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Isolated rat hepatocyte couplets: A primary secretory unit for electrophysiologic studies of bile secretory function

(bile canaliculi/Mg^{2+}-ATPase/microelectrode/electrical potential/input resistance)

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ABSTRACT **Hepatocyte couplets were isolated by collagenase perfusion from rat liver. Between adjacent cells, the bile canaliculus forms a closed space into which secretion occurs. As in intact liver, Mg^{2+}-ATPase is localized at the canalicular lumen, the organic anion fluorescein is excreted, and secretion is modified by osmotic gradients. By passing a microelectrode through one cell into the canalicular vacuole, a transepithelial potential profile was obtained. In 27 cell couplets the steady-state intracellular (-26.3 ± 5.3 mV) and intracanalicular (-5.9 ± 3.3 mV) potentials were recorded at 37°C with reference to the external medium. Input resistances were determined within the cell (86 ± 23 MΩ) and in the bile canalicular lumen (32 ± 17 MΩ) by passing current pulses through the microelectrode. These data define electrical driving forces for ion transport across the sinusoidal, canalicular, and paracellular barriers and indicate ion permeation across a leaky paracellular junctional pathway. These findings indicate that the isolated hepatocyte couplet is an effective model for electrophysiologic studies of bile secretory function.**

Several models of bile secretion have been proposed (1, 2), but validation of these models requires direct electrophysiologic measurement. Electrophysiologic techniques have not been widely used to study bile secretory function in the liver, because the small (1–2 μm) diameters of the bile canaliculi and their inaccessible location between adjacent liver cells have precluded micropuncture of the canalicular space. Thus, studies have been limited to measurement of intracellular electrical potentials and provide information about potential gradients across the sinusoidal membrane (3–5). Electrical potentials in the bile ducts have also been obtained to assess events from these more distal sites (6–8). Finally, the functional significance of the electrical potential has been studied with respect to the transport of organic anions in isolated plasma membrane vesicles from both sinusoidal and canalicular domains of rat liver (9–11).

During preparation of isolated rat hepatocyte suspensions by collagenase perfusion, Oshio and Phillips (12) observed that some cells remain attached to one another. These "couplets" adhere to glass coverslips, and the canalicular remnants between the two adjoining cells apparently form a closed space that can enlarge to more than 3–5 μm. Using time-lapse cinematography, Oshio and Phillips observed that these canalicular spaces expand and collapse periodically, as a result of continuous secretion. The possibility was then raised that the bile canalicular space between the couplet might be punctured with micropipettes to measure electrical potentials directly within this lumen (13).

In this report, we present evidence that these isolated rat hepatocyte couplets represent primary canalicular bile secretory units that are amenable to micropuncture and classical electrophysiologic approaches.

METHODS

Isolated rat hepatocytes were prepared and characterized according to Seglen (14), as modified (15). In this preparation, cell couplets were encountered infrequently. By decreasing the amount of collagenase (Sigma) to 0.05% in 150 ml of buffer, $0.7–2.2 \times 10^7$ hepatocytes per g of rat liver were obtained containing 17.2% ± 4.2% couplets; 86%–94% of the cells excluded the dye Trypan blue. Other characteristics were as described (15). Of the final cell suspension, 60–75 μl was injected into 35 × 10 mm tissue culture dishes containing 3 ml of Leibovitz-15 tissue culture medium, 335 mosmol/liter (16) (GIBCO), yielding $0.75–1.0 \times 10^5$ cells per ml. The medium contained 100 units per 100 μg of penicillin and streptomycin per ml. The cells were placed in an incubator at 37°C in an air atmosphere and allowed to settle onto 5 × 5 mm glass coverslips until studied 4–8 hr after isolation.

Optical Equipment. A Zeiss IM 35 inverted microscope was mounted on a vibration-free air-isolated table (Micro-g, Baker Loring, Peabody, MA) in a Faraday cage. Double-differential interference contrast (Nomarski) optics were used. In addition to high resolution, these optics provide a narrow depth of field, which allows precise localization of the electrode tip (along the vertical axis.) A Neofluar 63/1.26-oil objective and ×10 eyepieces were used. A Planachromat LD 40/0.60 objective was used as condenser to provide space above the microscope stage for manipulation of microelectrodes. For incident light fluorescence microscopy, we used a 100 W mercury lamp, neutral density filters, and a primary filter with a 450- to 490-nm window and a secondary filter with a cutoff at 515 nm. Photographs were obtained with Kodak Ektachrome 400 film. Experimental chambers for direct visualization of the cell couplets were built from a glass coverslip (diameter, 25 mm) glued to a cylindrical wall (height, 3 mm) obtained from a 25-mm diameter plastic beaker. Coverslips with attached cells and up to 1.5 ml of culture medium were placed into the chamber, which rested on a 1-mm-thick metal plate, heated by a circular Peltier element (Cambion, Cambridge, MA). The metal plate contained an 11-mm hole for insertion of the objective to visualize the cells from below the chamber. Heating current was controlled from a miniature probe in the chamber and maintained temperature within 37.0 ± 0.1°C. A coarse and fine micromanipulator with hydraulic drive (Narishige, Tokyo) were mounted on the microscope stage.

Electrical Measurements. Electrical potentials were recorded with conventional microelectrodes, pulled on a Narishige PD5 electrode puller from 1-mm OD Omega Dot glass tubing (Frederick Haer, Brunswick, ME). Electrodes were

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filled with 1 M KCl and had tip resistances of 120–180 MΩ. Electrodes were discarded if the tip potential was >3 mV. Electrodes were connected to a modified WPI Instruments M701 amplifier (Hamden, CT) that allowed determination of input resistances. These were measured by passing rectangular current pulses (0.5 nA; 50 msec) through the electrode and recording the resulting electronic membrane potential charges after compensation for the electrode resistance. A Ringer–agar/KCl/AgCl bridge served as a ground connection. Potentials, their electrotonic changes, and the injection current were recorded with a Gould 200 pen recorder (Cleveland, OH). Electrodes with larger tips were used for dye ejection by iontophoresis. When filled with 10 mM disodium fluorescein, the resistance was ≈200 MΩ. A maximal current of 10 nA could be passed through these electrodes, and with a few 100-msec current pulses sufficient dye could be ejected to obtain bright intracellular or intracanalicular fluorescence.

Histochemistry. Canalicular Mg^{2+}-ATPase activity was localized using the Wachstein–Meisel method (17), 10 min and 1, 2, 3, 4, and 5 hr after cells had been allowed to settle. Coverslips with adherent cells were fixed in cold Karnovsky's fixative (3% paraformaldehyde/0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) for 2 min, transferred into 0.1 M cacodylate buffer (pH 7.5), and then kept at 4°C in 0.1 M Tris maleate (pH 7.2). Coverslips from all time points were then simultaneously placed for 10 min at 37°C into freshly prepared incubation medium containing (mM): ATP, 0.83; Tris maleate, 80; $PbNO_3$, 3.6; $MgSO_4$, 10, pH 7.2. After extensive washing in distilled water the reaction product was developed in 1% $(NH_4)_2S$.

Experimental Procedures. *Fluorescent dyes.* Functional integrity of the organic anion transport system was qualitatively assessed by adding 2 μl of fluorescein (40 mg per ml of H_2O) and fluorescein diacetate (Sigma) (50 mg per ml of acetone) to the tissue culture dishes 4 hr after cell isolation. Adherent cells were examined by fluorescence microscopy in dye-free tissue culture medium 10 min to 4 hr after exposure to the dye.

Osmotic gradients. Cell size and diameter of canalicular spaces (vacuoles) were measured before and after increasing the osmolarity of the tissue culture medium by adding sucrose. Optical transections and photographs were obtained of a cell couplet at the largest diameter of the canalicular vacuole, ≈10 μm above the surface of the coverslip. The osmolarity of the bathing fluid was then increased by 100 mosmol/liter by adding an equal volume of culture medium to which 200 mM sucrose was added. Photographs were taken at 0.5, 1, 2, 5, and 10 min. Rough estimates of relative volume changes of the cells were obtained by measuring their diameter and assuming a spherical shape. The volumes of the canalicular vacuoles were estimated by measuring their length and width and assuming an ellipsoid shape. The width of the canaliculus between cells was usually smaller and subject to larger changes than the length and height.

Micropuncture technique. Micromanipulators were mounted to the right of the microscope stage so that the microelectrode shaft formed a 30° angle to the plane of the coverslip. The electrode tip was first placed in the center of the field of view ≈50 μm above the coverslip. In general, cell couplets were selected for micropuncture if the canalicular space was dilated; they then were oriented with one cell to the left and the other to the right. The cell couplet was placed within a few micrometers of the center of the field marked by a reticule cross. The cells were then focused so that the focal plane transected the canalicular vacuole. The electrode tip was moved downward to the same focal plane as the cells but precisely in the center of the field at the reticule cross so that the tip was adjacent to the canalicular vacuole, both being sharply in focus. From that position, the electrode was withdrawn by axial movement only, and the cell couplet was moved into the center of the field, so that the canalicular vacuole occupied the former position of the electrode tip. Under visual direction, the electrode was then advanced toward the vacuole by axial movement and electrical potentials were continuously recorded. The cell membrane dimpled as the electrode touched the right-hand cell, and it was penetrated by cautiously tapping the microscope stand. By further axial movement, the electrode tip was advanced within the cell to the canalicular vacuole, which was then frequently displaced by a few micrometers to the left prior to puncturing by again cautiously tapping the microscope. The electrode tip could usually then be seen within the canalicular lumen.

RESULTS

By decreasing the total amount of collagenase in the perfusion medium, a population of hepatocytes was isolated from rat liver that contained a significant increase in undissociated cells. Cell couplets were particularly prominent in these preparations, although larger groups of cells were also observed. We have focused on the bile secretory properties of these cells and, particularly, the electrophysiological characteristics of the hepatocyte couplets 4–8 hr after their isolation from the intact rat liver. Eight to 10 hr after initial plating, 89% ± 4% of the cells attached to the coverslip excluded Trypan blue. As observed by Oshio and Phillips (12), these cell couplets retain a canalicular space between the two adjacent hepatocytes, which apparently seals and expands as secretion proceeds.

As shown by the Mg^{2+}-ATPase stain, a histochemical marker for the canalicular domain of the hepatocyte, these membranes are present in their normal location, circling each cell in a belt-like arrangement, immediately after isolation from the collagenase-perfused liver (Fig. 1*A*). However, within 4 hr the reaction product is localized predominantly to a small portion of the cell-surface membrane between the two adjacent hepatocytes (Fig. 1*B*). These findings, together with electron microscopic observations that the spaces are bordered by tight junctions and lined with microvilli (18), provide morphologic evidence of the functional equivalent of bile canaliculi (19, 20).

These canalicular spaces demonstrated a secretory cycle characterized by periods of expansion followed by rapid col-

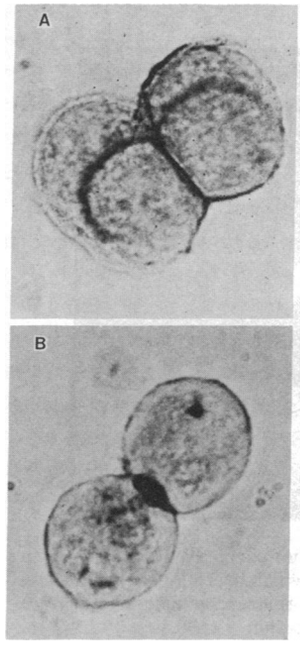

FIG. 1. Histochemical demonstration of Mg^{2+}-ATPase activity in isolated hepatocyte couplets. (*A*) Immediately after isolation; (*B*) after 5 hr in tissue culture. Note belt-like distribution of enzyme activity, reminiscent of the location of bile canaliculi on the surface of liver cells in intact tissue (*A*) (×740) and redistribution of enzyme activity to the canalicular vacuole formed during tissue culture (*B*). (×660.)

lapse, a process that was stimulated by adding taurocholate to the medium (21, 22).

These qualitative observations suggest that the isolated hepatocyte couplet functions as a primary bile secretory unit, a conclusion further supported by the ability of selected cell couplets to transport and concentrate organic anion fluorescent dyes from medium to the bile canaliculi (Fig. 2) (23–25). Hepatocellular uptake of these dyes occurs within a few minutes. Fluorescein diacetate must be hydrolyzed by hepatic esterase to fluorescein before fluorescence can be detected. Although hepatic uptake of fluorescein diacetate can occur passively, in contrast to fluorescein, which depends on an active transport mechanism, further concentrations of fluorescein in bile occurred as early as 10 min after addition of either dye to the medium, demonstrating that the canalicular excretory system is functionally intact. However, considerable variation in cell and canalicular fluorescence was observed. Accumulation of the fluorescent dye within the canalicular lumen also shows that the canalicular membrane and tight junction are relatively impermeable to these organic anions. Continued observations of fluorescent canaliculi often revealed spontaneous collapse of the space and rapid disappearance of the luminal fluorescence.

These canalicular spaces were also sensitive to hypertonic osmotic gradients imposed by adding sucrose to the medium. Maximum canalicular volume reductions were observed within 1 min (Fig. 3 *A* and *B*) in six cell couplets from three experiments (42.0% ± 13.4% of control values) compared to a smaller reduction in volume of the hepatocytes (79.3% ± 4.6% of control values) (mean ± SD). The changes in cell volume are consistent with the behavior of an ideal osmometer, because the decrease in volume was in proportion to the increase in osmolarity of the medium. In contrast, the change in canalicular volume exceeded this prediction. As bile secretion continued, the canalicular spaces again expanded as shown in Fig. 3*C*.

Since these bile secretory properties of the isolated cell couplets were similar to *in vivo* findings, we attempted electrophysiologic measurements. A representative experiment is shown in Fig. 4. After alignment of the microelectrode and cell couplet (see *Methods*), a continuous recording of the electrical potential was obtained. In the extracellular position, the electrotonic potential changes due to the electrode resistance and passage of 0.5-nA depolarizing current pulses were compensated. As soon as the electrode touched the surface of the hepatocyte a small, usually positive, deflection of the potential was recorded together with a large increase in the input resistance. After puncture of the cell membrane, a sudden negative deflection (A) of the potential was observed. Steady-state recordings of the intracellular potential (B) were slightly lower than the initial negative deflection and electrotonic potential changes due to passage of current assumed a constant amplitude, reflecting the resistance to current flow out of the cells. If stable intracellular recordings were not obtained, further attempts to puncture the canalicular space were not made. Otherwise, the electrode was advanced to touch the cytoplasmic surface of the canalicular membrane where an increase in input resistance was again observed. Successful puncture of the bile canaliculus (27/124 attempts) resulted in an immediate maximal drop in the negative electric potential (C), which then increased slightly to a steady state. Input resistances were

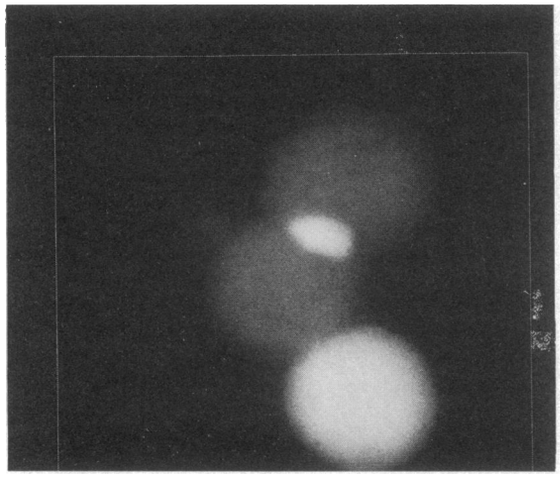

FIG. 2. Row of three liver cells; fluorescence microscopy. Two cells form a canalicular vacuole into which secretion of fluorescein occurs. Cells were exposed for 1 hr to fluorescein diacetate; picture was taken after 10 min in dye-free medium. (×690.)

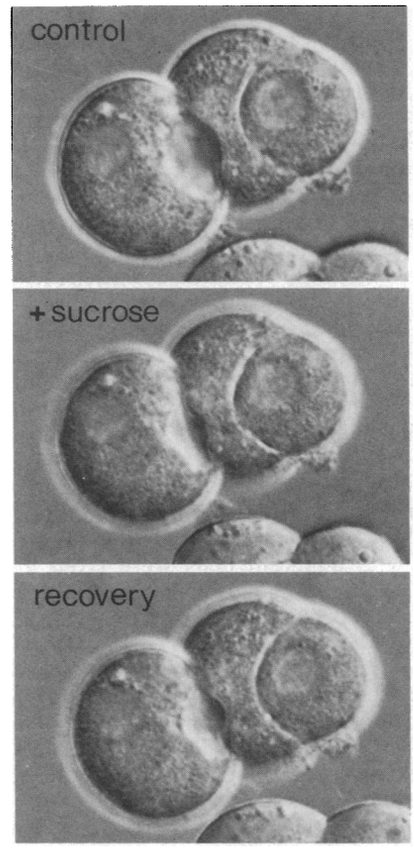

FIG. 3. A liver cell couplet with canalicular vacuole and one attached cell; double interference contrast. Note shrinkage of the canalicular spaces 1 min after exposure to hypertonic medium and partial recovery of volume after 5 min. (×830.)

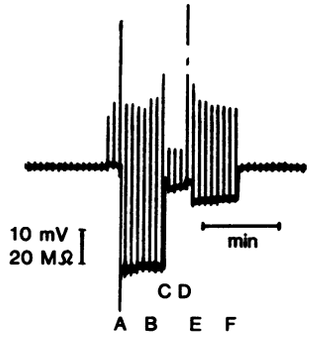

FIG. 4. Trace of electric potential recorded during puncturing one cell of a couplet (A), the canalicular space (C), and the second cell (E). Downward displacement is negative with reference to the bathing solution. Multiple upward deflections are due to 0.5-nA depolarizing current pulses measuring input resistance (10 mV ≙ 20 MΩ). A–F are explained in Table 1 and text. At the end of the experiment, the microelectrode was suddenly withdrawn.

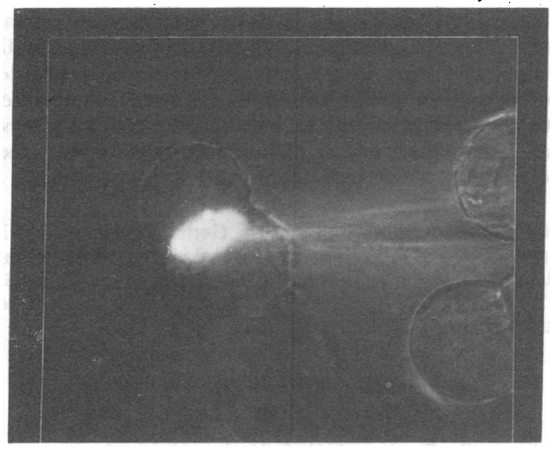

FIG. 5. Liver cell couplet and canalicular vacuole; fluorescence microscopy. Fluorescein was injected into the canalicular space by iontophoresis through a microelectrode inserted from upper right. (×660.)

considerably lower than intracellular recordings. Occasionally, small decrements in potential and input resistance were observed within the canaliculi. In one instance, the canalicular space spontaneously collapsed during the recording; this was associated with an abrupt abolishment of potential and a decrease in input resistance. The second cell was successfully punctured in 14/27 attempts. Again an increase in input resistance was observed as the electrode touched the canalicular membrane of the adjacent cell from the canalicular lumen (D). The electrical potential immediately after puncture of the second cell (E) and during steady-state recording (F) were always significantly lower than the values obtained with punctures from the first cell. However, it was difficult to puncture the second cell because further movement of the electrode tip resulted in considerable displacement of the canalicular vacuole into the cell. Thus, it is possible that these impalements were leakier than observed in the first cell or in the bile canaliculus. However, since these values were always larger than observed within the canalicular lumen, they served as an additional validation of the electrode position within the canaliculus.

Iontophoresis of fluorescein from the microelectrode was carried out on five occasions after puncture of the bile canaliculus. As shown in Fig. 5, fluorescence was retained within the canalicular space, demonstrating that these spaces could be punctured with the microelectrodes and did not freely communicate with the surrounding medium. A summary of these electrophysiological measurements from 27 cell couplets is presented in Table 1.

DISCUSSION

Isolated rat hepatocyte couplets are uniquely suited to direct electrophysiologic approaches. After insertion of a micropipette into one of two adjoining hepatocytes, the cell potential and input resistances can be recorded. The average steady-state potential values of −26.3 ± 5.3 mV are only slightly lower than the values of −30 to −40 mV obtained in the intact rat or mouse liver. The intracellular potential is a highly sensitive parameter of cell function, which can be influenced by multiple factors including the activity of Na^+/K^+-ATPase, transmembrane ion concentration gradients, relative membrane ion conductances, and hormonal influences. Thus, deviations from *in vivo* measurements could be due to multiple factors, including lack of hyperpolarizing hormonal effects such as glucagon or epinephrine (26, 27) or electrogenic uptake of amino acids present in high concentration in tissue culture medium (16, 28). Finally, as in other epithelia, potential differences across sinusoidal, canalicular, or tight junction barriers are interdependent (29, 30).

This study achieves a long sought goal: to micropuncture the bile canaliculus and directly record intracanalicular potentials from the liver. Localization of the microelectrode within the canalicular lumen was visualized directly with Nomarski interference contrast optics but was also confirmed by the injection of fluorescein into the canalicular space (Fig. 5). After an initial recording of −4.1 mV (Table 1) in the bile canaliculus, the potential usually increased slightly. This could be due to leakage of KCl from the microelectrode tip (31), in which case the initial reading will be more accurate. The origin of the canalicular potential is presently unknown, but it could be due to a diffusion potential generated by unequal distribution of permeant ions between canalicular lumen and bathing fluid. Alternatively, as shown in other epithelia, the potential could be due to current flow in the equivalent circuit composed here of the sinusoidal, canalicular, and tight junction barriers and their respective electromotive forces.

The potential difference between the hepatocyte and canalicular lumen may provide a driving force for organic anion excretion across a conductive pathway in the canalicular membrane. This potential difference could account for a 3-fold concentration gradient for monovalent anions such as taurocholate (1, 2), a conclusion also supported from studies in canalicular membrane vesicles (9). Since higher concentration gradients for organic anions may be achieved, other transport mechanisms presumably exist.

The present findings also provide information about the properties of the barrier between the canalicular lumen and the external media. Specifically, retention of fluorescent dyes within the lumen after either spontaneous excretion (Fig. 2) or direct ejection from the microelectrode (Fig. 5) confirm *in vivo* observations that the canalicular membrane and junctional barriers are relatively impermeant to these organic anions. These barriers must also be relatively impermeant to sucrose, as indicated by the rapid reductions in the canalicular space imposed by hypertonicity by sucrose in the medium (Fig. 3). The magnitude of this volume reduction in the canalicular lumen following addition of sucrose, which increased the osmolarity of the medium by 100 mosmol/liter, substantially exceeds the 23% shrinkage predicted by osmotic water flow alone out of a previously isotonic canalicular

Table 1. Electric potential and input resistance in liver cell couplets

	Electrode position					
	First cell		Canalicular space		Second cell	
	A	B	C	D	E	F
Electric potential, mV	−31.0 ± 5.7 (−15 to −42)	−26.3 ± 5.3 (−15 to −37)	−4.1 ± 2.6 (−1 to −12)	−5.9 ± 3.3 (−1 to −15)	−12.9 ± 5.4 (−8 to −23)	−16.8 ± 7.6 (−6 to −29)
Input resistance, MΩ		86 ± 23 (46 to 156)		32 ± 17 (8 to 68)		64 ± 32 (27 to 107)

Results are expressed as mean ± SD. Numbers in parentheses represent range. A–D, n = 27; E and F, n = 14.

space. This phenomenon can only be explained if permeant solutes also simultaneously leave the canalicular space by diffusion or solvent drag.

Finally, the low canalicular input resistance compared to the hepatocyte cell membrane indicates that the tight junctions must be permeable to small ions as suggested by isotope flux measurements (32) and junctional penetration of ionic lanthanum chloride (33) in intact rat liver. Canalicular input resistance should be higher than the intracellular input resistance if current flow were restricted to the series of resistances imposed by cellular membranes across the transcellular pathway.

Anatomically, this rat hepatocyte cell couplet system represents a primary bile secretory unit, because the bile canalicular lumen in the intact liver is bordered by at least two adjacent hepatocyte membranes that are sealed by the tight junction elements. In agreement with the reports of Phillips and his associates, these couplets remain viable for >8 hr after isolation and continue to secrete bile (12, 21, 22). After isolation, the junctional complexes between the two cells must undergo rearrangement, because the space should initially be in free communication with the surrounding incubation medium as a result of the dissociation of other hepatocytes that are contiguous and surround these two cells in the intact liver. The remaining junctions between the two hepatocytes evidently reseal, converting the space between into a "closed" lumen that retains the canalicular contents. The dynamics of this process are not completely understood. Mg^{2+}-ATPase reaction product initially outlines the "hemicanaliculi" around the entire hepatocyte in the couplets immediately after their isolation; yet, 4 hr later, the staining is rearranged entirely at the reformed canalicular lumen. Whether this phenomenon occurs by lateral diffusion of membrane components or is the result of internalization and reinsertion of *de novo* synthesis or canalicular membrane is unclear. The canalicular space (diameter, 1–2 μm at the time of isolation of the couplets) is now expanded.

Although this isolated cell couplet bile secretory unit exhibits several important features that are similar to secretory properties in the intact liver, there are notable differences. The canalicular lumen between adjacent cells is a closed space so that abnormal pressure may build up as secretion proceeds—a process that may increase permeability of the junctional barriers. Also, the secretory contents are released only periodically as the canalicular space collapses. Presumably, the junctional complexes are disrupted during this process and then rapidly reseal. Pericanalicular microfilaments and other cytoskeletal elements may maintain tension in the canalicular wall and/or periodically contract as suggested by Oshio and Phillips (12). Nevertheless, most of the secretory properties of the cell couplets studied so far appear similar to the process of bile formation *in vivo*. The response of canalicular vacuoles to osmotic gradients and their relative impermeability to organic solutes confirms that the osmotic theory of bile flow is applicable to these isolated secretory units. Bile formation thus proceeds by a process dependent on the accumulation of various permeant and impermeant solutes within the canalicular lumen that results in subsequent osmotic water flow. The secretion of fluorescent dyes, a property originally described by Hanzon (23–25), indicates that the organic anion transport system(s) remains intact, a finding also supported by the stimulation of the secretory cycles with taurocholate (21, 22). Finally, the permeability of the tight junctions to small ions supports the idea that the paracellular pathway allows equilibration of electrolytes between the blood and bile.

These isolated cell couplets provide unique opportunities for application of electrophysiologic techniques. In addition to the establishment of a transepithelial potential profile, as reported in this study, future experiments should define electrochemical gradients for individual ions across the sinusoidal, canalicular, and tight junction barriers and electrogenic components of individual transport mechanisms.

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