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Hepatic Bile Formation: Developing a New Paradigm

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***Abstract*——In 1959, Ivar Sperber contrasted bile formation with that of urine and proposed that water flow into the canalicular conduit is in response to an osmotic, not a hydrostatic, gradient. Early attempts to support the hypothesis using a bile acid, sodium taurocholate, and the hormone secretin to stimulate bile flow led to conflicting data and a moratorium on attempts to further develop the initial proposal. However, current data amplify the initial proposal and indicate both paracellular and transcellular water flow into hepatic ductules and the canalicular conduit in response to an osmotic gradient. Also, the need to further modify the initial proposal became apparent with the recognition that bile acid aggregates (micelles), which form in the canalicular conduit, generate lecithin-cholesterol vesicles that contain water unrelated to an osmotic gradient. As part of this development is the recent introduction of the fluorescent localization after photobleaching technique for direct determination of hepatic duct flow and clarification of the role of biomarkers such as mannitol and polyethylene glycol 900. With the new paradigm, these biomarkers may prove useful for quantifying paracellular and transcellular water flow, respectively.**

***Significance Statement*——It is essential to identify and characterize all the sites for water flow during hepatic bile formation to obtain more precision in evaluating the causes and possible therapeutic approaches to cholestatic syndromes. Updating the Sperber proposal provides a new paradigm that addresses the advances in knowledge that have occurred.**

I. Background

When Ivar Sperber introduced the concept that water flow into the canalicular conduit, referred to as "bile capillaries," is mediated by an osmotic gradient (Sperber, 1959), he was distinguishing this mechanism from the hydrostatic pressure gradient responsible for urine formation. Knowledge of urine formation was at a much more advanced state than bile formation, and his review entitled "Secretion of Organic Anions in Urine and Bile" focused mostly on renal function. Sperber did not describe the anatomy of the biliary tree, nor did he discuss the determinants of an osmotic gradient, other than reflecting that, as the predominant solute, bile acids were likely to play a major role.

Glutathione and other solutes are also present at higher concentrations in canalicular fluid than intracellularly and therefore add to the osmotic gradient. It became conventional to refer to osmotically generated water flow as either "bile acid dependent" or "bile acid independent."

Data developed in the decades following Sperber's hypothesis showed that the relationship of bile acids to osmolarity depends on the concentration at which they begin to aggregate, referred to as the critical micelle

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ABBREVIATIONS: FLAP, fluorescent localization after photobleaching; PEG-900, polyethylene glycol molecular weight 900; TDC, taurodehydrocholate.

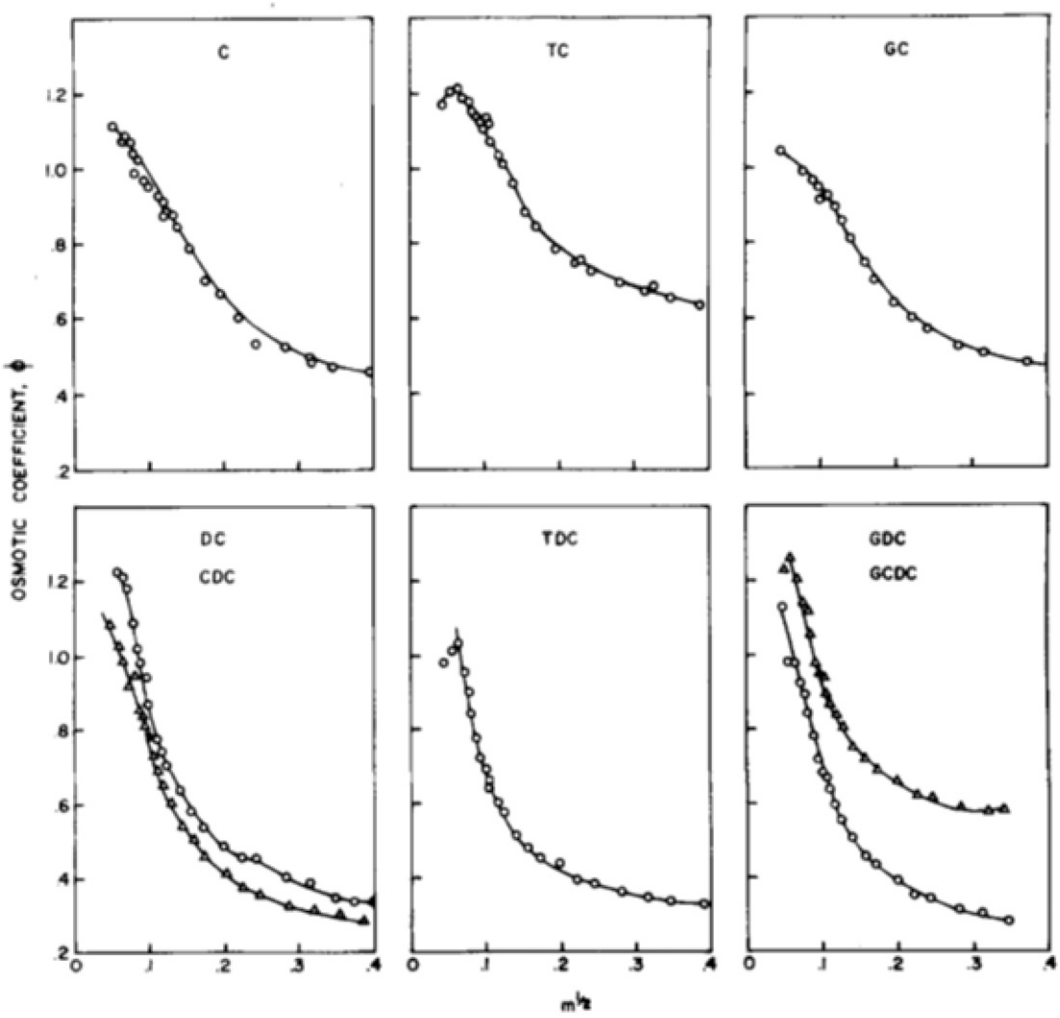

Fig. 1. Osmotic coefficients of bile acids in aqueous solution. Most naturally occurring bile acids have physical properties that cause them to aggregate in aqueous solution as their concentration increases, referred to as micelle formation. A decrease in osmotic coefficient indicates less water enters via aquaporin 8 and claudin 2 and proportionally more via lecithin-cholesterol vesicle formation. This relationship accounts for the findings in the absence of claudin 2. The decrease in the rate of osmotic equilibration results in an increase in bile acid concentration. Figure reproduced with permission from Carpenter (1969); copyright Springer Nature. C, cholate; CDC, chenodeoxycholate; DC, deoxycholate: GC, glycocholate; GCDC, glycochenodeoxycholate; GDC, glychodeoxycolate; TC, taurocholate; RDC, taurodeoxycholate.

concentration. As shown in Fig. 1, using a vapor pressure osmometer it was found that the osmotic gradient generated by micelle-forming bile acids varies inversely with the concentration; the osmotic coefficient is greatest at low concentration and decreases in a curvilinear fashion as the micelle concentration increases (Carpenter and Lindenbaum, 1979). The findings are in accord with the knowledge that osmolarity is determined by the number of molecules in solution and not by their molecular size. As molecular size increases because of micelle formation, the proportion of monomers decreases with a lowering of the osmotic coefficient. This relationship accounts for higher rates of bile flow mol for mol in comparing taurocholate, a micelle-forming bile acid, to taurodehydrocholate (TDC), a non–micelle-forming bile acid (O'Máille, 1980). Endogenous bile acids all have the property of forming aggregates of varying molecular size (Coello et al., 1996). Separately, micelle formation was shown to be related to the secretion of lecithin-cholesterol vesicles into the canalicular conduit (Cohen and Carey, 1990; Marzolo et al., 1990; Cohen et al., 1992).

An attempt by Bradley, who trained in the Homer Smith laboratory at NYU School of Medicine (Bradley, 1995), and his co-workers, to quantify hepatic bile flow used mannitol (Wheeler et al., 1968), a biomarker that had been validated to quantify glomerular filtration rate (Kiss et al., 2018). The utility of mannitol for quantifying water flow was a surprise because even though it has a relatively low molecular weight (mol wt = 182), it yielded urine/plasma concentration ratios greater than 100, not significantly different from inulin (mol wt = 5000) and polyethylene glycol (PEG-900) (mol wt 900). While structurally distinct, these three compounds share important characteristics: they are not metabolized, filter with water across the glomerular membrane, and do not undergo significant reabsorption

as they flow past the proximal and distal tubules of the nephron and the loop of Henle.

In exploring the use of mannitol as a biomarker of hepatic bile flow, bile/plasma concentration ratios of slightly greater than 1 in the dog were interpreted as evidence for the absence of significant water reabsorption (Wheeler et al., 1968). When the bile/plasma concentration ratio of mannitol was found to decrease when hepatic bile flow was increased through secretin administration, which is known to increase water flow from the hepatic ductules, mannitol was further proposed as a biomarker for canalicular water flow. (Wheeler et al., 1968)

However, several subsequent studies did not support the proposal that the bile/plasma concentration ratio of mannitol differs significantly depending on whether water flow is stimulated by bile acids or secretin. (Hardison and Norman, 1969; Barnhart and Combes, 1978; Nicholls, 1979). Also, it was found that mannitol will diffuse for the hepatic ductules (Peterson and Fujimoto, 1977). Therefore, interest waned in the use of mannitol as a biomarker to quantify hepatic bile flow.

Since these attempts, much has been learned at the cellular level that informs our understanding of water flow. Specifically, it is now understood that aquaporins embedded in the basolateral and apical surfaces of hepatocytes and cholangiocytes govern water and solute flow and that the tight junctions contain proteins that modulate the movement of water and some solutes.

Although there remain gaps in knowledge regarding the functional anatomy of the biliary tree and with limitations in methodologies to elucidate water flow in the context of hepatic bile formation, data and methods developed since the Sperber proposal support a new paradigm for defining the multiple sites at which it occurs and their different determinants.

II. Canalicular Conduit: Role in Normal Hepatic Bile Formation

The claudin 2 knockout mouse led to a change in the prevailing concept that canalicular water flow was predominantly transcellular, by establishing that approximately 50% of such flow is paracellular (Matsumoto et al., 2014).

If the claudin 2 protein, expressed in the tight junctional apparatus (the intercellular semipermeable barrier between hepatocytes and between cholangiocytes) is the only paracellular route for water flow, then transcellular water flow accounts for approximately 50% of total hepatic bile flow. Other claudin proteins in the tight-junctional apparatus such as claudin 1 and claudin 3 normally function as barriers to water and solute movement (Tanaka et al., 2018; Huang et al., 2021).

Claudin 2 is normally expressed only in the pericentral region of the canalicular conduit (Tanaka et al., 2017). Bile acids returning from their enterohepatic circulation after meals are mostly transported into the periportal region, and it is reasonable to expect that micellar concentrations would occur mostly in this region. A study in rats 24 hours after partial hepatectomy (Vos et al., 1999) (when there is no significant change in bile acid pool size) found that bile flow per gram of remaining liver was significantly greater in the partially hepatectomized animals than in the sham-operated control animals. Cholesterol and lecithin output was not significantly different. The investigators concluded that the increased bile acid load on the remaining liver increased bile acid transport in the pericentral region of the canalicular conduit where claudin 2 is expressed.

Transcellular canalicular water flow occurs via aquaporin 9 on the basolateral surface and aquaporin 8 on the apical surface of the hepatocyte. Aquaporin 9 is often referred to as an aquaglyceroporin in recognition of its glycerol transport function during hepatic triglyceride metabolism, a key role of the liver (da Silva et al., 2022). In contrast to aquaporin 8 on the apical surface, aquaporin 9 is not considered rate-limiting with respect to water flow.

Studies of aquaporin 8 in animals given either cholestatic agents (ethynyl estradiol, or estradiol 17-glucuronide) or a choleretic agent (glucagon) for at least several days established a significant correlation between water flow and apical expression of that protein (Carreras et al., 2007; Soria et al., 2009). Taurocholate output, when determined, did not vary significantly. It was therefore reasonable to attribute the reduction in flow to the apical expression of Aquaporin 8.

However, in acute studies after single injection of estradiol 17-glucuronide aquaporin 8 expression was unchanged (Mottino et al., 2006). Using horseradish peroxidase as a biomarker for paracellular flow, an increase in paracellular permeability was identified (Kan et al., 1989). Direct knowledge of the expression of claudin proteins after single and prolonged doses will define further the full range of effects of these agents.

Another source of water entry into the canalicular conduit unrelated to an osmotic gradient became apparent with the finding that "quasi-elastic light scattering and electron microscopy, when performed within 30 min of collection, revealed unilamellar vesicles in all biles" (Cohen and Carey, 1990). By 24 hours, vesicles in taurochenodeoxycholate-rich and taurocholate-rich biles had dissolved into mixed micelles (Cohen and Carey, 1990; Cohen et al., 1992). The report is also supported by the other observations (Crawford et al., 1997, 1995).

Lecithin-cholesterol vesicles (referred to as liposomes), when prepared in vitro, are unilamellar structures containing water (Akbarzadeh et al., 2013) and provide a source of canalicular water unrelated to osmolarity.

In summary, an update to the Sperber proposal specifies that water entry into the canalicular conduit in response to an osmotic gradient is mostly via aquaporin 8 and claudin 2 and also via lecithin-cholesterol

vesicle formation regulated by micelle-forming bile acids that is independent of an osmotic gradient.

A. Observational Studies of Canalicular Events during Hepatic Bile Formation

Four fluorescent markers have been used to characterize water flow in the canalicular conduit: fluorescein (uranin), carboxyfluorescein diacetate, cholyl-lysyl fluorescein, and bis-5-carboxymethoxy and 2-nitrobenzyl ether (CMNB-caged fluorescein).

Over a period of several years, Hanzon (Hanzon, 1952) studied the excretion of uranin (sodium fluorescein) into "bile capillaries" of the normal rat liver and after injury by UV light, hypoxia, hypothermia, or stasis. In normal liver, the transfer of uranin into bile "capillaries" increased its concentration 500-fold and generated an increase in bile flow, thereby visually confirming the Sperber hypothesis.

Each type of injury had a different effect on uranin excretion. Brief exposure to UV light can cause membrane injury with diffusion of uranin from the bile "capillaries" even though by light microcopy the liver cells appear normal. Only in studies where biliary pressure was increased by elevating the height of the bile duct cannula was movement of fluorescein from the canalicular conduit back into the sinusoids observed.

A subsequent study of fluorescein excretion extended the observations of Hanzon. It was observed that the canalicular conduit underwent rhythmic contractions that appeared to move the fluid unidirectionally (Watanabe et al., 1991). The observation led to detailed studies that identified a cytoskeleton containing actomyosin filaments (Tsukada and Phillips, 1993) that surround the conduit and subserve a peristaltic function. It was also noted that the conduit is filled with numerous microvilli that greatly increase the surface area for carrier-mediated solute transport.

The role of motility to propel the fluid toward the hepatic bile ductules was confirmed in a study utilizing carboxyfluorscein diacetate (Meyer et al., 2017). The nonfluorescent compound taken up by hepatocytes is hydrolyzed to fluorescent carboxyfluorscein, which is rapidly secreted into the canalicular conduit. The experimental approach eliminates the possibility of fluorescence occurring in hepatocytes that might be considered as being in the canalicular conduit. Mathematical modeling of their observations led to the conclusion that motility has an important role in propelling fluid generated in the canalicular conduit toward the hepatic ductules and that an increase in the rate of flow also occurs. Canals of Hering were not specifically identified and mechanisms accounting for the calculated increase in the rate of flow were not discussed.

A more recent observational study utilizing fluorescein, cholyl-lysyl-fluorescein and caged CNMB-fluorescein led to some conclusions that differed from the previous studies (Vartak et al., 2021).

This study used nonfluorescent caged molecules to generate fluorescent molecules by photoactivation (cleavage of the nitro benzyl ether group) using a beam of light focused on different regions within the liver lobule. The method, known as fluorescent localization after photobleaching (FLAP) (Ishikawa-Ankerhold et al., 2012), is used to track the movement of specific molecules within cells, but the technique is applicable to their movement in extracellular fluid.

The newly synthesized fluorescent molecules within the lumen diffuse down their concentration gradient independent of the rate of osmotically generated water flow. A rate of change in concentration greater than the calculated diffusion coefficient indicates the rate of water flow.

The FLAP technique is analogous to a drop of concentrated sweetener that falls into a cup of tea. The molecules will diffuse from higher to lower concentration based on their molecular weight and the composition of the tea. Stirring the tea increases the rate at which the molecules will disperse and subtracting the diffusion coefficient from the total dispersion rate indicates the rate of stirring (flow).

Using the FLAP technique, the investigators identified ductular water flow in response to an osmotic gradient generated by taurocholate and secretin. Their finding confirms previous reports from several laboratories using mannitol as a biomarker for water flow (Hardison and Norman, 1969; Barnhart and Combes, 1978; Nicholls, 1979).

The FLAP technique applied to the canalicular conduit did not generate data supporting the existence of osmotically determined canalicular water flow. The investigators therefore proposed that only diffusion of bile acids from higher to lower concentration occurs in the canalicular conduit and that osmotically generated flow by bile acid monomers begins in the ductules. Since no data are reported with the use of other bile acids, a more conservative conclusion could have been that osmotically determined flow was below their limits of detection with infusion of taurocholate.

Considering the structure of the canalicular conduit with the presence of numerous microvilli and the formation of cholesterol-lecithin vesicles and mixed micelles, the FLAP approach may not be applicable. The investigators stated: "We next measured the loss of fluorescence in a circular photoactivated region in the bile canalicular network, since the diameter of individual canaliculi approaches the optical resolution limit and is too small to precisely define a region within the lumen" (Vartak et al., 2021). Thus, in contrast to the studies of flow in ductules, the photoactivating beam was not focused directly on the lumen of the canalicular conduit but rather on a region considered equivalent but not further defined. No indication is given on the location of the caged molecules that were photoactivated.

Are they incorporated with the cholesterol-lecithin vesicles or mixed micelles or dissolved in the fluid surrounding them? This information is required for the determination of the diffusion coefficient, essential for calculating the rate of osmotically determined flow. The investigators state that "diffusion coefficients in the canalicular network and interlobular ducts ranged between 2.4–6 μm2/s" but provide no explanation for the variations that were found. By contrast, the diffusion coefficient remained constant during their studies of ductular water flow.

Another consideration in evaluating the utility of the FLAP technique to determine canalicular water flow is the effect of the photoactivating beam on hepatocytes. This problem was specifically addressed in studies by Hanzon.(Hanzon, 1952) UV light, depending on the intensity and duration, changed vectorial flow of fluorescein in the canalicular conduit to a diffusion pattern into hepatocytes that appeared normal by light microscopy. Validation that the photoactivation did not alter the permeability of hepatocytes is lacking.

The "diffusion hypothesis" also does not address the movement of cholesterol and lecithin from the canalicular conduit into the hepatic ducts. Their concentration in hepatic bile exceeds their aqueous solubility, and therefore they are solubilized within the mixed micelles that form and augmented by osmotic flow generated by bile acid monomers, glutathione, and other solutes.

Uncertainty regarding application of the FLAP technique to diameters too small for direct photoactivation and whether the diffusion-based concept can account for the flow of other constituents indicates the need for further evidence before its acceptance as a tool for evaluating canalicular events.

Nevertheless, the FLAP technique provides direct determination of the rates of flow in hepatic ductules and ducts and therefore can provide novel insights into the relative rates of flow in these conduits and the likelihood of significant water reabsorption.

B. Other Biomarkers for Water Flow in the Canalicular Conduit

When erythritol, mannitol, and PEG-900 were introduced in studies of hepatic bile formation, the focus was on transcellular flow across the apical membranes. Since mannitol, erythritol, and PEG-900 were known to distribute rapidly into hepatocyte cell water and have an intracellular concentration equal to plasma, solvent drag (Krugliak et al., 1994) across the membrane was proposed as a major mechanism to move with water transcellularly. When PEG-900 was found to have a plasma-to-bile ratio (P/B ratio) approximately 31-fold greater (Friman et al., 1988; Javitt, 1982), the paradigm was no longer tenable.

The findings that PEG-900 and mannitol clearances increase with taurocholate infusion (Wheeler et al., 1968; Friman et al., 1993), but that only PEG-900 clearance decreases with TDC infusion (Roma et al., 1991) provides a novel insight into its site of entry into the canalicular conduit. It is known that polyethylene glycols have significant lipid solubility in contrast to mannitol and other sugars that are virtually lipid insoluble (Ukabam and Cooper, 1984). Therefore, PEG-900 can enter when cholesterol-lecithin vesicles form, which accounts for its relationship to taurocholate excretion.

Designating mannitol as a biomarker for osmotically determined flow raises a question regarding the route(s) of entry. Since mannitol diffuses rapidly into hepatocytes (Schanker and Hogben, 1961), the apical aquaporin 8 channel is a possibility.

A study of the properties of aquaporin 8 states that both mannitol and sucrose are impermeant solutes (Liu et al., 2006). Also, there is abundant evidence that secretin-stimulated hepatic duct flow when "hyperosmolarity" does not occur will not increase mannitol clearance. Therefore, it is reasonable to conclude that mannitol is a biomarker for paracellular flow via claudin 2 into the canalicular conduit and the hepatic ducts. The equatorial diameters of mannitol (6.3A), and PEG-900 (8.3A) (Lane et al., 1996) compared with that of claudin 2 (6.5–8.0A) (Laghaei et al., 2016) further support a paracellular route for mannitol and transcellular route for PEG-900.

Although more studies are needed before PEG-900 can be fully validated as a biomarker for formation and excretion of mixed micelles and mannitol as a biomarker for paracellular water flow, existing studies, not specifically designed to test the hypothesis, are supportive.

A study reported a number of years ago (Fig. 2), in retrospect, also supports the direction of a new paradigm. Using a large variety of naturally occurring bile acids, their effect on hepatic bile flow and erythritol clearance, a lower molecular weight surrogate for mannitol, was determined. It was found that although each intravenously administered bile acid increased hepatic bile flow, erythritol clearance varied widely. Since paracellular water flow was not considered to provide a significant contribution to hepatic bile flow, the investigators suggested that each of the bile acids affected the transcellular permeability of erythritol.

In retrospect, the data fully support the concept of bile acids generating both transcellular and paracellular water flow. Thus, TDC, considered a non–micellar-forming bile acid, caused a parallel increase in erythritol clearance and water flow, implying a paracellular route and in agreement with the increase in mannitol clearance that occurred in human studies(Boyer and Bloomer, 1974).

The increase in water flow with taurocholate caused a progressive fall in erythritol clearance, in keeping with the view that as the micellar aggregates increased water flow was proportionally mostly transcellular and did not contain erythritol.

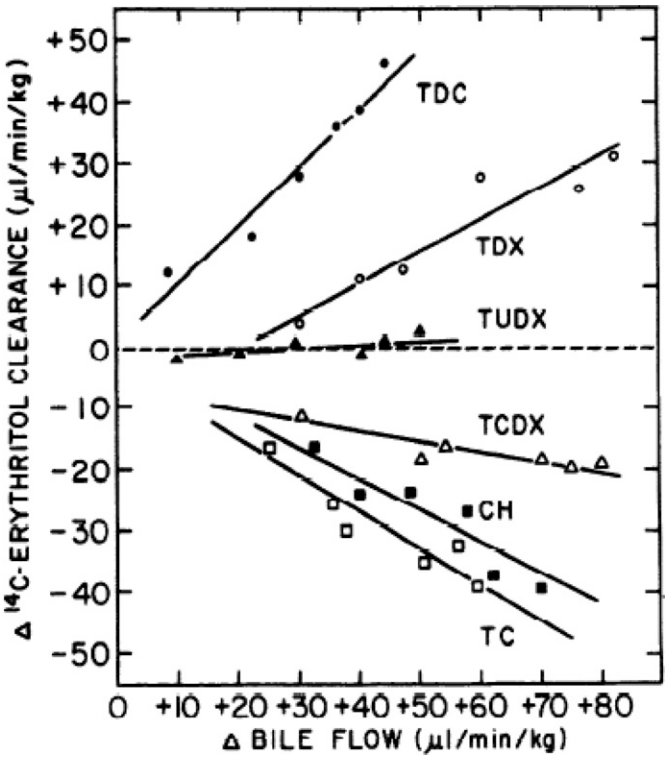

Fig. 2. Bile flow and erythritol clearance after intravenous infusion of bile acids. Erythritol, a lower molecular weight surrogate for mannitol, is a biomarker for water flow. However, it was found that although erythritol clearance increased in parallel with water flow when TDC was infused, it decreased progressively when taurocholate was infused. Initially the findings were attributed to changes in permeability of the membrane to erythritol caused by the different bile acids. Alternatively, with the recognition of paracellular flow via claudin 2 and the relationship of lecithin-cholesterol vesicle secretion to bile acid micelle formation, it is reasonable to propose that total hepatic bile flow for each bile acid is a composite to transcellular and paracellular flow. Erythritol clearance is a biomarker for paracellular flow. Figure reproduced with permission from Tavoloni (1984); copyright 1984 The American Physiologic Society. CH, cholate; TC, taurocholate; TCDX, taurochenodeoxycholate; TDX, taurodeoxycholate; TUDX, tauroursodeoxycholate.

The slopes of the erythritol clearances vary with each of the bile acids administered, indicating that the increase in water flow is a composite of paracellular and transcellular sources. The latter depends on the critical micelle concentration that is different for each of the bile acids.

III. Canals of Hering: Role in Hepatic Bile Formation

Data on possible modifications in the composition of the fluid entering from the canalicular conduit and flowing through the Canals of Hering could not be found. The most recent description is that they are "troughlike structures that arise within the lobule and drain bile from the bile canaliculi into the terminal bile ductule" (Saxena and Theise, 2004). Although the Canals of Hering have been referred to as hepatic preductules and hepatic ducts (Steiner and Carruthers, 1961), clarity is best maintained by considering the Canals of Hering as a "continuum between the hepatocyte canaliculus and the ductules" (Banales et al., 2019).

IV. Hepatic Ductules and Ducts: Role in Hepatic Bile Formation

The expression of the claudin 2 protein in the tight junctional apparatus, the intercellular barrier between cholangiocytes resolves perplexing data regarding the role of the duct system in generating additional water inflow.

The transcellular inflow of water in response to secretin occurs via Aquaporins 4 and 1 in the basolateral and apical membrane of cholangiocytes, respectively, has been established (Marinelli et al., 1997, 1999, 2000).

The puzzle was why in some studies secretin-stimulated hepatic bile flow increased mannitol clearance and in others no change occurred with a decrease in its bile/plasma concentration ratio. Insight into the puzzle was obtained when it was shown in vitro that adding a solution of inorganic electrolytes to a micellar solution of sodium taurocholate, both at the same osmolarity, yielded a solution of greater osmolarity, referred to as "hyperosmolarity" (Hardison and Norman, 1969). The mixture increased the proportion of bile acid monomers in solution. Since osmolarity depends on the number rather than the molecular weight of solutes, the increase is predictable.

This phenomenon was not widely recognized and therefore studies of hepatic bile formation using sodium taurocholate and secretin infusions did not control for the concentration of taurocholate in bile that determines formation of aggregates and their size. Therefore, the effect of the inorganic electrolyte solution added to bile by secretin administration was variable regarding the occurrence of hyperosmolarity. In a study where osmolarity was monitored, a relationship to mannitol clearance was found (Preisig et al., 1962).

Thus, secretin has two different effects on hepatic bile flow: transcellular flow via aquaporins 4 and 1 and a variable hyperosmotic effect that provides additional water and mannitol. This latter inflow is paracellular, probably via claudin 2, since mannitol has very low permeability to aquaporin 1 (Drake et al., 2015).

Cholangiocytes also have carriers for bile acids on their apical and basolateral surfaces.(Ballatori et al., 2005) They can return bile acids to the space of Disse and therefore to hepatocytes. This potential for an additional mechanism for augmenting canalicular water flow, referred to as cholehepatic shunting, is difficult to evaluate quantitatively with respect to normal hepatic bile formation.

V. Synopsis

The Sperber proposal in 1959 that water flow into "bile capillaries" is determined by an osmotic gradient can be updated to a new paradigm that specifies the multiple sites for water entry and identifies biomarkers

that may be useful for quantifying their role. In the canalicular conduit the gradient is generated mostly by bile acid monomers, supplemented by other determinants such as glutathione and occurs mostly via aquaporin 8 and claudin 2. In addition, bile acid aggregates (micelles) promote water entry via lecithin-cholesterol vesicle formation and secretion. No data exist on changes that may occur in canalicular fluid composition as it flows thru the Canals of Hering to hepatic ductules. In the hepatic ductules water inflow is transcellular via aquaporins 4 and 1 in the basolateral and apical membranes of cholangiocytes, respectively, and also paracellular via claudin 2 in response to an increase in osmolarity that can occur when secretin-stimulated flow increases the proportion of monomers derived from bile acid aggregates. The FLAP technique can directly determine rates of water flow in the ducts. Mannitol and PEG-900 are candidates as biomarkers for paracellular and micelle-regulated transcellular water flow, respectively.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Javitt.

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