculate the relative osmolarities of the secreted and luminal fluids, it is necessary to know the concentrations of all solutes and the appropriate osmotic coefficients. The concentrations of Na, K, Cl, sucrose, and raffinose were known from direct measurement. In the experiments in which glucose was determined, it was found to be secreted at 17 per cent of its luminal concentration, and this was assumed also to be true of experiments in which glucose was present but not analyzed. No calcium or magnesium determinations were performed, and one must make a guess about their behavior, although they are present in such low concentrations that a wrong guess will introduce only a very small error. Some calcium diffuses out of the gall bladder *in vivo* during the reabsorption of bile, and when one considers the electrical potential difference present across the canine gall bladder *in vivo*, the concentration of calcium in canine gall bladder bile is about that expected from the Nernst equation if calcium had diffused out until electrochemical equilibrium was attained. On the basis of the present meager evidence, then, it seems simplest to assume for purposes of the calculation that Ca^{++} and Mg^{++} appeared in the secreted fluid at the same concentration as in the lumen, though this is surely an overestimate. Since the anions (mainly chloride) were monovalent except for $SO_4^{=}$ at only 1.2 mM, all cations were assumed to be accompanied by monovalent anions.

The osmotic coefficient Φ for glucose is 1.00 for the low concentrations used. Osmotic coefficients for sucrose were taken from the tables of Robinson and Stokes (1959) and fell between 1.000 and 1.015 over the concentration range encountered in the experiments. Raffinose was assumed to have the same osmotic coefficient as sucrose. Since NaCl was the major salt present, all salts

were assumed to have the osmotic coefficient of NaCl at the same total salt concentration. NaCl osmotic coefficients at 37°C were obtained by interpolation from the tables of Robinson and Stokes and ranged from 0.949 to 0.920. The osmolarity of each sample was then obtained by multiplying the concentration and the osmotic coefficient of salt, glucose, and sucrose or raffinose, then adding the products. The validity of this procedure was confirmed by the fact that it predicted within experimental error the freezing point depressions of the Ringer's solutions as measured by the Aminco osmometer.

Fig. 8 shows the relation between the calculated osmolarities of the secreted and luminal fluids. The fact that all points lie close to the line of 45° slope

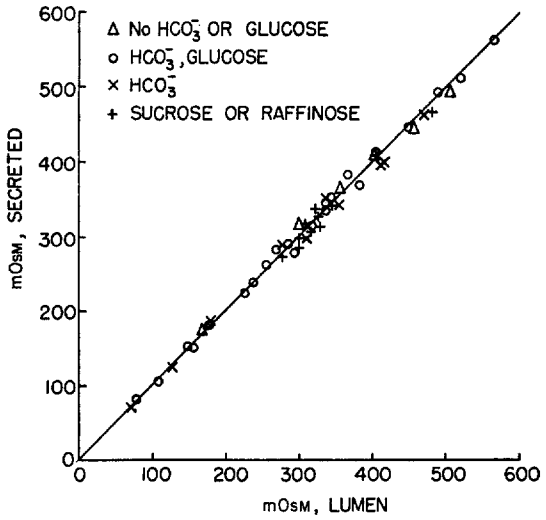

FIGURE 8. Osmolarity of the secreted fluid as a function of the osmolarity of the luminal bathing solution. For symbols, see legend to Fig. 5. The straight line is the line of 45° slope.

quantitatively confirms the conclusion that the secreted fluid is isotonic to the luminal fluid under all conditions tested. The actual ratio of the osmolarity of the secreted fluid to the osmolarity of the luminal fluid is 0.987 ± 0.025 (average value and standard deviation) for thirteen experiments involving sucrose or raffinose, and 1.003 ± 0.016 for forty-one experiments without sucrose or raffinose. The secreted fluid is thus isotonic within experimental error, and the standard deviation of the osmolarity ratio is of the order that one would expect from the analysis of experimental errors. However, there are two additional sources of error that reduce the precision with which the osmolarity ratio can be stated. In the first place, on the basis of indirect evidence the most reasonable simple guess that could be made about calcium and magnesium was that they appeared in the secreted and luminal fluids at the same concentration. At worst, if $[Ca]_{secreted}$ and $[Mg]_{secreted}$ are in fact 0, then the secreted fluid will actually be 3 per cent more hypotonic than calculated. Second, the weight of secreted fluid collected was on the average 2.8 per cent less than the

weight of fluid lost by the gall bladder, and this difference was assumed to be due to evaporation of secreted fluid while opening the Goetz tube. If in fact some or all of this difference represents fluid that evaporated directly from the surface of the gall bladder before falling into the Goetz tube, then at worst the secreted fluid may be up to 2.8 per cent more hypertonic than calculated. The two additional sources of error are thus in opposite directions, but it is not known to what extent they will cancel.

Because of these two sources of error the precision of the experiment is less good than the standard deviation of 1.6 to 2.5 per cent, but the most pessimistic estimate of the precision would be 5 per cent. Within this margin of error the secreted fluid is isotonic to the luminal fluid from 68 to 578 mOsM. Thus, when filled with 250 mM NaCl, the gall bladder secretes 250 mM NaCl; when filled with 50 mM NaCl, it secretes 50 mM NaCl; and when filled with 100 mM NaCl plus 150 mM sucrose, it secretes 175 mM NaCl.

DISCUSSION

Validity of the Unilateral Preparation

Before the interpretation of the results can be considered, it must first be made clear on what grounds the use of the unilateral preparation for studying the mechanism of water transport is justified:

1. Only after the gall bladder had equilibrated with experimental solutions on both sides for *ca.* 40 minutes (*ca.* 14 minutes during the cannulation, a further equilibration period of 15 to 20 minutes, and *ca.* 8 minutes while the luminal solution was renewed) did the collection of secreted fluid begin. While the experimental solutions often differed markedly from rabbit plasma in osmolarity and salt concentration, this time must have sufficed for the cells of the gall bladder wall to reach osmotic equilibrium with the solutions and attain an ionic steady state, since no detectable shifts of water, Na, or Cl between the gall bladder wall and the solutions occurred while secreted fluid was being collected.

2. Before the actual collection of secreted fluid began, the gall bladder was allowed to secrete into another Goetz tube for 10 minutes. During this preliminary period the former outer bathing solution was pushed out of the serosal connective tissue space and replaced by secreted fluid, so that the first drops obtained in the collection period were already secreted fluid.

3. The secreted fluid was found to contain virtually no sucrose, raffinose, inulin, phenol red, or Evans Blue when these were present in the lumen. One is therefore dealing with a semipermeable membrane and the specific transported fluid elaborated by the activity of gall bladder epithelial cells. Transport cannot involve a bulk passage (pinocytosis) of luminal fluid.

4. In the absence of sucrose and raffinose the rate of water transport was found to be the same whether there was a bathing solution outside or whether

the gall bladder was functioning as a unilateral preparation. This observation is now seen to be a trival consequence of the fact that the secreted fluid has the same osmolarity as the bathing solution. It thus makes no difference whether the fluid at the serosal surface of the epithelial cells was placed there by the experimenter or elaborated by the gall bladder itself, since its composition is about the same in either case. In experiments with sucrose in the lumen the transport rate was lower with an outer bathing solution than in the unilateral preparation. This is because the unilateral preparation generated an isotonic secretion, whereas the outer bathing solution lacked sucrose and would be hypotonic, creating an unfavorable osmotic gradient. A hydrostatic pressure of about 4 cm water is present across the unilateral preparation that is absent in the preparation with outer bathing solution, but the filtration constant of the gall bladder is so low that this would increase the rate of water movement by only 0.2 per cent.

5. In a few experiments on conventional preparations with an outer bathing solution identical to the luminal bathing solution, the osmolarity of the transported fluid was calculated from the loss of salt and water from the lumen and found to be isotonic to the bathing solution between 257 and 358 mOsM. In both the unilateral and conventional preparations there is specific transport of isotonic NaCl, stimulation by bicarbonate, preferential transport of chloride over bicarbonate, no requirement for glucose, and retention of sucrose and larger molecules. Hence the conventional preparation yields the same conclusions as the unilateral, but the latter permitted these conclusions to be reached with greater accuracy.

For these reasons it is valid to regard the fluid collected from a unilateral preparation as the pure fluid elaborated by the norml transport processes of the gall bladder.

Interpretation of the Results

The findings that must still be explained to obtain an understanding of water transport in the gall bladder are the behavior of potassium and the isotonicity of the secreted fluid.

THE BEHAVIOR OF POTASSIUM The appearance of K in the secreted fluid raises three problems.

1. *Why Does* $[K]_{secreted}$ *often Exceed* $[K]_{lumen}$ *in the Presence of Sucrose or Raffinose?* At first sight this fact might suggest active transport of K as well as of Na and Cl. However, previous work made it fairly certain that the movement of K through the gall bladder is governed only by passive forces, as was demonstrated for fish (Diamond, 1962 *a*, 1962 *b*) and rabbit (Wheeler, 1963; Dietschy, 1964) gall bladders. The key to the present findings is given by Dietschy's explanation why [K] in rabbit gall bladder bile is higher than

[K] in rabbit plasma. A diffusion potential (lumen-negative) was found to exist across the wall of the bile-filled gall bladder because of the Na and Cl concentration gradients ([Na] higher and [Cl] lower in the lumen than in plasma). A lumen-negative potential difference would lead to K accumulation in the lumen, and when both the K concentrations and the electrical potential difference were taken into account, K was found to be at electrochemical equilibrium according to the Nernst equation. Dietschy was able to show that the K concentration gradient across the gall bladder wall oriented itself passively according to the electrical potential under a variety of other circumstances as well.

In the present experiments, when sucrose or raffinose was present in the lumen, $[Na]_{secreted}$ and $[Cl]_{secreted}$ were found to be higher than $[Na]_{lumen}$ and $[Cl]_{lumen}$, and since the chloride permeability is considerably lower than the sodium permeability in rabbit gall bladder (Wheeler, 1963; Dietschy, 1964; Pidot and Diamond, 1964), this NaCl concentration gradient must have produced a diffusion potential, lumen-positive. This electrical potential difference was of the correct orientation to account for $[K]_{secreted}$ exceeding $[K]_{lumen}$, and calculation from the Nernst equation showed that it would be of the correct size. Hence the fact that $[K]_{secreted}$ often exceeded $[K]_{lumen}$ in the presence of sucrose or raffinose does not indicate active K transport across the gall bladder and is compatible with K entering the secreted fluid passively.

2. *Why Does* $[K]_{secreted}$ *Decrease with Osmolarity in the Absence of Sucrose or Raffinose (Fig. 5)?* It was found that $[Na]_{secreted}$ was scarcely greater than $[Na]_{lumen}$ in the absence of sucrose or raffinose, hence there could have been no diffusion potential. Under these circumstances, as one would expect from the passive behavior of K, there was no instance in which $[K]_{secreted}$ exceeded $[K]_{lumen}$. One might then suppose that the apparent decrease in $[K]_{secreted}$ with osmolarity means that the permeability to K decreases with osmolarity, although there is not yet direct evidence about possible changes in permeability with osmolarity. Alternatively, since the rate of fluid transport was also found to vary with the osmolarity, it seemed possible that the true correlation of $[K]_{secreted}/[K]_{lumen}$ was with the fluid transport rate, and accordingly the relation between these two variables is plotted in Fig. 9. $[K]_{secreted}/[K]_{lumen}$ drops to low values at low secretion rates, and since Fig. 9 contains fewer aberrant points than Fig. 5, $[K]_{secreted}$ might depend directly on the fluid transport rate, and its apparent decrease with osmolarity would be because higher osmolarities tend to yield lower fluid transport rates. If the appearance of K in the secreted fluid were determined solely by the rate at which K diffused through the gall bladder, then $[K]_{secreted}$ should have been highest at the lowest rates of fluid transport, when the maximum time was available for equilibration. In fact, $[K]_{secreted}$ was lowest at the lowest secretion rates, and this might suggest that the gall bladder epithelial cells have a mechanism at their serosal

surface which takes up K from the secreted fluid at a constant rate, so that they would be able to reabsorb a greater fraction of the K in the secreted fluid at the lowest secretion rates. The presence of such a mechanism is implied by the high K concentration in the epithelial cells of the gall bladder and by analogy with frog skin, urinary bladder, and kidney proximal tubule.

Thus, the apparent decrease of $[K]_{secreted}$ with osmolarity either means that the permeability to K decreases with osmolarity, or that at the low transport

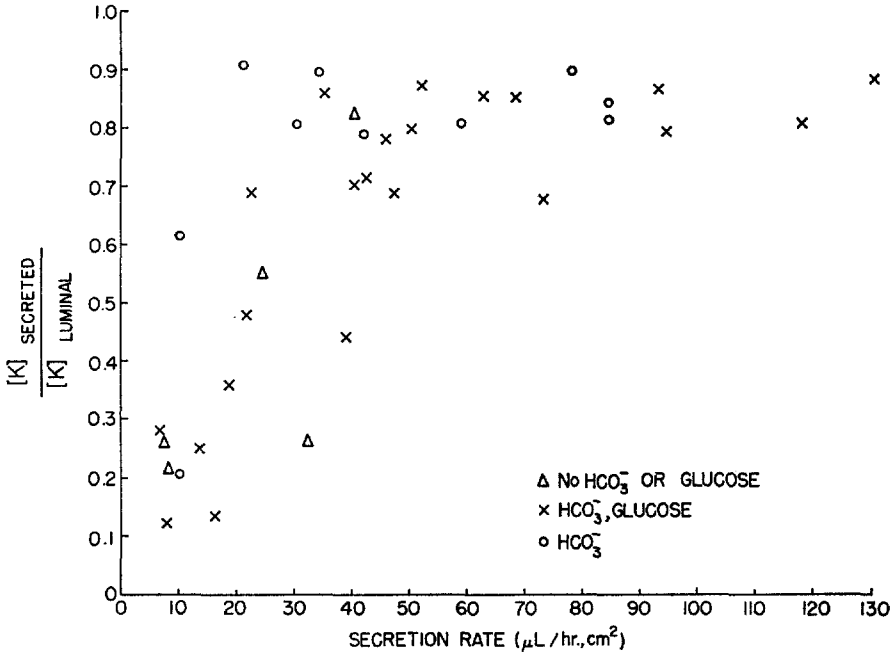

FIGURE 9. $[K]_{secreted}/[K]_{lumen}$ as a function of the rate of fluid transport. For symbols, see legend to Fig. 3.

rates prevailing at high osmolarities the epithelial cells return much of the K in the secreted fluid to the lumen.

3. *How Does* K *Get into the Secreted Fluid?* If it could be shown that the passive permeability of K (P_K) were sufficiently high to account for the observed rate at which K enters the secreted fluid, then the presence of K in the secreted fluid could be attributed to simple diffusion, and there would be no need to postulate special mechanisms. The following calculation will demonstrate that P_K is in fact sufficiently high.

Average values of relevant parameters for experiments in the range 180 to 350 mOsM without sucrose or raffinose are: $[K]_{lumen}$ 8.2 mM, $[K]_{secreted}$ 6.6 mM, fluid transport rate 56 μl/cm², hour. The K secretion rate is therefore (6.6) (56) (10^{-3}) = 0.37 μmole/cm², hour. If a flux of 0.37 μmole/cm², hour is

to be generated by diffusion down the concentration gradient between solutions at 8.2 and 6.6 mM, the required permeability is $0.37/(8.2 - 6.6) = 0.23$ cm/hour $= 6.4 \times 10^{-5}$ cm/sec. From radioactive flux measurements Wheeler (1963) found the partial conductivity of K at 5.9 mM to be roughly 0.0031 ohm^{-1}/100 mg gall bladder wet weight. Expressed as an absolute permeability coefficient (calculated as $\frac{RTG}{z^2F^2C}$, where G is the partial conductivity and C the concentration of potassium, and R, T, z, and F have their usual meanings) and related to surface area (1 cm^2 = 54 mg wet weight), this comes out 7.4×10^{-5} cm/sec. The actual value of P_K (7.4×10^{-5} cm/sec.) is therefore of the same size as the value of P_K required to generate the observed secretion of K by diffusion (6.4×10^{-5} cm/sec.). Although this does not exclude the possibility that some other mechanism is involved, there is at present no need to postulate anything other than diffusion.

To summarize the conclusions arrived at in this discussion of the behavior of K: the observed values of $[K]_{secreted}$ can always be explained by a passive entrance of K into the secreted fluid, although the epithelial cells themselves may reabsorb some secreted K. Whatever the mechanism and channels by which the gall bladder secretes water, K could then diffuse into the secreted water through the usual channels for passive K movement.

THE MECHANISM OF ISOTONIC WATER TRANSPORT The secreted fluid was found to be isotonic to the luminal fluid within at least 5 per cent under all conditions—that is, over a more than eightfold range of osmolarities, 12-fold range of Na concentrations, 27-fold range of chloride concentrations, in the presence and absence of glucose or bicarbonate, and in the presence of sucrose or raffinose concentrations which increased the luminal osmolarity by up to 62 per cent. Of the theories of water transport discussed in the introduction, these findings are what the simpler theory, local osmosis, predicted; but are incompatible with the double membrane effect, which predicted anisotonic transported fluids at osmolarities other than that of plasma. In addition, these results are in conflict with a theory of water transport based on codiffusion, as suggested in a previous study (Diamond, 1962 *c*), since codiffusion would also have predicted anisotonic transported fluids at osmolarities other than that of plasma. Any attempt to reconcile the results with the double membrane effect or codiffusion by assuming concentration-dependent membrane parameters or other correction factors seems unpromising because it would take an extraordinary constellation of correction factors to make the secreted fluid fortuitously isotonic within 5 per cent over an eightfold range of osmolarities, and there is already a simpler theory which fits the results without *ad hoc* assumptions. The only convincing way of accounting for isosmolar transport under all conditions is osmotic equilibration. It is therefore concluded that the mech-

anism of isotonic water transport in rabbit gall bladder is neither the double membrane effect nor codiffusion, but must involve local osmosis.

THE ROUTE OF ISOTONIC WATER TRANSPORT Given that water transport depends upon local osmotic equilibration of actively transported solute in the vicinity of the cell membrane, the problem remains as to exactly where in the cell membranes this water flow occurs. The simplest possibility would be that the water movement linked with active NaCl transport takes place in the same channels through which water and water-soluble molecules traverse the cell membrane under passive conditions, *i.e.*, in response to solute concentration gradients in the external bathing solutions. These passive channels (sometimes pictured as "pores") are relatively long and narrow (Diamond, 1962 *c*), impermeable to molecules of the size of sucrose or larger, and bear negative fixed charges (Pidot and Diamond, 1964). However, two kinds of evidence indicate that solute-linked water transport does not follow these channels and that special structures at the cell membrane must be postulated: evidence from osmotic permeability and from streaming potentials.

(*a*) *Osmotic Permeability* If the gall bladder were almost impermeable to water, then clearly osmosis resulting from active solute transport could not pull enough water through the passive channels, and additional factors would have to be postulated. For any finite value of the osmotic water permeability P_{osm} (defined as the water flow per unit area, time, osmotic pressure difference; variously termed the filtration constant, hydraulic conductivity, etc.), then at low rates of secretion the actively transported solute would have time to equilibrate completely and pull water along in virtually isotonic proportions; but at higher rates of secretion equilibration would become progressively more incomplete, and the transported fluid more hypertonic. The question is thus a quantitative one: given the measured solute transport rate, is the measured P_{osm} of the gall bladder (*i.e.* of its passive channels) sufficient to account for the observed degree of osmotic equilibration of the transported fluid? The following calculation will demonstrate that the measured P_{osm} is grossly insufficient.

In the Appendix it is shown that if local osmosis caused by actively transported solute is the sole force behind water transport, then the osmolarity C of the transported fluid should be:

$$C = \frac{0_o}{2} + \sqrt{\frac{0_o^2}{4} + \frac{M_o}{RTP_{osm}}} \qquad (1)$$

where 0_o = osmolarity of the bathing solution

M_o = rate of active solute transport

R = gas constant, T = absolute temperature.

With low secretion rates (M_o) or high values of P_{osm}, C approaches 0_o, *i.e.*

isotonicity, while for larger M_o or lower P_{osm}, C becomes progressively more hypertonic. The derivation assumes (*a*) that the membrane is completely impermeable to actively transported solute, (*b*) that all the hydrostatic resistance of the membrane resides in the osmotically discriminating barriers characterized by P_{osm}, and (*c*) that osmotic equilibration of actively transported solute can proceed across all the regions of the membrane where passive water flow is possible. Deviations from any of these assumptions would make C higher than the value given by equation 1, which therefore sets a lower limit on the transported osmolarity.

If one performs the calculation at a bathing solution osmolarity of 300 mOsM ($0_o = 300 \times 10^{-3}$ mOsM/cc), the average fluid transport rate is 47 μl/cm^2, hour (Fig. 2), and the transport rate of solute (93 per cent of it being actively transported solute) is (300) (47) (10^{-6}) = 1.41×10^{-2} mOsM/cm^2, hour (M_o). P_{osm} was estimated as 7.3×10^{-3} cm/hour, atmosphere (0.16 μl/cm^2, hour, mOsM) from measurements of rates of osmosis when both sides of the gall bladder initially had identical bathing solutions and an impermeant non-electrolyte (mannitol, sucrose, or raffinose) was then added to one bathing solution. RT is 26 atmospheres—cc/mM at 37°C. Inserting these values into equation 1, one obtains as the predicted value of the transported osmolarity

$$\frac{300 \times 10^{-3}}{2} + \sqrt{\frac{(300 \times 10^{-3})^2}{4} + \frac{1.41 \times 10^{-2}}{(7.3 \times 10^{-3})\ (26)}} =$$

461×10^{-3} mOsM/cc = 461 mOsM. This would be hypertonic by 161 mOsM (3.6 atmospheres), completely beyond the range of experimental error within which the transported fluid was found to be isotonic. Alternatively, one may ask how permeable the gall bladder would have had to be for the hypertonicity of the transported fluid to fall within the range of experimental error (±5 per cent). For C = 315 mOsM (105 per cent of 0_o) and 0_o and M_o as before, equation 1 yields $P_{osm} = 115 \times 10^{-3}$ cm/hour, atmosphere, or 16 times the measured value. The same calculation at 0_o = 200 mOsM requires that P_{osm} be 45 times its measured value.

Thus, the channels for passive water and solute movement would be grossly insufficient to permit complete osmotic equilibration of the transported fluid, since the measured P_{osm} for passive water flow is too low by 16 to 45 times. This measured P_{osm} remained much too small whether or not active transport was going on and regardless of the direction of water flow or the side on which the impermeant test molecule was placed.

Application of equation 1 to published values of M_o and P_{osm} for other tissues predicts a transported fluid hypertonic by 116 mOsM for rat small intestine (M_o and P_{osm} from work of Parsons and Wingate, 1961), by 20 mOsM for the proximal tubule of the kidney (from Whittembury, Oken,

Windhager, and Solomon, 1959), by 95 mOsM for frog stomach (from Durbin, Frank, and Solomon, 1956, and Hogben, 1955), and by 47 mOsM for the cerebrospinal fluid system (from Heisey, Held, and Pappenheimer, 1962). All these tissues actually transport fluid isotonically. If the mechanism of water transport in these cases, as in the gall bladder, proves to be local osmosis, then their passive water permeabilities are also grossly insufficient.

Osmotic evidence thus indicates that solute-linked water transport in the gall bladder does not follow the passive channels in the cell membranes. This conclusion is reinforced by the failure of solute-linked water transport to produce the electrokinetic effects that occur in these channels, as described in the next section.

(*b*) *Streaming Potentials* are the electrical potential differences (PD's) that arise across charged membranes through which water is flowing. Qualitatively they are due to the fact that the aqueous channels through the membrane contain an excess of mobile ions of opposite sign to balance the fixed charge. Water flow through these channels will then carry along the mobile ions and give the side of the membrane towards which water is moving an electrical potential of sign opposite to that of the fixed charge. The gall bladder must contain negative fixed charges, since it is much more permeable to cations than to anions (Wheeler, 1963; Dietschy, 1964). When water flows through it passively (*i.e.*, in response to a solute concentration gradient in the external bathing solutions), a streaming potential is set up in which the side towards which water moves goes positive, confirming the presence of negative fixed charges (Diamond, 1962 *c*; Pidot and Diamond, 1964). The proportionality coefficient is approximately 1 mv for a flow of 7.5 μl/cm^2, hour. However, the water flow associated with active NaCl transport gives no streaming potential. When osmosis and active NaCl transport occur simultaneously, the streaming potential is proportional only to that component of the water flow due to osmosis, and if that osmotic gradient is chosen which balances active transport and yields a net water flow of 0, the streaming potential expected for osmosis is still observed (Pidot and Diamond, 1964). Thus, isotonic water transport must occur in channels distinct from those for passive water flow. Calculations based on Dietschy's (1964) experiments yield the same conclusion. He observed isotonic water transport at 43 μl/hour, cm^2, which should have given a streaming potential of 43/7.5 = 5.7 mv, lumen-negative; but the actual PD was only 0.7 mv, lumen-positive. Similarly, the transport rate of 165 μl/hour, 100 mg = 89 μl/cm^2, hour obtained by Wheeler (1963) should have yielded a streaming potential of 11.9 mv, lumen-negative, whereas in fact the lumen was slightly positive. Actually, water traversing the gall bladder must cross two cell membranes, the luminal and serosal membranes of the epithelial cells, both of which have approximately the same relative permeabilities. The complete absence of a streaming potential thus means that water associated with actively transported NaCl crossed neither of

these membranes by passive channels. Similarly, the finding that the transported fluid is isotonic means that local osmotic equilibration must be the mechanism of water transport at both membranes.

Since solute-linked water transport does not follow the passive channels through the cell membrane, special structures in the cell membrane must be postulated to explain where local osmosis occurs, and two kinds of possibilities will be suggested. (*a*) Osmotic equilibration of actively transported NaCl may occur in permeable regions of the cell membrane which molecules crossing the gall bladder passively cannot reach. The effective value of P_{osm} to insert into equation 1 would then be much greater than the measured value. There would have to be some valve-like morphological or mechanical arrangement to keep passively diffusing solutes away from these hypothetical permeable regions and to ensure that NaCl arrive there only *via* the active transport mechanism. For example, the serosal membranes of the epithelial cells in the gall bladder, as in many other tissues, have deep, narrow infoldings. If NaCl were actively transported into the bottoms of these microcrypts and they had a high water permeability, the stream of isotonic NaCl passing out the mouths of the crypts would prevent passively diffusing solutes in the serosal bathing solution from entering the crypts while they were distended, and the crypts could not contribute to measurements of passive osmotic water flow. However, equating the hypothetical permeable regions with the microcrypts fails to explain what prevents passively diffusing solutes from reaching the regions from the luminal bathing solution, and microcrypts could not provide the answer at the mucosal membranes of the epithelial cells, where no such crypts are seen. (*b*) Osmotic equilibration of actively transported NaCl might occur in some restricted region (*e.g.*, by secreting NaCl into a newly generated vesicle, etc.) before the cell membrane is traversed, and isotonic NaCl might then be transported across the osmotic barrier in the cell membrane physically, *e.g.* by membrane contractile proteins, breaking and rejoining of membrane, mitochondria, etc. The physical transfer of an isotonic NaCl solution, resulting from active NaCl transport, across the cell membrane is not to be confused with pinocytosis, which involves bulk uptake of fluid and is completely unable to account for the specificity of the transport process.

The present evidence, then, demands the existence of some special channels but is inadequate to decide which, if either, of the two kinds of special structures described might be involved in local osmosis.

In summary, the mechanism of water transport in rabbit gall bladder can be neither codiffusion nor the double membrane effect, since the transported fluid is isotonic under all conditions. Water transport cannot follow the channels for water flow under passive conditions, since it produces no streaming potential and these channels are grossly insufficient to permit osmotic equilibration. The mechanism of isotonic water transport in rabbit gall

bladder is therefore local osmosis in the cell membrane at special sites distinct from the channels for passive flow.

APPENDIX

Local Osmosis

The problem is to calculate the osmolarity of the transported fluid if active solute transport produces water transport by local osmosis in a restricted region of the cell membrane. The situation is illustrated diagrammatically in Fig. 10.

Consider a membrane separating two identical bathing solutions at the same temperature and pressure. Solute is actively transported at a rate M_o (moles/sec.)

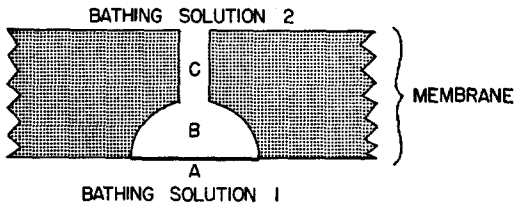

FIGURE 10. Diagrammatic representation of how local osmosis, in association with active solute transport, might carry water across a membrane separating identical bathing solutions. Active solute transport from bathing solution 1 across the osmotically discriminating barrier A maintains a raised concentration of solute in B, a restricted space within the membrane. Water continually enters B from bathing solution 1 by osmosis. The resulting raised hydrostatic pressure in B continually forces water and solute out through C, the remaining hydrostatic resistance in the membrane between B and bathing solution 2.

from bathing solution 1 across an osmotically discriminating barrier A characterized by a hydraulic conductivity P_{osm} (cc/sec., atm) into a restricted region B. P_{osm} may actually arise from a series of barriers and represents the minimum osmotic resistance which water must overcome to cross the membrane. The osmotically discriminating barrier A is assumed impermeable to solute, and the restricted region B is assumed small enough to have uniform concentrations throughout. The total hydrostatic resistance between the restricted region and bathing solution 2 is characterized by the hydraulic conductivity P_R (cc/sec., atm). Bathing solutions 1 and 2 have the same osmolarity 0_o (mOsM/cc), and active solute transport maintains the steady-state osmolarity of the restricted region at some higher value C (mOsM/cc).

Then water will enter the restricted region across the osmotic barrier by osmosis at a rate $RTP_{osm}(C - 0_o)$ and build up a steady-state hydrostatic pressure X (atmospheres) in the restricted region. Water is forced back from the restricted region across the osmotic barrier to bathing solution 1 by filtration at a rate XP_{osm}, but no solute is forced back because the osmotic barrier is impermeable to solute. Fluid is forced out of the restricted region towards bathing solution 2 at a rate XP_R and carries solute at a rate CXP_R(mOsM/sec.).

The equation for conservation of water is:

$$XP_R = RTP_{osm}(C - 0_o) - XP_{osm} \quad (1)$$

and for the solute:

$$M_o = CXP_R \quad (2)$$

Solving equation (1) for X:

$$X = \frac{RT(C - 0_o)\,P_{osm}}{P_R + P_{osm}}$$

Substituting this in equation (2):

$$RTP_R\,P_{osm}\,C^2 - RT0_o\,P_R\,P_{osm}\,C - M_o(P_R + P_{osm}) = 0.$$

Solving the quadratic for C:

$$C = \frac{0_o}{2} + \sqrt{\frac{0_o^2}{4} + \frac{M_o(P_R + P_{osm})}{RTP_R\,P_{osm}}} \quad (3)$$

If $P_R \gg P_{osm}$ (*i.e.*, if most of the hydrostatic resistance of the whole membrane is in the osmotically discriminating barrier), then equation (3) reduces to:

$$C = \frac{0_o}{2} + \sqrt{\frac{0_o^2}{4} + \frac{M_o}{RTP_{osm}}} \quad (4)$$

Equation (4) gives the transported osmolarity C which would result by local osmosis for a given bathing solution osmolarity 0_o, solute transport rate M_o, and osmotic permeability P_{osm}. In the limit where $M_o \to 0$ or $P_{osm} \to \infty$, $C \to 0_o$ (*i.e.*, C is isotonic); otherwise $C > 0_o$ (transported fluid hypertonic).

There are three limitations on the usefulness of equation (4). (1) To get from equation (3) to equation (4), it was assumed that P_R is very high compared to P_{osm}. Inspection of equation (3) shows that for a given value of P_{osm}, C increases with decreasing P_R. That is, a high hydrostatic resistance (low P_R) beyond the restricted region will make C more hypertonic. (2) If the membrane is not absolutely impermeable to solute, as assumed (*i.e.*, if the reflection coefficient σ of the osmotically discriminating barrier is less than 1), then water will enter the restricted region by osmosis at a rate less than $RTP_{osm}(C - 0_o)$, and C will be more hypertonic. (3) If the active transport mechanism can utilize only a part of the water-permeable area of the membrane for equilibration, then the effective value of P_{osm} will be lower, and C more hypertonic.

Since all three deviations are in the direction of increased hypertonicity, equation (4) sets a lower limit on the transported osmolarity.

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