The Ultrastructural Route of Fluid Transport in Rabbit Gall Bladder

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ABSTRACT The route of fluid transport across the wall of the rabbit gall bladder has been examined by combined physiological and morphological techniques. Fluid transport was either made maximal or was inhibited by one of six physiological methods (metabolic inhibition with cyanide-iodoacetate, addition of ouabain, application of adverse osmotic gradients, low temperature, replacement of Cl by SO_4, or replacement of NaCl by sucrose). Then the organ was rapidly fixed and subsequently embedded, sectioned, and examined by light and electron microscopy. The structure of the gall bladder is presented with the aid of electron micrographs, and changes in structure are described and quantitated. The most significant morphological feature seems to be long, narrow, complex channels between adjacent epithelial cells; these spaces are closed by tight junctions at the luminal surface of the epithelium but are open at the basal surface. They are dilated when maximal fluid transport occurs, but are collapsed under all the conditions which inhibit transport. Additional observations and experiments make it possible to conclude that this dilation is the result of fluid transport through the spaces. Evidently NaCl is constantly pumped from the epithelial cells into the spaces, making them hypertonic, so that water follows osmotically. It is suggested that these spaces may represent a "standing-gradient flow system," in which osmotic equilibration takes place progressively along the length of a long channel.

INTRODUCTION

The physical mechanism by which epithelial tissues such as the intestine, kidney proximal tubule, liver, and gall bladder form secretions or absorbates isotonic to plasma has been considerably clarified in recent years. As a result

of evidence that water movement across epithelial membranes is a secondary consequence of active solute transport (Curran and Solomon, 1957; Windhager et al., 1959; Diamond, 1962 *c*, 1965), the problem has become one of understanding how passive water fluxes and active solute fluxes are coupled.

Osmotic forces seem to be involved in such coupling. For example, the rabbit gall bladder transports a fluid which is always osmotically equilibrated with the bathing solution within its lumen, regardless of the latter's absolute osmolarity (Diamond, 1964 *b*). Nevertheless, osmotic pressure differences between the bulk solutions bathing opposite sides of the transporting epithelium cannot be responsible for isotonic transport: osmotic gradients between the transported fluid and the parent bathing solution or plasma are small or nonexistent in many organs, and furthermore all epithelia tested have proved able to move water uphill against considerable external osmotic gradients. From this and other evidence, it has been concluded that complete osmotic equilibration must take place within the epithelium itself and that the physical basis of water transport may be described as *local* osmosis (Diamond, 1964 *b*, 1965). According to this concept, the osmotic gradients responsible for fluid transport are local ones within the tissue, established by active transport of solute into restricted regions adjacent to the epithelial cell membrane.

These physiological considerations immediately raise several structural problems: What route do substances crossing an epithelium follow, and where are the local osmotic gradients established? What is the detailed geometry of the transporting region? The latter question is critical, because solute transport across a flat surface into an unstirred layer of the thickness encountered in epithelia could not yield an isotonic fluid: solute would diffuse away from such a surface before osmotic equilibration was complete, causing the transported fluid to be grossly hypertonic.[1] Even in frog skin, a tissue which actually does transport hypertonically, diffusion delays in a flat unstirred layer would not retard solute sufficiently to explain the observed rate of water flow (Dainty and House, 1966).

The present report is an attempt to answer such questions for the case of the rabbit gall bladder. This organ is particularly suitable for combined physiological and morphological studies, because of its durability in vitro, its high rate of solute transport, and its physiological and anatomical simplicity. The epithelial cells are in direct contact with the luminal bathing solution, so that rapid and uniform fixation can be achieved. Isotonic fluid transport is driven by an electrically neutral NaCl pump, and can proceed against

[1] A calculation for this can be made by inserting the values of solute transport rate, water permeability, and unstirred layer thickness which have been experimentally measured for rabbit gall bladder into equation 18 of Dainty and House (1966). The result is that transport in the gall bladder, if it occurred across a flat surface, would produce a fluid hypertonic by 1320%, which is grossly incompatible with the isotonicity actually observed.

osmotic gradients of two or three atmospheres (Diamond, 1962 *a*, *b*, *c*, 1964 *a*, *b*; Wheeler, 1963; Dietschy, 1964; Whitlock and Wheeler, 1964).

The principle of our experiments was to vary the rate of fluid transport in the gall bladder experimentally, then to fix the organ rapidly, and to examine it by light and electron microscopy. The results show that fluid transport in the gall bladder proceeds via structures of a distinctive geometry, which have analogues in other epithelia. The second paper of this series (Diamond and Bossert, 1967) discusses the physiological consequences of this geometry in the light of a mathematical analysis. A preliminary report of some of our light microscopic findings has been presented elsewhere (Diamond and Tormey, 1966), and some similar experiments have also been described by Kaye, Wheeler, Whitlock, and Lane (1966).

TABLE I
COMPOSITION OF EXPERIMENTAL SOLUTIONS

	$NaCl$-HCO_3 Ringer's solution	NaCl Ringer's solution	Na_2SO_4 Ringer's solution	Sucrose Ringer's solution
	mM	*mM*	*mM*	*mM*
NaCl	110.0	140.0	—	—
$NaHCO_3$	25.0	—	—	—
KCl	7.0	7.0	—	—
$CaCl_2$	2.0	2.0	—	1.0
Glucose	11.1	—	11.1	—
NaH_2PO_4	1.2	0.375	0.375	—
Na_2HPO_4	—	2.125	2.125	—
$MgSO_4$	1.2	—	1.2	—
Na_2SO_4	—	—	118.0	—
K_2SO_4	—	—	3.5	—
$CaSO_4$	—	—	8.0	—
Sucrose	—	—	—	257.0
KH_2PO_4	—	—	—	0.375
K_2HPO_4	—	—	—	2.125

METHODS

Techniques for obtaining cannulated in vitro preparations of gall bladder and for measuring the rate of fluid transport gravimetrically were similar to those described previously by Diamond (1962 *a*, 1964 *a*). New Zealand white rabbits weighing 3 to 4 kg were used. The dissection and experiment were generally carried out in a cold room at 4°C, and different experimental temperatures were obtained by placing the beaker containing the gall bladder in a thermostatically controlled water bath within this room. The various Ringer solutions in which the preparations were bathed are described in Table I. All were designed to be isotonic with respect to rabbit plasma and had a pH of 7.4–7.6.

After transport rates had been measured gravimetrically for approximately 1 hr, the gall bladder was prepared for microscopic examination. The Ringer solution within the lumen of the preparation was rapidly removed and replaced by two quick changes of fixative; then the fixative-filled preparation was transferred to a vial of the fixative. A 1% solution of osmium tetroxide in 0.15 M phosphate buffer at pH 7.4 was the fixative usually employed. (In a few cases 3% glutaraldehyde in 0.1 M phosphate buffer with postfixation in osmium tetroxide was used, and results similar to those with osmium tetroxide alone were obtained. Glutaraldehyde was not used routinely because of evidence that in some tissues (Maunsbach, 1966; Tormey, unpublished observation) aldehyde fixatives can cause cell swelling and/or shrinkage.) The tissue was fixed for 1 hr at 4°C, dissected into small pieces, dehydrated through

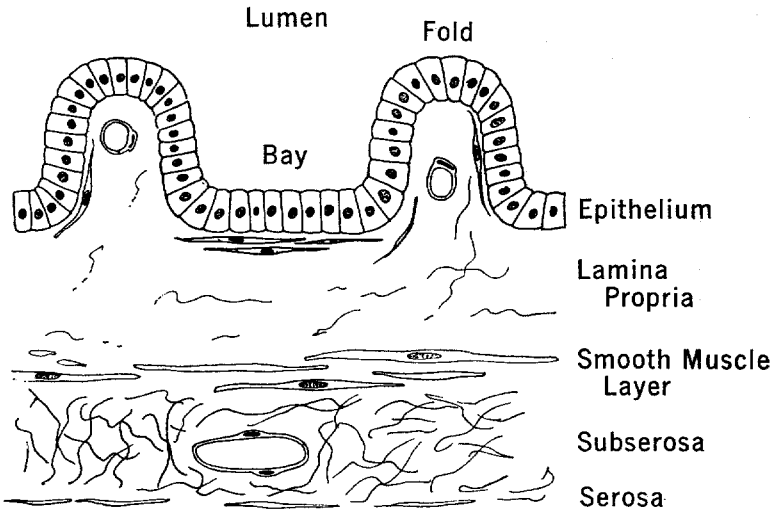


FIGURE 1. Diagram illustrating the component layers of the gall bladder wall, as described in the text. *Not drawn to scale.*

a graded ethanol series to propylene oxide, and embedded in Epon 812. At the time of dissection the fundus and neck of the gall bladder were avoided, and only material from the body region was embedded in order to minimize sampling variation. The tissue was embedded in such a way that nearly perfect cross-sections through the gall bladder wall could be obtained. Unstained 2 μ thick sections were examined in a light microscope equipped with Zeiss Neofluar phase contrast optics, and measurements of cell dimensions were made with an eyepiece screw micrometer. All quantitative data were obtained from osmium-fixed material, and measurements were made through the 100× (NA = 1.3) objective. In spite of the thickness of the sections, the shallow depth of field of this lens permitted reasonably accurate measurements of dimensions down to 0.2–0.5 μ. Sections 60–80 mμ thick, stained with heavy metals, were examined in a RCA EMU 3-F electron microscope.

RESULTS

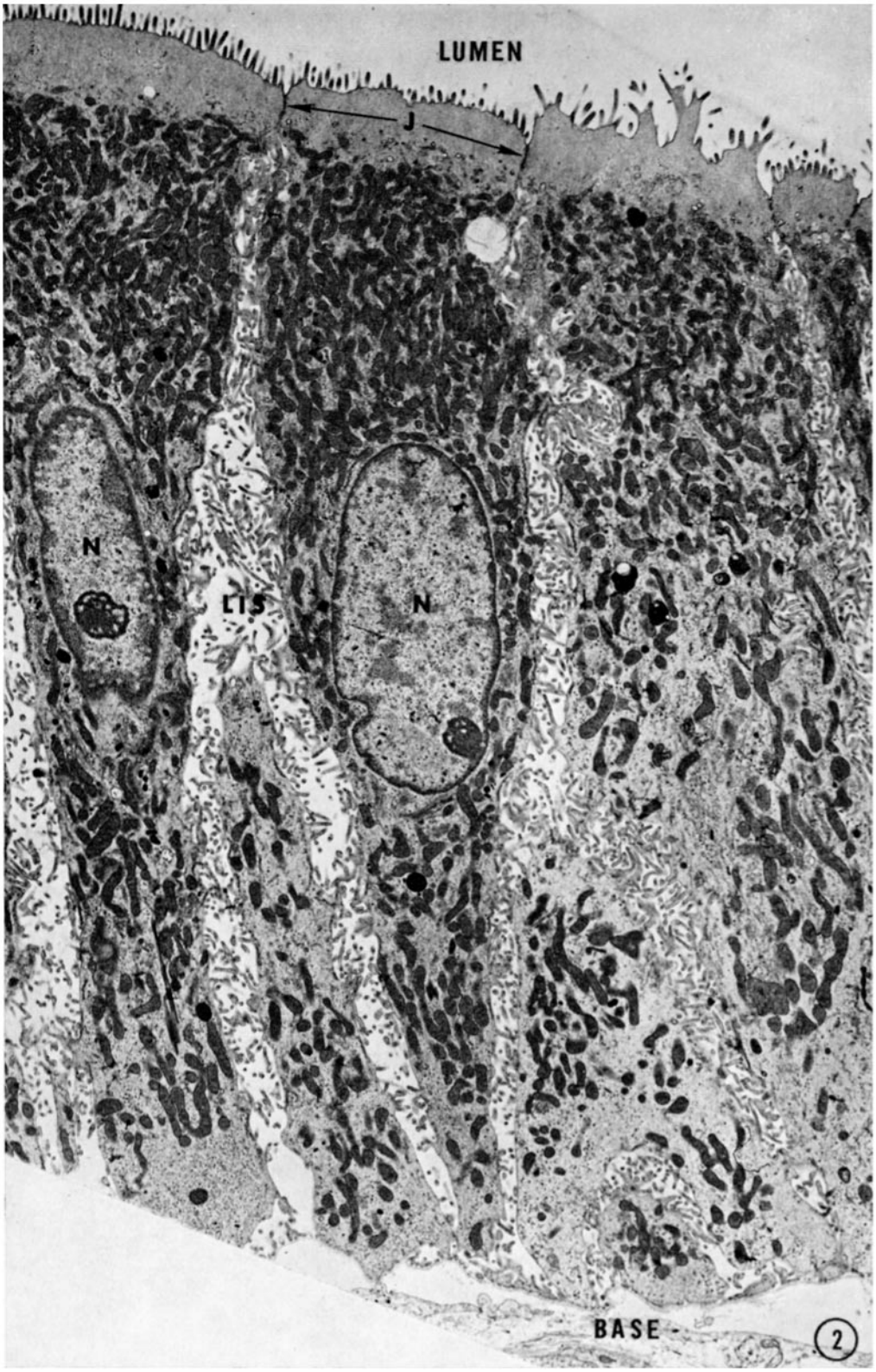
General Structural Features

In order to understand the results of these experiments, it is first essential to appreciate the salient structural features of the rabbit gall bladder. The following description is based on the structural features which we have observed to be common to both transporting and nontransporting gall bladders in vitro, and it provides the framework within which physiological interpretations of changes in structure will be made.

LIGHT MICROSCOPY The gall bladder is essentially an epithelium-lined sac. Proceeding from the lumen outwards, one finds several layers, as illustrated diagrammatically in Fig. 1, viz.: (*a*) a mucosa consisting of a surface epithelium and a lamina propria; (*b*) a layer of smooth muscle fibers; (*c*) a subserosal layer of connective tissue; and (*d*) a partial layer of serosa. The mucosal layer is thrown into numerous folds, varying in height from 100 to 500 μ or more, which intersect one another to form a reticular pattern visible to the naked eye, and which demarcate pits or bays of varying breadth and depth. The thickness of the gall bladder wall as measured from the bottoms of the bays to the serosal surface in transporting preparations is relatively constant, averaging about 350 μ, with the combined muscular and subserosal layers accounting for about two thirds of this dimension.

The epithelium consists of a single layer of tall columnar cells with remarkably uniform appearance. They possess on their apical surface a delicate microvillous border barely discernible by light microscopy. (The "apical" surface of the epithelial cells is that facing the lumen of the gall bladder; the "basal" surface is that facing the connective tissue and muscular layers; the direction of fluid transport is from apex to base.) The nuclei are located at about midheight in the cells, and the cytoplasm contains small granular organelles of several types. The cells are separated from one another by intercellular spaces which in some cases are a micron or more in width. These spaces, when visible, usually appear patent down to the basal surface of the epithelium but are invariably closed off near the luminal surface, where the cells always remain in close mutual contact. We have observed no other cell type as a regular component of epithelium from the body of the gall bladder.

The lamina propria, which underlies the epithelium, is composed of very loosely woven connective tissue. At some points fibroblasts are piled up several layers deep immediately beneath and parallel to the epithelium, but more commonly they are either loosely arranged or virtually absent for considerable distances along the mucosa. Capillaries and venules are common and tend to

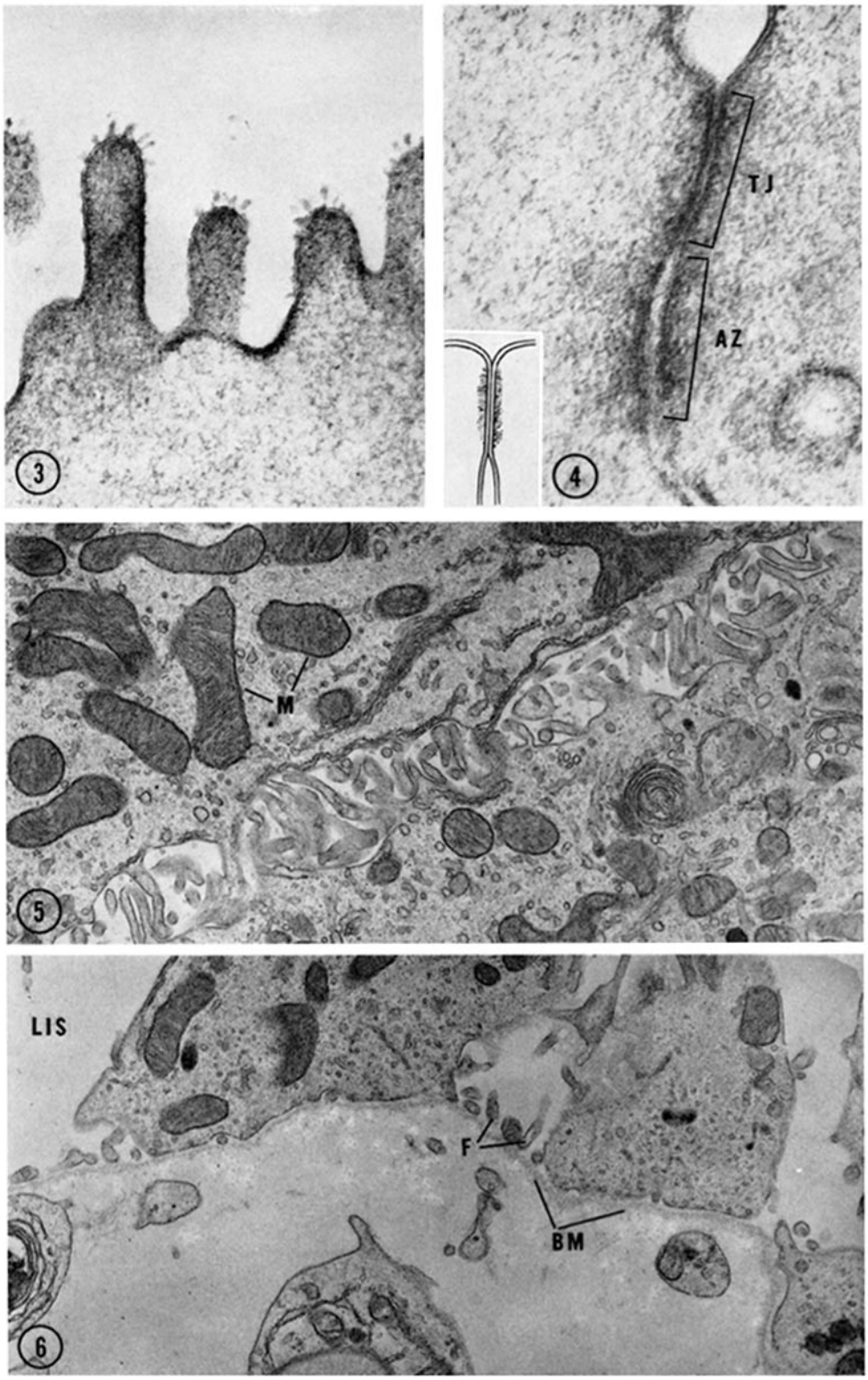


lie near the epithelium. The muscular layer contains small bundles of smooth muscle fibers irregularly interspersed with loose connective tissue. The subserosal layer is packed with close-knit bundles of collagen fibers. In places the subserosal layer merges directly with the liver parenchyma, and elsewhere it is bounded by a serosa of simple squamous epithelium (mesothelium) which in our preparations appears discontinuous. NaCl and water are transported out of the gall bladder lumen by the epithelium and pass (in vitro) across these other layers.

ELECTRON MICROSCOPY OF THE EPITHELIUM The over-all architecture of the gall bladder epithelium is illustrated by low power electron microscopy in Fig. 2; similar structure is present also in Figs. 8–10, which typify selected experimental conditions. Several regions of the cells, viz., the apical surface, terminal web, intercellular junctions, lateral surface, basal surface, and cytoplasm, will now be considered in turn.

The apical (luminal) surface is characterized by the presence of numerous microvilli, such as are shown at high magnification in Fig. 3. These are similar in structure to those which constitute the brush or striated borders of other transporting epithelia, such as those of kidney or intestine, but here they are smaller and more sparsely distributed than in these other organs. The "unit" plasma membrane which constitutes their outer surface is covered by very fine fibrous projections, which are probably associated with the histochemically demonstrable mucopolysaccharide coating found in this part of the cell. (Our test for mucopolysaccharide was the periodic acid-Schiff reaction carried out on 2 μ sections of Epon embedded material.)

FIGURE 2. Low power electron micrograph of gall bladder epithelium. Three tall columnar cells can be seen extending from the luminal (apical) surface (*Lumen*) of the epithelum to its basal surface (*Base*). (Portions of several other cells are present but cannot be followed all the way to the apical surface because of the slightly oblique plane of section.) The luminal surface is studded with numerous microvilli (better shown in Fig. 3). Immediately beneath this surface is a relatively empty zone of cytoplasm known as the terminal web. At the level of the terminal web the plasma membranes of adjacent cells come together to form intercellular junctions (*J*); these areas of cellular contact appear here merely as small zones of slightly increased density; their nature is revealed by high power micrographs (compare Fig. 4). Below the intercellular junctions, adjacent cells are separated by clear intercellular spaces (*LIS*); into these long, narrow spaces project numerous finger like cytoplasmic evaginations (better seen in Fig. 5); the continuity of the intercellular spaces with the underlying connective tissue at the basal surface of the epithelium is better demonstrated in Fig. 6. Nuclei (*N*) lie at approximately mid-height within the cells. Above the level of the nuclei, the cytoplasm is almost completely filled with mitochondria, mainly in the form of short rods; below the nuclear level, mitochondria are still the dominant cytoplasmic component, but they are less closely packed. Transporting gall bladder. Uranyl acetate/lead citrate staining. Magnification $\times$ 4,600.



Underlying the apical surface is the terminal web, a zone several microns deep, that differs from the cytoplasm of the rest of the cell in that it consists essentially of a net of extremely fine fibers and contains no organelles aside from a few vesicles. A possible physiological significance of microvilli and the

FIGURE 3. High magnification detail of several microvilli at the luminal surface of the epithelium. The plasma membrane, which constitutes the outer surface of these cytoplasmic evaginations, is visualized as a typical "unit membrane"; i.e., it appears as two dense lines separated by a clear space. Short, fine filaments project outward from the unit membrane near the tips of the microvilli. The cytoplasm within both the cores of the microvilli and the underlying terminal web contains very fine fibrous material; the longitudinal arrangement of the fibers within the microvilli is not shown as clearly here as in glutaraldehyde-fixed preparations. Uranyl magnesium acetate/lead citrate staining. Magnification × 75,000.

FIGURE 4. High power electron micrograph showing a junction between two cells near the luminal surface. In the uppermost portion of the picture the unit plasma membranes of the two cells approach one another and then fuse to form a tight junction (*TJ*) about 2,000 A long. Along the length of the tight junction, the outer dense lines of the two unit membranes appear to fuse together into a single intermediate line; and also the fibrillar ground material of the terminal web is condensed at the inner (cytoplasmic) surface of the junctional membranes, causing an increase in their apparent-width and density. (These two features of the tight junction are also illustrated diagrammatically in the insert. The micrograph lacks the clarity of the diagram primarily because the thickness of the microscopic section is more than an order of magnitude greater than the widths of the individual components of the unit membranes.) Below the tight junction is the adhering zonule (*AZ*); here the adjacent membranes are spaced apart and electron-dense material is found in the gap separating them. The lateral intercellular space begins below the adhering zonule. The tight junction is thought to be an effective seal between the contents of the gall bladder lumen and the intercellular space. Uranyl magnesium acetate/lead citrate staining. Magnification × 100,000.

FIGURE 5. Medium magnification micrograph showing portions of two cells and the lateral intercellular space separating them. The width of this intercellular space is nearly the minimum observed. Numerous finger-like folds project into the space and tend to interdigitate, thereby rendering the space exceedingly tortuous. The continuity of these folds with the cytoplasm is evident in several places. A number of mitochondria (*M*) with typical internal structure are evident as the predominant constituent of the cytoplasm. Also seen are scattered membranous profiles of endoplasmic reticulum, Golgi complexes, and vesicles. Transporting gall bladder. Lead citrate staining. Magnification × 16,000.

FIGURE 6. Detail of basal epithelial surface. A delicate, amorphous basement membrane (*BM*) is seen at the boundary between the epithelium and the underlying connective tissue. The basal surface differs from the other surfaces of the epithelium in being relatively devoid of membrane folds and hence essentially planar. Nevertheless some fingerlets of cytoplasm (*F*) do project down to contact the basement membrane. Although the lateral intercellular spaces (*LIS*) are here widely dilated, the cell bases and cytoplasmic fingerlets appear to remain firmly adherent to the basement membrane. As a result, fluid must exit from the intercellular spaces through gaps several hundred Angstroms wide between cell processes. Transporting gall bladder. Lead citrate staining. Magnification × 15,000.

terminal web is considered in the following paper (Diamond and Bossert, 1967).

Close to the apical surface, the lateral plasma membranes of adjacent cells join to form typical, so-called "tight junctions." As illustrated in the high power micrograph of Fig. 4, these junctions appear to be areas where the unit plasma membranes of adjacent cells come together and fuse their outer dense lines into one. Such areas are invariably present in this region of the cell, and in favorable tangential sections they may be seen to gird the entire cell periphery. Therefore, the tight junctions form continuous hoops around the cells (or "occluding zonules," in the terminology of Farquhar and Palade, 1963). Proceeding down the lateral intercellular space from the apical surface, the cell membranes are fused into tight junctions for a distance of some 2,000–3,000 A. Directly below these junctions is found a second type of junction, the so-called "adhering zonules" (Farquhar and Palade, 1963), where adjacent plasma membranes are spaced uniformly apart and the intercellular space shows enhanced electron density. These two types of intercellular junction hold the cells firmly together, as is especially evident in those places where adjacent cells are otherwise separated by as much as several microns, but invariably remain in intimate contact in the junctional region (as in Fig. 8). These junctions in addition form a morphological seal between the lateral intercellular spaces and the gall bladder lumen. The degree to which they are also physiological seals is discussed on p. 2054.

The extensive lateral cell surface is greatly increased in area by numerous finger-like evaginations of the plasma membrane, beginning a short distance below the junctional area and continuing to the basal surface. They are best illustrated in Fig. 5. Although resembling microvilli in shape, they differ from them in several ways: they are longer and thinner; their cytoplasmic cores do not contain the longitudinally oriented fibers which are present within microvilli; they appear to be considerably more flexible inasmuch as they are often found bent and folded; their unit membranes are slightly thinner and are not coated with fine fibrous projections, and they do not give a positive histochemical reaction for mucopolysaccharide.

The basal surface of the epithelium is underlaid by a continuous so-called basement membrane about 300 A thick (Fig. 6). This structure shares nothing in common with the plasma membrane except its name, as is evident from both its typically amorphous appearance and its demonstrable permeability to molecules up to at least 100 A in diameter, such as ferritin (Tormey, unpublished observation). The basal surface (plasma membrane) of each cell is essentially flat and unadorned with folds over most of its area; but, near the lateral borders of the cell base, fingerlets of cytoplasm frequently reach out to contact the basement membrane (Fig. 6). Sometimes these fingers project under the main body of the cell, and less frequently the cells partially undercut one another with broad leaflets of cytoplasm.

Although closed off from the lumen by cell junctions, the lateral intercellular spaces are in open communication with the basement membrane and connective tissue layers underlying the epithelium. The spaces are quite variable in width and devoid of electron scattering material. In many instances the cells are narrowly separated by a gap of only 150–200 A, and the cytoplasmic evaginations from adjacent cells then tend to interlock (Fig. 5). Where cell separation is moderately increased, a looser interdigitating pattern is found (Fig. 2). The interdigitations enormously amplify the membrane surface area and increase the tortuosity of the path which would be traversed by any molecule transported into the intercellular space across the plasma membrane. The over-all length of the intercellular space in transporting gall bladders is about 30 μ, but because of the membrane folds the effective diffusion path must be considerably longer.

Mitochondria in large numbers are the most prominent cytoplasmic feature of these cells. Except for the terminal web region, most of the cytoplasmic volume is filled with these organelles. They are more concentrated towards the luminal pole of the cells: the population density of mitochondria in the basal half of the cells varies from about 50 to 80% of that found in the apical half (Figs. 2 and 8–10). At any given level they are distributed uniformly from one side of the cell to the other and do not bear a particularly close spatial relationship to any other structure. This lumen-to-base gradient of distribution may be of physiological significance (Diamond and Bossert, 1967).

Most types of cytoplasmic organelle found in animal cells are represented in the gall bladder epithelium, but, except for the mitochondria, they are unimpressive in their quantity and spatial distribution. Thus, rough surfaced endoplasmic reticulum is usually found as short, isolated cisternae interspersed among the mitochondria. Smooth surfaced reticulum is similarly scanty. Several discrete juxtanuclear Golgi complexes can usually be found in each cell, but they are invariably small. Lysosomes are scattered about in highly variable fashion. Relatively few ribosomes are seen.

The presence of vesicles is of particular interest because of the question of whether pinocytosis might be involved in transport in the gall bladder. Grim (1963), for instance, suggested that bulk pinocytosis might "pump" fluid across the epithelium, but this notion can be ruled out by physiological experiments (Diamond, 1964 *a*). One might still wonder about the possibility of a different type of vesicular mechanism: concentration of NaCl within cytoplasmic vesicles which could subsequently accumulate water (local osmosis) and discharge their contents at the lateral or basal cell surface. Modest numbers of vesicles are found scattered through most of the cytoplasm, and some are occasionally found attached to the plasma membrane. Their sparsity, however, argues against any form of pinocytosis being a significant factor in water movement. Vesicles are particularly scarce in the terminal web region; solitary vesicles are sometimes found in contact with the plasma membrane

between microvilli, but this is rare. There was no sign of pinocytotic uptake of protein tracer in two experiments in which gall bladders pumped a Ringer solution containing 4% ferritin for 1 hr and then were fixed with glutaraldehyde and examined by electron microscopy. Large, unit membrane-bound vesicles or vacuoles are found sporadically in the apical region; but on the basis of histochemical data, they most likely contain mucous material absorbed from the gall bladder lumen. In short, the morphological evidence is inconsistent with pinocytosis playing a major role in fluid transport in this organ.

The ultrastructure we have observed in rabbit gall bladders is in many but not in all regards similar to that reported for other mammalian species,

TABLE II

DIMENSIONS OF EPITHELIAL CELLS IN VARIOUS PHYSIOLOGICAL STATES

Experiment	Transport rate	Intercellular space width	Cell height × width
	$\mu l/hr$	μ	μ
Normal transport	388	0.88 (477)	32×4.9 (70)
Inhibited transport			
1. Ouabain	39	0.02 (113)	42×5.1 (47)
2. CN-iodoacetate	−5	0.01 (66)	38×5.5 (26)
3. Adverse gradient	−5	0.17 (214)	31×4.7 (53)
4. Cold	71	0.04 (101)	38×4.8 (52)
5. Na_2SO_4	28	0.30 (337)	26×4.1 (41)
6. Isotonic sucrose	−1	0.03 (108)	25×3.6 (49)

All values are arithmetical means. Figures in parentheses after cell dimensions are number of measurements made. All space widths under conditions of inhibited transport differ from normally transporting widths at a statistical significance level of $p < 0.01$ or better. All cell heights under conditions of inhibited transport (except for adverse gradient experiments) differ from normally transporting cell heights at a statistical significance level of $p < 0.05$ or better.

namely, human (Evett, Higgins, and Brown, 1964; Chapman et al., 1966), mouse (Yamada, 1955; Hayward, 1962 *a*), guinea pig (Hayward, 1962 *b*), and dog (Johnson, McMinn, and Birchenough, 1962). It is also consistent with the description of rabbit gall bladder given by Kaye, Wheeler, Whitlock, and Lane (1966). Several details described here have not been reported previously, most notably the differences between the membranous folds on the apical and lateral surfaces, and the gradient of mitochondria from apex to base. Also the rabbit appears to differ from the other species reported in possessing especially well developed lateral intercellular spaces and particularly large numbers of mitochondria.

Experimental Changes in Structure

Gall bladders fixed in various functional states showed striking structural changes, most of them clearly visible in the light microscope. Quantitation

of these changes appeared highly desirable, especially since the structural appearances were by no means uniform. Indeed, in most cases it would be difficult to be certain whether a randomly selected electron micrograph was of a transporting or a nontransporting gall bladder. Because light microscopy makes feasible the sampling of large populations of cells, we decided to rely mainly on it for quantitation. Although electron microscopy makes possible measurements of dimensions below the resolution of the light microscope and allows greater precision of *individual* measurements, it is seriously handicapped by the possibility of sampling errors due to the relatively small numbers of cells which can be conveniently examined. The results obtained by light microscopy are summarized in Table II, and will be presented first, to be followed by additional, largely qualitative, observations by electron microscopy.

TRANSPORTING GALL BLADDERS In five experiments gall bladders were filled with NaCl-HCO_3 Ringer's solution and incubated in a beaker containing an identical solution at 37°C for 1 hr before fixation. This solution has been shown to be optimal for in vitro pumping (Diamond, 1964 *a*). Fluid transport out of the gall bladder lumen occurred at the mean rate of 388 μl/hr, or 19.7% of each gall bladder's luminal volume in an hour.

The epithelium of these preparations always demonstrated widely dilated lateral intercellular spaces. In some cases, particularly when the spaces were very wide, there was a marked tendency for them to widen progressively toward the base of the epithelium. Much more often, however, approximately the basal two-thirds of the lateral spaces were essentially constant in width. The greatest separations were invariably found between cells near the tips of the mucosal folds, and zones of minimal separation were usually found in the depths of the bays.

For quantitative analysis of cell separation the following procedure was adopted. Intervals of 75 μ were measured off along the basal surface, and the width of the intercellular space nearest each interval was measured at a point halfway between the luminal and basal surfaces of the epithelium. A distance of 150 μ measured along the length of the mucosal folds from their tips was found to be the approximate dividing line between the more widely separated cells and the less widely separated; note was therefore made of whether the cells measured lay more or less than 150 μ from the tips, and the two populations thus obtained were analyzed separately.

In pumping gall bladders the mean intercellular space width was 0.88 μ. Cells within 150 μ of the tips of the folds were separated on the average by 1.52 μ, while those at greater distance were separated by 0.57 μ. A small number of cells near the tips exhibited extreme separation of several microns. Fig. 7 shows the frequency distribution of the space widths, and demonstrates that the very widely spaced cells, which are the exception rather than the rule, cause a marked skew to the right.

Cell heights and widths were also measured; every tenth or twentieth cell which could be seen in cross-section; i.e., extending intact from the basal to the apical surface of the epithelial layer, was used. These dimensions under the various physiological conditions studied are listed in Table II. The pumping cells averaged 32 μ high by 4.9 μ wide.

GALL BLADDERS WITH FLUID TRANSPORT INHIBITED 21 experiments were performed in which transport was inhibited by one of six methods.

1. *Ouabain Poisoning* Two gall bladders were bathed at 4°C for 1 hr in NaCl Ringer's solution to which had been added 10^{-3} M ouabain, and were

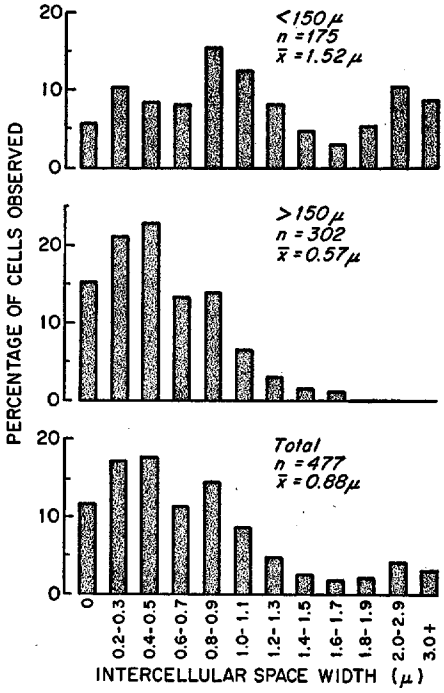


FIGURE 7. Frequency distribution of intercellular space widths in transporting gall bladders. The top graph considers only the population of cells lying within 150 μ of the tips of the mucosal folds, while the middle graph considers only the cells farther than 150 μ from the tips. These two populations are combined and analyzed as one in the bottom graph. Space widths were measured to the nearest 0.1 μ.

then warmed to 37°C to measure transport rates. (This ouabain concentration was chosen in order to inhibit transport completely and consistently, since previous work had shown that relatively high concentrations are necessary for the ouabain effect in gall bladder (Diamond, 1962 *a*; Dietschy, 1964).) A third gall bladder, after pumping at a normal rate for 1 hr in NaCl-HCO_3 Ringer's solution at 37°C, was transferred to the same Ringer solution with 10^{-3} M ouabain added, and transport was followed until ouabain inhibition set in. The mean rate of fluid efflux was 39 μl/hr, or, referred to the luminal volume, 2.4%/hr. This was only 10–12% of the rate in the normal gall bladder. The epithelial cells in all cases appeared swollen (compare Table II), and

the mean intercellular space width by light microscopy was 0.02 μ. Since the resolving power of our light optics was approximately 0.2 μ, any spacing less than 0.2 μ was observed as zero. The figure 0.02 μ therefore indicates that *at least* 90% of the spaces observed were narrower than 0.2 μ by an indeterminate amount. (Fig. 9 is an electron micrograph of ouabain-treated cells.)

2. *Cyanide-Iodoacetate Poisoning* In two experiments the NaCl-HCO_3 Ringer solution contained 3 mM sodium cyanide and 3 mM iodoacetic acid. After both surfaces of the gall bladder had been bathed in this solution for 1 hr at 4°C, the temperature was raised to 37°C, and transport was found to be completely inhibited (see also Diamond, 1962 *a* and 1964 *a*): the mean rate of fluid efflux was -5 μl/hr, and the mean rate relative to luminal volume was -0.6%/hr. Virtually no intercellular spaces could be demonstrated in the epithelium by light microscopy (mean space width, 0.01 μ). The cells were swollen, and demonstrated signs of necrotic degeneration in patchy areas.

3. *Adverse Osmotic Gradients* When the solution within the gall bladder lumen is made increasingly hypertonic by addition of some impermeant solute, such as sucrose, water transport continues at decreasing rates against the resultant osmotic gradient. When a certain gradient (generally 120–150 mM) is reached, net water outflow drops to zero (Wheeler, 1963; Dietschy, 1964; Diamond, 1964 *a*). Further increases in gradient produce a net inflow of water. Five gall bladders were bathed at 37°C in NaCl-HCO_3 Ringer's solution on the outside while the lumen was filled with the same solution to which had been added 120 or 150 mM sucrose. In two experiments with the 120 mM gradient a slight fluid outflux remained, while the three experiments with the higher gradient produced a slight influx. The mean fluid transport rate was -5 μl/hr, or, relative to luminal volume, -2.2%/hr. In all cases the intercellular spaces were collapsed as compared with the normally pumping preparations, although some separation was still visible in the light microscope: mean observed width was 0.17 μ. Unlike the poisoned preparations, the cells in these osmotic experiments did not appear changed in volume as compared with normally transporting gall bladders. Kaye, Wheeler, Whitlock, and Lane (1966) reported one experiment in which an adverse gradient of 115 mM apparently caused a similar change in spacing.

4. *Low Temperature* When four gall bladders bathed in NaCl-HCO_3 Ringer's solution were maintained at 4°C, the transport rate was only 14–18% of that at 37°C. The mean absolute rate observed was 71 μl/hr, and the mean relative rate was 2.8%/hr. In two of the experiments, transport rates were measured at 4°C immediately after dissection at the same temperature. The other two gall bladders were allowed to pump at 37°C for about 35 min before being run in the cold for 50 min. The two gall bladders which were continually

maintained at 4°C showed very little cellular separation: average intercellular space width was 0.04 μ. The pair which first pumped at 37°C showed much greater separation, which approached but was less than that seen in normally transporting preparations. Thus, the changes in cell geometry occurring during pumping in vitro were not completely reversible under the conditions of this experiment. However, in two other experiments in which the gall bladder was allowed to pump in vitro and pumping was then inhibited by ouabain or isotonic luminal sucrose (see below), complete reversal of cell separation in less than an hour was observed.

5. *Na_2SO_4 Ringer's* In four experiments gall bladders were run in Na_2SO_4 Ringer's solution, which differed from that employed in normal pumping experiments in that Cl and HCO_3 were completely replaced by SO_4. Since the pumping of Na and Cl by the gall bladder is coupled in an obligatory fashion, and since SO_4 is a poor substitute for Cl in the pump (Diamond, 1962 *a*; Dietschy, 1964; Martin and Diamond, 1966), this Ringer's solution was transported at a rate only 7% of that for NaCl-HCO_3 Ringer's solution. The observed mean rate was 28 μl/hr, and the mean relative rate was 1.3%/hr. The intercellular spaces were found collapsed as compared to pumping epithelium, but not as completely collapsed as in any of the other experiments with inhibited transport. The mean space width observed was 0.30 μ. The difference between this and the mean space width of transporting gall bladders (0.88 μ) was found to be statistically significant at the 1% probability level. In addition, the cells appeared shrunken compared with transporting gall bladders. The result differs from that of Kaye, Wheeler, Whitlock, and Lane (1966) who stated that in two experiments SO_4 substitution for Cl caused intercellular spaces (as observed by electron microscopy) to narrow to 200 A; in the absence of more precise quantitative data for their experiments, the significance of this apparent discrepancy is uncertain. Kaye et al. (1966) also reported that replacement of Na by the nonabsorbed cation tetraethyl ammonium (TEA) caused similar narrowing of the spaces.

6. *Isotonic Sucrose* In three experiments gall bladders maintained at 37°C were bathed on their serosal surface by NaCl Ringer's solution and on their mucosal (luminal) surface by sucrose Ringer's solution. The two solutions were mutually isotonic, but the inert nonelectrolyte sucrose completely replaced NaCl in the mucosal solution. In one of the experiments, the preparation was first allowed to pump normally in NaCl-HCO_3 Ringer's for half an hour. Fluid transport was found to be completely inhibited by the absence of luminal NaCl, the mean rate being -1 μl/hr (see also Diamond, 1962 *a*; Dietschy, 1964). The morphological results were particularly striking in that the epithelial cells appeared greatly shrunken and yet virtually no cellular

separation was visible: mean intercellular space width was 0.03 μ. (Fig. 10 is an electron micrograph of these cells.)

The pattern running consistently through all these experiments is that the intercellular spaces are widely dilated when fluid transport occurs and are collapsed to varying degrees when fluid transport is inhibited. This is prima facie evidence that these spaces play a role in the transport process.

ELECTRON MICROSCOPY Electron microscopic observations confirm and extend the results obtained by light microscopy.

The most spectacular feature of transporting gall bladders observed in the electron microscope was variation in the geometry of the intercellular spaces (Fig. 8). Not infrequently cells were separated from one another by a distance of only 200 A all the way from the terminal bar to the basement membrane; yet in other cases, sometimes only a few cells away, the intercellular spaces were several microns wide. Accompanying extreme cell separation of several microns was an obvious decrease in cell volume, indicated by a marked decrease in cell width without a compensatory increase in height. In spite of wide dilation of the intercellular spaces, the cell bases invariably appeared to remain affixed to the basement membrane. The resulting arrangement is well demonstrated in Fig. 6, where several cytoplasmic "foot" processes are shown interposed between gaping intercellular spaces and the basement membrane. Between such "feet" were intercellular passages varying in width from several hundred up to about a thousand Ångstroms.

Since the epithelium at the tips of the mucosal folds more often possessed broad intercellular spaces than did that in the bays between the folds, the ultrastructure of cells from these two regions was compared for other differences as well. It was found that cells with widely dilated intercellular spaces often possessed far fewer lateral membrane folds than did those with narrower spaces (compare Figs. 2 and 8). Aside from this, our comparison revealed no consistent structural variations.

The possibility that the increased separation found on the folds might be an artifact arising from "unnatural" conditions peculiar to the in vitro preparations seems to be ruled out by our observations on several gall bladders which were fixed within 1 or 2 min of removal from the body. Their appearance was generally similar to that of in vitro pumping preparations, and in particular their intercellular spaces were dilated with the greatest separation tending to occur at the tips of the folds. However, separation was only about half as great as that observed in vitro, which is consistent with the assumption that they were absorbing fluid in vivo but at a less than maximal rate. This assumption is reasonable since the in situ gall bladders were probably filled with bile whose salt concentration had already been much reduced. It is not suprising that gall bladders maintained in vitro for periods of about 80 min in physiological fluids differ so little in appearance from those freshly excised,

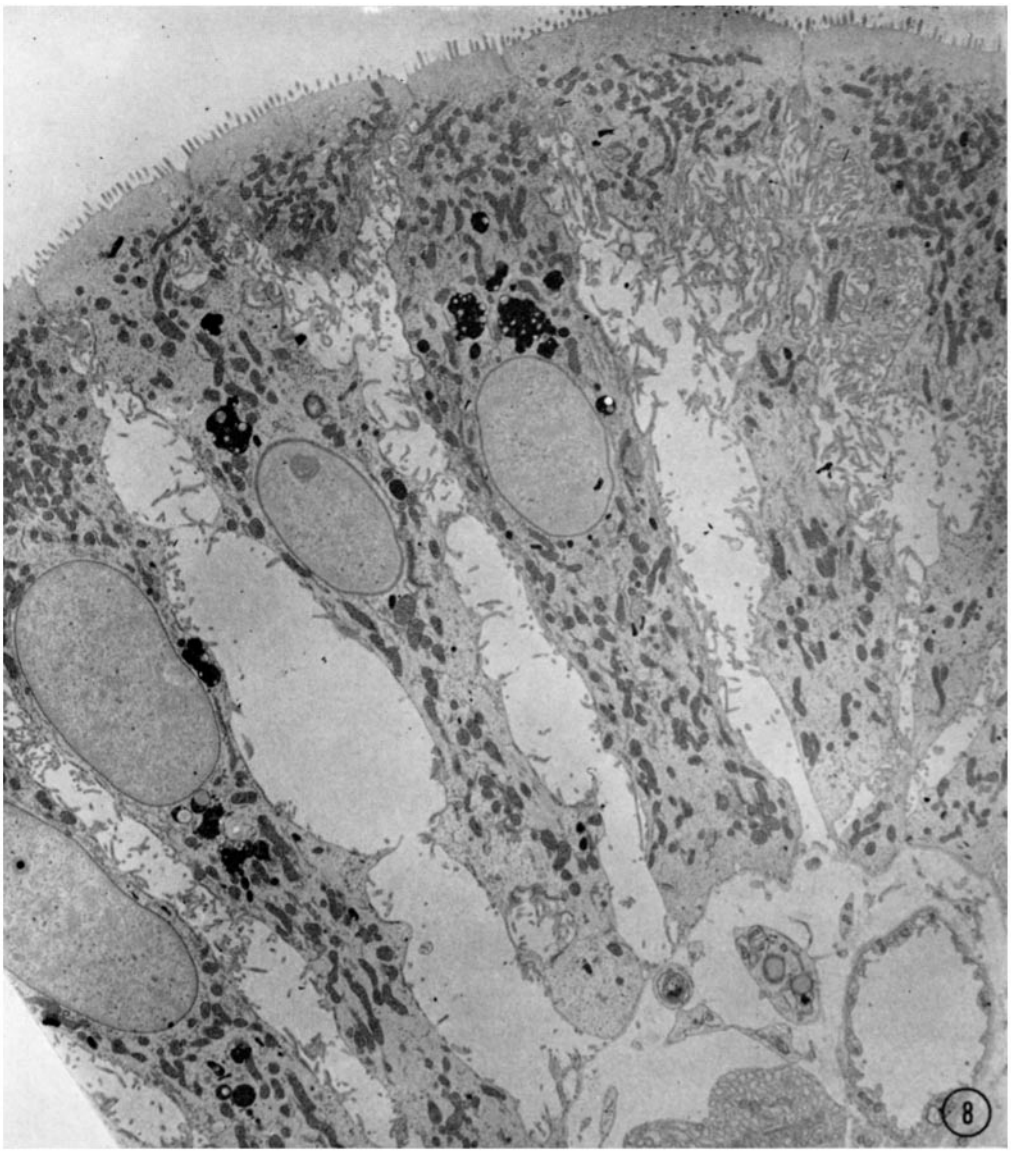


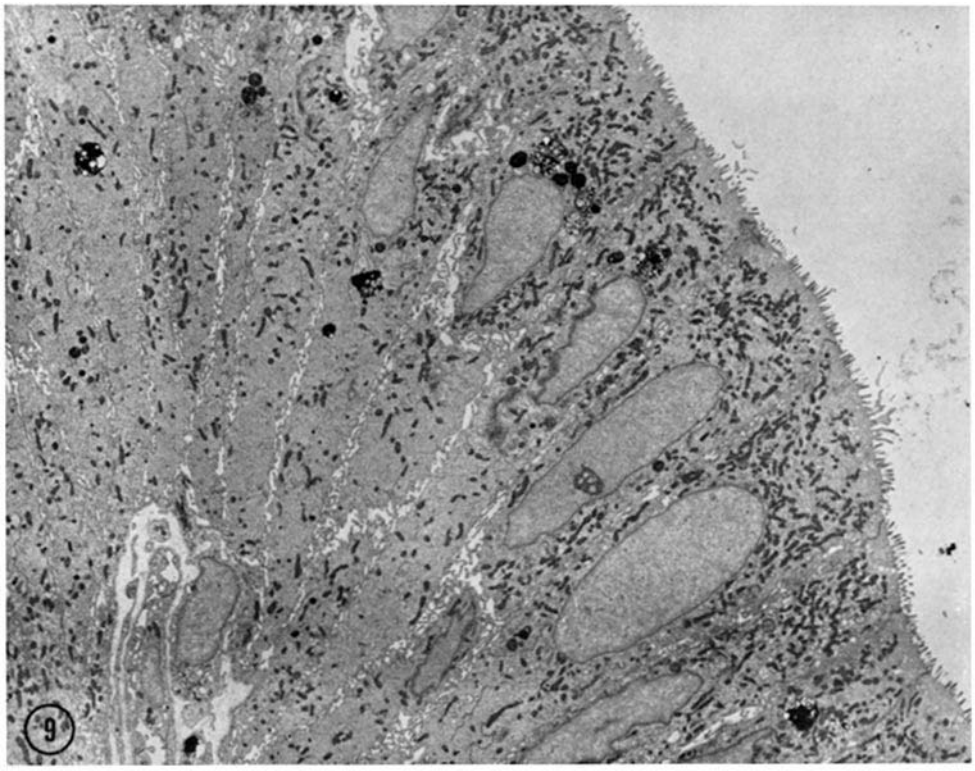
FIGURE 8. Low power electron micrograph of normally transporting gall bladder, taken near the tip of a mucosal fold. (Compare with Fig. 2, which is also of a transporting gall bladder but taken further from the tip of a fold.) The most striking feature here is the considerable dilation of the lateral intercellular spaces. In spite of this extreme dilation, the cytoplasmic foot processes remain in place at the base of the epithelium (compare Fig. 6), and the cells remain adherent to one another at the intercellular junctions. Membrane folds into the intercellular space appear reduced in number in the areas with greatest dilation. Aside from these features, no consistent differences are found between widely separated cells and those less widely separated. The very dense, pleomorphic bodies in the neighborhood of the nuclei are presumably lysosomes; these are an incosntant feature of the gall bladder epithelium. Lead citrate staining. Magnification × 3,400.

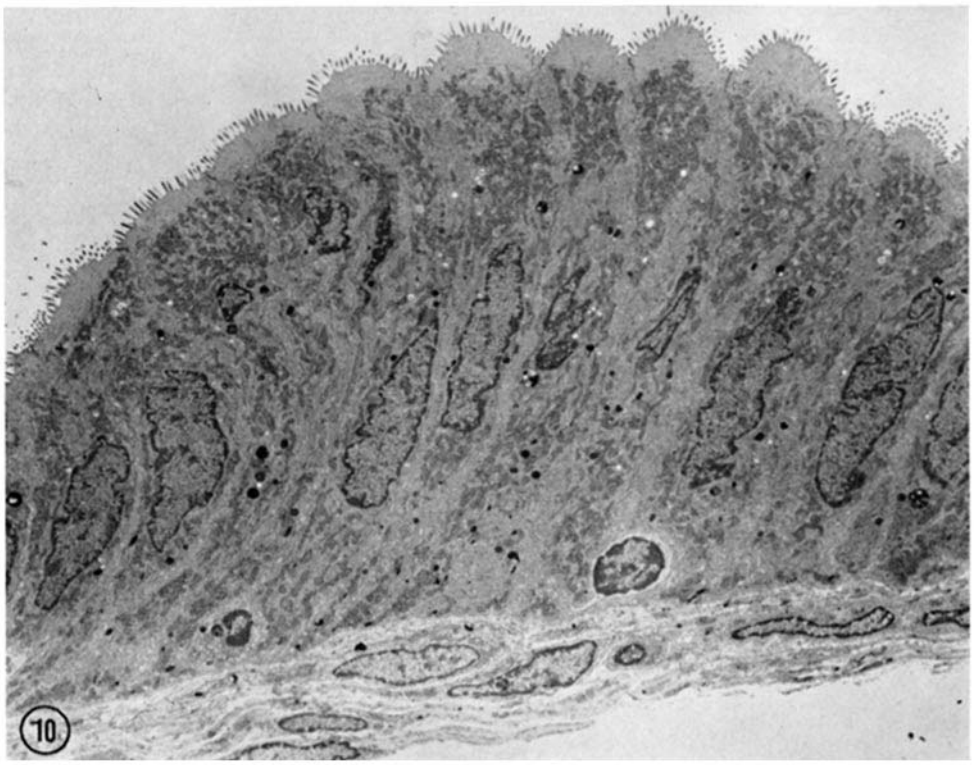
when one considers that in vitro gall bladders can pump many hours without decline in transport rate or change in permeability.

Although no attempt has been made to quantitate and compare all the details of structure evident in various preparations, examination of electron micrographs has thus far revealed no significant structural alterations in addition to those which would be expected on the basis of the light microscopic results. As an example, in the electron as in the light microscope, the epithelial cells of ouabain-treated preparations (Fig. 9) appeared swollen, with relatively empty looking cytoplasmic ground substance and organelles spaced much further apart than in pumping cells. Otherwise, the general appearance was suprisingly "normal"; the intercellular spaces were for the most part collapsed and the majority were only several hundred Ångstroms wide. At the other end of the spectrum, cells exposed to isotonic luminal sucrose solution (Fig. 10) appeared considerably shrunken, with extremely dense cytoplasmic ground substance and closely packed organelles; the intercellular spaces were the narrowest observed in any preparation, being a mere 150 A wide, which is the minimal distance normally found separating epithelial cells in most organs. The other preparations showed intermediate changes in cytoplasmic density, and their intercellular space widths were consistent with those measured by light microscopy.

HYDROSTATIC PRESSURE EXPERIMENTS Any fluid pumped across the gall bladder epithelium in vitro must traverse a considerable width of connective tissue before leaving the wall of the gall bladder. This connective tissue has a finite hydraulic resistance, which would cause the development of hydrostatic pressure in the lamina propria at the base of the cells. We wondered whether this pressure could be large enough to force the cells apart, thereby misleading us into concluding that the fluid originated between the cells. This possibility was tested in the following series of experiments.

Gall bladders were everted (following the method of Dietschy (1964) and of Diamond and Harrison (1966)) so that the epithelium faced the outside of the resulting sac. A long cannula was inserted into the everted sac, and both sides of the preparation were bathed in NaCl or NaCl-HCO_3 Ringer's solution at 4°C, so that fluid transport was inhibited. A hydrostatic pressure was then applied to the base of the epithelium by elevating the height of the column of Ringer's solution inside the cannula. After either 10 or 60 min at a given pressure, each gall bladder was placed in osmium tetroxide solution and fixed. Two gall bladders maintained at a pressure of zero cm H_2O showed virtually no cell separation and thus corresponded exactly to standard (noneverted) preparations run at 4°C. Five gall bladders maintained at pressures of 2, 4, or 5 cm H_2O showed little increase in separation, although at the higher pressures there was a tendency for the cells at the tips of the folds to separate





several tenths of a micron. A pressure of 10 cm H_2O caused the preparation to become grossly leaky; considerable dilation at the tips of the folds was apparent, and in some places the epithelium was ruptured. Since Dietschy (1964) found that similarly applied pressures in the range of 1–5 cm H_2O rapidly caused irreversible damage to transport function, the pressures actually existing in the lamina propria of transporting preparations must be considerably lower than those we applied.[2] Hence the finding of minimal changes in intercellular spaces even after unphysiologically high back-pressures of several centimeters means that hydrostatic pressures generated outside the epithelium cannot explain the appearance of transporting gall bladders.

DISCUSSION

Interpretation of the Morphological Findings Several earlier workers (Hayward, 1962 *a*; Johnson et al., 1962) noted the presence of somewhat distended lateral intercellular spaces in the gall bladders of mice and dogs, and they suggested that these spaces might be involved in fluid transport. Kaye, Wheeler, Whitlock, and Lane (1966) recently arrived at the same conclusion with rabbit gall bladders on the basis of combined physiological and morphological studies. In the present experiments all six experimental conditions employed to reduce or abolish fluid transport caused relative closure of the lateral intercellular spaces.

The fact that the lateral spaces are open during transport and closed in the absence of transport suggests *but does not prove* that fluid transport proceeds through the spaces. This interpretation may be safely made only when other

[2] In the noneverted orientation the gall bladder withstands pressures of at least 20 cm H_2O applied from mucosa to serosa (Diamond, 1962 *c*). Eversion per se does not make the gall bladder more susceptible to damage, as shown by the normal transport rates (Dietschy, 1964) and very stable permeability characteristics (Diamond and Harrison, 1966) of everted preparations. Evidently for purely mechanical reasons the cells are more easily damaged by pressures applied from the connective tissue than from the lumen.

FIGURE 9. Low power picture of epithelium of a gall bladder in which transport was inhibited by ouabain. The lateral intercellular spaces are nearly completely collapsed. The cells are considerably taller than transporting cells (note magnification difference in comparing with Fig. 8). As a consequence of the increase in cell volume, the cytoplasm appears somewhat rarefied and mitochondrial separation is increased. The difference in mitochondrial population density between the apices and bases of the cells is particularly striking here. Lead citrate staining. Magnification × 2,400.

FIGURE 10. Low power electron micrograph of epithelium from gall bladder in which transport was inhibited by sucrose Ringer's solution in the lumen. These cells are considerably shrunken as compared with both ouabain-treated and normally transporting epithelia, and their cytoplasm is extremely dense. In spite of the shrinkage, the intercellular spaces are so collapsed as to be invisible at this magnification. The small round cell visible within the epithelium near its basal surface is a migratory lymphocyte. Uranyl acetate/lead citrate staining. Magnification × 2,400.

explanations for such cell separation have been sought and ruled out. Our experiments render two alternative possibilities unlikely. First, the dilation might a priori be attributable to hydrostatic pressure built up within the lamina propria and transmitted back to the epithelium. This possibility is eliminated by our demonstration that grossly unphysiological pressures would be required to produce the degree of dilation observed in pumping gall bladders. Second, spaces might develop between pumping cells merely because the cells shrank during transport and as a result pulled apart from one another; spaces between nontransporting cells would be closed because inhibition of transport caused the cells to swell. The measurements of average cell height and width shown in Table II allow an evaluation of this possibility. Although such measurements are too crude to permit an exact calculation of cell volume, substantial relative changes in volume under different conditions are nevertheless obvious. Experiments may be ranked in the following order according to cell dimensions and volume, beginning with the conditions producing the largest cells: ouabain $\geq$ cyanide-iodoacetate $\geq$ low temperature $\gg$ normal (transporting) $\simeq$ adverse osmotic gradient $\gg$ sulfate $\simeq$ isotonic sucrose. This ranking shows that spaces between transporting cells cannot be due simply to cell shrinkage, since nontransporting cells may be considerably shrunken, unchanged in volume, or swollen compared to normal cells, depending on the method used to inhibit transport. Thus, one returns to the most obvious interpretation for the dilation of the intercellular spaces, namely, that they are the route of fluid transport.

The observation that dilation of the intercellular space is far more pronounced at the tips of the mucosal folds than elsewhere presents a difficult problem in interpretation. One possibility is that there is a direct proportion between the degree of dilation and the rate of transport of any given cell. This view is implicit in the interpretations of Kaye and collaborators (Kaye and Lane, 1965; Kaye, Maenza, and Lane, 1966). It entails that the cells at the tips of the folds are doing most of the work, and that those relatively few cells (compare Fig. 7) with extreme dilation are by far the most active. However, one hesitates to follow this line of argument if one accepts the principle that cell structure and cell differentiation are closely related, because the cells with extreme separation are otherwise virtually indistinguishable from cells with minimal separation. An additional consideration is that, like the intestinal epithelium, the cell population of the gall bladder epithelium is constantly turning over: cells are continually dividing in the depths of the bays, and as these cells age, they are pushed towards the tips of the folds where they must eventually slough off to make room for younger cells (Kaye, Maenza, and Lane, 1966). The cells near the tips are thus the oldest; their appearance might equally well be a sign that they are moribund as that they are especially active.

An alternative explanation for the extreme dilation near the tips of the folds is that these older cells are simply more susceptible to mechanical deformation under a given force. Experimental support for this is provided by our observations on nonpumping everted gall bladders. When hydrostatic pressures were applied to the serosal side of these preparations, such dilation of the intercellular space as was found was virtually restricted to the tips of the mucosal folds. Since under the conditions of this experiment the applied hydrostatic pressure was the same for all cells, it must be concluded that the cells at the tips are especially liable to deformation.

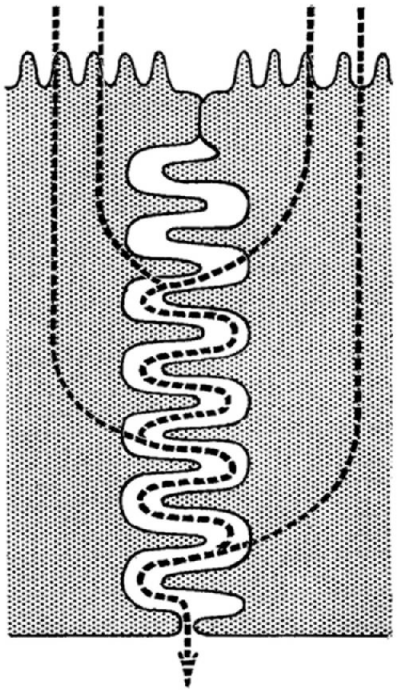


FIGURE 11. Diagrammatic representation of our concept of the route of fluid transport across the gall bladder epithelium. Salt and water first enter the cells across their luminal surfaces by a yet to be determined mechanism. They then pass through the cytoplasm and cross the lateral cell membrane to enter the lateral intercellular space. The geometry of this channel provides an explanation for the coupling between active salt transport and passive water fluxes, as is discussed in the text. The lateral intercellular space is presumably rendered hypertonic by active salt transport and supports a standing osmotic gradient, with the osmolarity decreasing progressively from the luminal to the basal end. Osmotic equilibration can then take place along the length of the channel, so that the fluid which eventually emerges at its open, basal end is essentially isotonic.

In summary, the separation between epithelial cells of transporting gall bladders seems to be the result of fluid transport proceeding through the intercellular spaces. The most likely explanation for the differences in separation found between the tips of the mucosal folds and the depths of the bays is that these differences are the consequence of regional variations in the passive, mechanical properties of the cells.

The Route of Fluid Transport Fig. 11 depicts the route of fluid transport across the gall bladder deduced from these experiments. Fluid enters the epithelial cells from the gall bladder lumen across the apical cell membrane, then crosses the lateral cell membranes to enter the lateral intercellular spaces,

and finally passes out the open basal ends of the spaces. Presumably NaCl is being actively transported from the cells into the spaces, making the spaces hypertonic and pulling water along osmotically.

A priori, salt and water might enter the lateral spaces in either of two ways: by entering the cells across the luminal cell membrane and then crossing the lateral cell membrane into the spaces (as assumed in Fig. 11); or by moving directly from the lumen across the intercellular junctions into the spaces, bypassing the cells entirely. While direct evidence on this important point is lacking, two lines of evidence make the transcellular route much more likely.

1. Experimental evidence for the route of salt transport comes from experiments (Diamond, 1962 *a*) in which NaCl was suddenly replaced in the luminal bathing solution by the impermeant nonelectrolyte sucrose. After the change, fluid continued to enter the serosal bathing solution for about 10 min even though there was no longer any luminal solute capable of active transport. During this transient the volume of the extracellular space, measured by inulin, remained unchanged, but the epithelial cells shrank, showing that the fluid pumped during the transient came from the cells themselves. The cells normally contain moderate concentrations of NaCl and evidently continued to pump these ions (plus water) across the lateral surface, against their concentration gradients, until most of the NaCl was removed from the cytoplasm. This suggests that when NaCl is present in the luminal bathing solution, salt and water are continually entering the cells across the luminal membrane as fast as they are being pumped out of the cells into the lateral spaces.

2. Any postulate that water enters the lateral spaces by passing directly through the intercellular junctions must be reconciled with the apparent fusion of cell membranes seen at the tight junction. In the electron microscope the fusion appears to be complete, and any gap of 10 A or greater should be detectable, if present. A more secure basis for an evaluation of the tightness of this structure is provided by the physiological observation that the gall bladder is virtually impermeable to water-soluble molecules with diameters above about 6 A (Wright and Diamond, personal communication), which means that any gap at the tight junction must actually be less than 6 A. Application of Poiseuille's law to tight junctions, taking their length as 2000 A, their maximum possible diameter as 6 A, and their total area as 0.02% of the mucosal surface area, shows that pressure differences of at least several hundred atmospheres would be necessary to drive fluid across the tight junctions at the range of absorption rates observed experimentally in the gall bladder. Although the applicability of Poiseuille's law to water flow through channels as narrow as 6 A is dubious, this calculation sets a conservative lower limit on the required pressure; deviations from this law and from the bulk viscosity of water expected in such narrow channels (Henniker, 1949; Derjaguin, 1965) would increase this estimate. These required pressures would

be considerably greater than the maximum possible osmotic driving force, namely, the osmotic pressure of a saturated NaCl solution. Thus, it is improbable that water bypassing the cells via the tight junctions could contribute appreciably to the observed fluid transport rate in the gall bladder. The possibility, of course, exists that in some other epithelia cell junctions may be found to be leaky, as has been suggested for the liver (Revel and Karnovsky, 1967), which also appears leaky from physiological evidence (Schanker and Hogben, 1961).

Although more direct evidence on the question of the permeability of gall bladder tight junctions would be desirable, these two arguments seem strong ones. Therefore, it seems reasonable to conclude that salt and water normally enter the cells across the luminal membrane and that they are then pumped out of the cell across the lateral membrane. The mechanism by which water might first enter the cells at the luminal surface is discussed in the following paper (Diamond and Bossert, 1967) where some speculation is offered on the possible role of the microvillous border in water-solute coupling at the luminal cell membrane.

Some transport might also conceivably occur across the flat basal surface of the epithelium rather than into the lateral intercellular spaces. But on physiological grounds it is unlikely that more than a small fraction of the total transport could occur this way, since solute could diffuse away from the basal membrane immediately and a hypertonic transport fluid would result.

After leaving the intercellular space, the transported fluid in the in vitro preparation must traverse several hundred microns of connective tissue before passing out of the gall bladder wall across its serosal surface. In vivo fluid would be carried away by capillaries close to the epithelial cells. It is possible that under in vitro conditions part of the fluid is also carried by these vessels, even though they are disconnected from the rest of the circulatory system. However, there is no compelling evidence for this. While dilated vessels are frequently found in transporting preparations, they are similarly found in nontransporting preparations.

The experimental evidence suggests no direct role for the basement membrane and connective tissue layer in osmotic equilibration of actively transported NaCl, and a role seems unlikely on several grounds. First, measured half-times for changes in transepithelial electrical potential differences after a change in the serosal bathing solution permit one to calculate the total resistance between the serosal bathing solution and the cell membranes at the serosal face of the epithelium. These measurements show that diffusion coefficients in the connective tissue are only 23% below those in free solution, and that the total resistance to ions between the cells and the serosal solution (i.e., the resistance of the connective tissue plus the basement membrane) is only 3-9% of the total epithelial resistance (Diamond, 1966). Second, if the

cell layer (possibly including the basement membrane) is scraped off the gall bladder, the connective tissue sac that is left accounts for only about 6% of the whole gall bladder resistance to ions as judged by tracer fluxes (Diamond, 1962 *b*) and electrical resistance measurements (Wright, personal communcation). Third, the absorbate of the gall bladder is isotonic not only in vitro but also under in vivo conditions, when transported fluid traverses only a small fraction of the connective tissue (Ravdin et al., 1932). Finally, the basement membrane is demonstrably permeable to molecules up to at least the size of ferritin (100 A). These four lines of evidence suggest that the relative significance of the basement membrane and connective tissue as a barrier to salt and water movement is slight, and that osmotic equilibration must take place almost entirely within the lateral spaces. The function of the basement membrane is probably a purely structural one in relation to the epithelium which rests on it.

The Geometrical Significance of the Lateral Intercellular Spaces The conclusion that the route of fluid transport across the gall bladder proceeds through the lateral intercellular spaces is of particular significance, because the geometry of these spaces may provide the explanation for isotonic water-to-solute coupling. As discussed in the Introduction, if solute were transported across a flat membrane, it would tend to diffuse away and yield a grossly hypertonic absorbate, since no further osmotic equilibration could take place once solute was out of contact with the membrane. Consider, however, what should happen when solute is transported out of the gall bladder epithelial cells across the lateral cell membranes and dumped into the lateral spaces, making them hypertonic. The length of the channels would retard the loss of solute by diffusion, and since the channels are bounded by cell membranes, osmotic equilibration by entry of water from the cytoplasm could take place along the entire channel length. There would thus be a progressive approach to osmotic equilibrium, with more and more water entering the channels as solute moved down them, until the solution emerging from the open end towards the connective tissue could be virtually isotonic. Thus, a standing osmotic gradient would be maintained in the channels by active solute transport, with the fluid at the closed end (near the tight junction) most hypertonic, and with the osmolarity decreasing along the length. Such a gradual approach to osmotic equilibrium would explain how osmotic forces drive water flow, even though the final transported fluid itself is isotonic within experimental error. The osmotic gradient responsible for water flow would be due to the high concentrations of NaCl near the closed end of the channel, and not to the slight or nonexistent hypertonicity of the final absorbate emerging from the open end. This explanation of solute-water coupling may be termed the standing-grad-

ient osmotic flow model. A somewhat similar explanation has been suggested for osmotic coupling in the ciliary body of the eye (Ballintine, 1959).

Whitlock and Wheeler (1964) have suggested that their physiological observations on transported osmolarities in rabbit gall bladder could be fitted to a somewhat different model system consisting of three compartments in series (Curran and MacIntosh, 1962; Patlak, Goldstein, and Hoffman, 1963). Kaye et al. (1966) have sought structural analogies between the gall bladder and this series system. As has been discussed in detail elsewhere (Diamond, 1967), however, the significance of the fit obtained by Whitlock and Wheeler is difficult to assess, because the experimental osmolarities were being fitted to equations involving eight theoretical parameters, values for the majority of which were chosen arbitrarily. For example, the value of one model parameter ($\sigma_1 = 0.9$) was stated to have been selected completely arbitrarily (Whitlock and Wheeler, 1964, p. 2258) and was then used to calculate two further parameters (called P_2 and P_2'); specific arbitrary assumptions were made about values of four other parameters ($\sigma_2 = 0 = \sigma_2'$, $\sigma_1' = 1$, L very large) because these assumptions were shown to be necessary for obtaining agreement with experimental results; and the value used for the eighth, electrical parameter (the diffusion potential set up by a NaCl concentration gradient) is now known to be 2.6 times the correct value. The assumption of the water permeability L being very large conflicts with the experimental fact which is one of the basic quantitative difficulties in explaining isotonic fluid transport; viz., that the water permeabilities measured for epithelia are actually much too low in relation to their fluid transport rates if one assumes a well stirred middle compartment (Auricchio and Bárány, 1959; Diamond, 1964 *b*). The hypertonic transported fluids observed by Whitlock and Wheeler probably arose from their use of serosal solutions with salt partially replaced by nonelectrolytes, since under these circumstances there inevitably must have been and apparently was considerable secondary modification of transported osmolarities due to salt diffusion down its resultant concentration gradient in the connective tissue. Finally, their mathematical analysis set out from the assumption that the lateral spaces are perfectly stirred. This assumption is almost certainly invalid, and as shown in the following paper (Diamond and Bossert, 1967), the lack of stirring may be of crucial importance in permitting formation of an isotonic absorbate. These criticisms of Whitlock's and Wheeler's theoretical calculations aside, their qualitative findings are largely in agreement with ours; namely, that solute-water coupling involves osmotic equilibration in a restricted region and that this region may be identified with the lateral intercellular spaces.

Long, narrow channels are common structural features in a variety of transporting epithelia and occur in several forms in addition to lateral intercellular spaces. These include basal infoldings such as are found in kidney tubular

epithelium, and intracellular canaliculi such as are found in parietal cells. Such channels may well play a role similar to that postulated for the intercellular spaces of the gall bladder. A more detailed discussion of the physiological significance of long, narrow channels will be found in the following paper (Diamond and Bossert, 1967), in which the consequences of standing osmotic gradients are analyzed mathematically.

It may be noted in conclusion that distended lateral spaces have recently been observed in several epithelia other than the gall bladder; namely, small and large intestine (Kaye and Lane, 1965), rat proximal tubule (Molyneux and Thorburne, personal communication; Leyssac, personal communication), and frog skin (Voute and Ussing, personal communication). It must be emphasized, however, that micrographs showing distension of a certain structure do not constitute ipso facto proof that the structure is the route of fluid transport. Several other criteria should be met before this conclusion can be safely drawn for a particular case: (*a*) It should be shown, using different physiological conditions but identical fixation procedures, that the structure is distended during transport and collapsed or less distended after exposure to several different agents which inhibit transport. (*b*) The structural changes should be quantitated using an unbiased sampling procedure. This is necessitated by the enormous variability usually present in samples of tissue. (*c*) Alternative explanations for the observed structural changes should be sought and ruled out; especially to be guarded against are changes which might be secondary to alterations in cell volume.

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