



Published in final edited form as:

*Compr Physiol.* 2013 July ; 3(3): 1035–1078. doi:10.1002/cphy.c120027.

## Bile Formation and Secretion

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### Abstract

Bile is a unique and vital aqueous secretion of the liver that is formed by the hepatocyte and modified down stream by absorptive and secretory properties of the bile duct epithelium. Approximately 5% of bile consists of organic and inorganic solutes of considerable complexity. The bile-secretory unit consists of a canalicular network which is formed by the apical membrane of adjacent hepatocytes and sealed by tight junctions. The bile canaliculi (~1  $\mu\text{m}$  in diameter) conduct the flow of bile countercurrent to the direction of portal blood flow and connect with the canal of Hering and bile ducts which progressively increase in diameter and complexity prior to the entry of bile into the gallbladder, common bile duct, and intestine. Canalicular bile secretion is determined by both bile salt-dependent and independent transport systems which are localized at the apical membrane of the hepatocyte and largely consist of a series of adenosine triphosphate-binding cassette transport proteins that function as export pumps for bile salts and other organic solutes. These transporters create osmotic gradients within the bile canalicular lumen that provide the driving force for movement of fluid into the lumen via aquaporins. Species vary with respect to the relative amounts of bile salt-dependent and independent canalicular flow and cholangiocyte secretion which is highly regulated by hormones, second messengers, and signal transduction pathways. Most determinants of bile secretion are now characterized at the molecular level in animal models and in man. Genetic mutations serve to illuminate many of their functions.

### Introduction

Bile formation is a unique function of the liver which is vital to survival of the organism. Knowledge of the mechanism of bile formation has progressed rapidly in recent years and has provided the basis for further diagnosis and treatment of cholestatic disorders. Here, we review historical milestones in these developments and summarize current knowledge in this field.

Bile is a complex aqueous secretion that originates from hepatocytes and is modified distally by absorptive and secretory transport systems in the bile duct epithelium. Bile then enters the gallbladder where it is concentrated or is delivered directly to the intestinal lumen. Bile consists of ~95% water in which are dissolved a number of endogenous solid constituents including bile salts, bilirubin phospholipid, cholesterol, amino acids, steroids, enzymes,

porphyrins, vitamins, and heavy metals, as well as exogenous drugs, xenobiotics and environmental toxins (76). Bile serves a number of important functions. (i) Bile is the major excretory route for potentially harmful exogenous lipophilic substances, noted above, as well as other endogenous substrates such as bilirubin and bile salts whose molecular weights are >300 to 500 daltons and not readily filtered or excreted by the kidney. (ii) Bile salts are the major organic solutes in bile and normally function to emulsify dietary fats and facilitate their intestinal absorption. (iii) Bile is the major route for elimination of cholesterol. (iv) Bile protects the organism from enteric infections by excreting immune globulin A (IgA), inflammatory cytokines, and stimulating the innate immune system in the intestine. (v) Bile is an essential component of the cholehepatic and enterohepatic circulation, and finally, (vi) many hormones and pheromones are excreted in bile, and contribute to growth and development of the intestine in some species and provide attractants for the weaning of non-human vertebrates.

The importance of bile secretion to the health of the organism becomes most evident when this secretion is impaired by developmental, genetic or acquired cholestatic diseases. This is most dramatically demonstrated by children born with biliary atresia who develop progressive cholestatic liver injury, biliary cirrhosis, and ultimately liver failure and death.

## Historical Aspects

Although the importance of bile has been recognized since antiquity (60) little was known about the fundamental mechanisms that produced this vital secretion until the middle of the 20th century. Knowledge lagged far behind the understanding of other body fluids such as urine. Indeed because bile is a “hidden” secretion, sampling required a surgical laparotomy and cannulation of the common bile duct. Yet the primary secretion is formed by hepatocytes at the level of the bile canaliculus whose ~1  $\mu\text{m}$  microscopic dimensions are only clearly visualized by electron microscopy. Thus for many years the scientific literature was largely limited to reports of the chemical composition of bile (508). Since there was no ability to sample or assess the primary source of bile, these analyses were the combined result of hepatocyte bile that was modified further by secretory and absorptive properties of the bile duct epithelium (74).

A mechanistic understanding of biliary secretion in the modern era began with earnest with the work of Ralph Brauer and Ivar Sperber. Brauer was a physiologist who worked for the US Navy. Using the isolated perfused rat liver, he demonstrated that bile was secreted against pressures that exceeded the vascular perfusion pressure (90). Thus bile was not formed by hydrostatic filtration as was urine. This landmark study clearly demonstrated that the formation of bile was an energy dependent process, findings that were later confirmed using metabolic poisons that resulted in inhibition of bile production (65, 70).

Sperber was the first to articulate the “osmotic theory of bile formation” in a classic review entitled “Secretion of organic anions in the formation of urine and bile,” published in *Pharmacological Reviews* in 1959 (513). Sperber was a Swedish physiologist who worked at the Royal College of Agriculture in Uppsala. His report was heavily influenced by prior work of many different investigators, particularly early renal physiologists but included his

own studies of the secretion of phenol red in urine and bile in the anesthetized chicken. Sperber observed that when organic solutes, bile acids and other “cholephiles” were injected intravenously, they were concentrated in bile and stimulated bile flow. A few years earlier, in 1953, Pappenheimer had proposed that water could flow across a semipermeable membrane as a result of the creation of osmotic gradients (429). It is reasonable to assume that Sperber was influenced by this publication and concluded that the concentrative transport of solutes in bile created osmotic gradients that then stimulated the passive diffusion of water (and electrolytes) across the semipermeable canalicular membrane into bile (77). This “Milestone” publication, albeit primarily a review of the work of others, established the following paradigm for the emerging field of biliary secretion upon which all further studies have been based.

“It appears quite possible to assume osmotic filtration as a factor in bile formation. The primary event of bile formation would be the active transfer (from cells or through cells of bile acids and possibly other, though quantitatively less important compounds) into the bile capillaries.”

Subsequent investigators began to define the basic mechanisms that generated this secretion. Henry Wheeler and his colleagues (585) were the first to measure the biliary clearance of radiolabeled inert solutes such as erythritol or mannitol. These small molecules were believed to enter bile at the level of the bile canaliculus, either across the tight junction barrier or through rapid transcellular pathways in the hepatocytes. Using these techniques of solute clearances, which were originally adopted from studies of renal clearances, Wheeler and subsequent investigators were able to characterize canalicular bile production and distinguish it from fluid secretion formed more distally by the bile duct epithelium (586). These studies led to the recognition that there were both “bile salt-dependent” [bile salt-dependent bile flow (BSDF)] and “bile salt-independent” [bile salt-independent bile flow (BSIF)] components of hepatocellular canalicular bile formation, and that there were considerable species differences with respect to the relative contribution of fluid secretion from the bile duct epithelium (76). Initially, these solute clearances were performed in dogs and rats with biliary fistulae, but were subsequently used to quantify the components of bile in patients with in-dwelling t-tubes following routine cholecystectomy (80). These studies estimated that man produced ~750mL of bile daily and that ~75% was formed at the level of the bile canaliculus in the adult. Approximately 50% of canalicular bile in man was found to be bile salt-dependent while a variable fraction (~25%) of the daily total production of bile came from the bile ducts in response to the release of secretin induced by meals (80).

At this point, attention was focused on the mechanism of the bile salt-independent component of canalicular bile. There was considerable interest in the role of sodium transport and many biliary physiologists believed that BSIF might be generated by active extrusion of sodium ions into the canalicular lumen. This concept was disproven when histochemical techniques demonstrated that the sodium pump ( $\text{Na}^+, \text{K}^+$ -ATPase) was localized at the basolateral membrane of the hepatocyte (66, 318, 541) similar to other classical epithelia. This milestone discovery indicated that the liver was physiologically similar to other polarized epithelia with respect to the location of specific transport proteins, despite the unique localization of the apical canalicular membrane encircling the hepatocyte

like a belt (71). This finding led to the realization that the inwardly directed sodium gradient, generated by the sodium pump, could be utilized as a secondary driving force when coupled to other solutes and provided a mechanistic explanation for the previously demonstrated dependence of hepatic uptake of conjugated bile salts on the presence of sodium ions (29, 567). Thus, as in other cells, the sodium gradient was coupled to move solutes “up-hill” energetically from plasma to the cell interior.

The ability to separate hepatocyte secretory events from those at the level of the cholangiocyte was greatly enhanced by the recognition in 1981 that some hepatocytes, when isolated from the liver by collagenase perfusion, remained attached and formed couplets or triplets which retained apical polarity between the adjacent cells (423). These canalicular lumens expanded with time in culture and provided a novel *in vitro* model that enabled studies of bile secretion to be made without the confounding effects of blood flow and pressure or the contribution of the bile ducts (177, 178, 196). Electrophysiological studies, including measurements of the transcanalicular membrane electric potential, confirmed that the sodium pump was electrogenic (197) and that together with potassium channels in the basolateral membrane, generated both chemical and electrical driving forces that could be used for transmembrane transport of organic solutes.

At the same time, the localization of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase to the basolateral membrane of the hepatocyte led to its use as a biochemical marker of this membrane domain that facilitated the purification of canalicular membrane subfractions from rat liver (365, 369). It was a significant advance to be able to isolate purified canalicular membrane subfractions that were relatively uncontaminated by the basolateral membrane because the apical canalicular membrane represents only ~10% to 15% of the surface membrane of the hepatocyte. This technical advance then enabled transport functions to be characterized in canalicular membrane vesicles (82), an approach that ultimately led to another major milestone, the recognition that bile salts and other solutes were transported into bile largely by adenosine triphosphate (ATP)-dependent transport mechanisms (3, 393, 411, 524) rather than driven by the cellular electrical potential as originally believed (367, 583).

Cell isolation techniques also led to the isolation of purified populations of cholangiocytes (12, 502, 533, 605) and resulted in the finding that they could form spheroids with enclosed lumens when placed in culture (372). These isolated bile duct units (IBDUs) enabled physiologic studies to be performed that characterized the role of hormones such as secretin, bombesin, vasoactive peptide (VIP), and others, as well as the function of ion transporters, in the generation of bicarbonate secretion from this epithelia (16, 74, 116, 117).

Since the 1990s molecular cloning techniques and cellular expression systems revolutionized our understanding of the mechanisms of bile formation and the molecular causes of cholestasis (554). Today most of the major membrane transport proteins that determine both the hepatic uptake of organic solutes as well as bile salt-dependent and bile salt-independent canalicular and cholangiocyte excretion are now characterized at the molecular level. What follows is a summary of the current state of knowledge in this field based largely on these historical milestones.

## Anatomical Determinants of Bile Secretion

The hepatocyte is a highly polarized cell, whose apical excretory domain represents about 10% to 15% of the cell surface area (Fig. 1A). The remaining major portions of the cell membrane consist of the basal membrane that faces the blood sinusoids and contains many microvilli, and the smooth lateral membrane that lines the intercellular space (Fig. 1B). The bile lumen (canaliculus) is very small (~ 1  $\mu\text{m}$ ) and is formed between two adjacent hepatocytes whose adjoining apical membranes are sealed by tight junctions (zonula occludens) (26) (Fig. 2A). This is the only physical barrier between the blood and the canalicular lumen (Fig. 1B) and as such determines “paracellular permeability” between blood and bile (26). The structure of the tight junction is best visualized in freeze fracture replicas, which reveal a series of four to five cross-linked parallel strands (Fig. 3). These strands are primarily composed of globular proteins known as occludins and claudins (363, 386) (Fig. 2B). Tight junctions hold the hepatocytes together, as well as provide a barrier that prevents bile acids and other large solutes from diffusing from bile, while at the same time allowing the passage of small ions.  $\text{Ca}^{2+}$  is needed to maintain this seal. Claudins and occludins are connected to cytoskeletal proteins (ZO-1 and ZO-2) on the cytoplasmic side of the membrane as part of the tight junction complex. This intercellular barrier is negatively charged which facilitates the passage of small ions, particularly sodium, but is impermeant to molecules the size of proteins. During cholestatic liver injury, the tight junction may be disrupted, resulting in regurgitation of bile contents into the intercellular space and dissipation of the intracanalicular osmotic gradients upon which the secretion of bile depends (72).

The apical canalicular membrane surface area is amplified by many microvilli where a number of transport proteins function to export various constituents from the hepatocyte into bile. Many of these export pumps are ATP dependent and members of the ATP-binding cassette (ABC) super family (407, 554). Unlike a classical epithelial cell, the apical bile canalicular membrane and bile canaliculus encircle each hepatocyte in belt-like fashion, whose interconnections with other cells can be visualized in three dimensions as a “chicken-wire”-like mesh (Fig. 1). Encircling the bile canaliculus on the cytoplasmic submembranous surface are an electron dense array of actin-based microfilaments that are structurally linked to the zonula adherens in the lateral cell membrane and the canalicular microvilli (440,442). Together with myosin-V these actin filaments function both to stabilize the apical pole of the hepatocytes as well as to enable periodic contractions (canalicular peristalsis) (441). Microtubule arrays extend from apically localized organizing centers to submembranous regions of both basolateral and apical plasma membrane domains. Vesicles (10–20 nm) are prominent in subapical regions of the hepatocyte and move across the cell between early (Rab4) and late (Rab11) recycling endosomal compartments attached to microtubules. The myosin-like motor dynein facilitates movement of vesicles toward the minus ends of microtubules at the apical pole, whereas kinesin motors move vesicles toward the positive ends near the basolateral domain (357). Members of the ABC family that reside on the canalicular domain such as the bile salt export pump appear to move from the endoplasmic reticulum (ER) through the Golgi by a direct pathway (248, 279), whereas proteins having glycosylphosphatidylinositol anchors or single transmembrane domains initially target to the

sinusoidal plasma membrane from where they proceed by transcytosis on microtubules to the canalicular domain (240, 481).

Hepatocytes are located within a functional unit of the hepatic lobule where bile is formed countercurrent to the direction of blood flow. Hepatocytes that are present in periportal zones of the lobule are exposed to the highest concentrations of bile salts and so are the primary cells involved in bile salt-dependent bile formation. In contrast, more distal cells in the lobule secrete bile that is comparatively bile salt independent. This results in smaller diameters of the canalicular lumen in central zones of the lobule, which increase in size as they approach the portal tracts (320). There they connect with the initial branches of the biliary tree, the canals of Hering. Once secreted into the biliary tree, biliary solutes are exposed to cholangiocytes that form the lining of the bile duct epithelium in the biliary tree. Cholangiocytes are classic epithelial cells but are highly heterogeneous both in structure and function (12, 54). Cells in the most proximal ducts are small and cuboidal, while their cytoplasmic/nuclear ratios and size increase in more distal bile ducts. The biliary tree contains ~12 branches from the common bile duct to form the right and left hepatic ducts, which branch to form the segmental and area ducts and, finally, the small septal ducts (>100  $\mu\text{mol/L}$  diameter), the smaller interlobular ducts (15–100  $\mu\text{mol/L}$ ), the ductules (<15  $\mu\text{mol/L}$ ), and the Canals of Hering that drain the bile from the bile canaliculi (54) (see Fig. 9). Transport proteins at the luminal apical membrane of cholangiocytes function to both secrete a bicarbonate-enriched fluid as well as to reabsorb fluid and other solutes from the primary secretion of hepatocytes (74,261,535). The final secretory product flows to the gallbladder and intestine. Considerable species differences exist with respect to the function of cholangiocytes in modifying hepatocyte bile (74, 262).

The hepatic artery is the primary blood supply to the bile duct epithelium and surrounds the biliary branches with an anastomosing plexus. While there are variations in cholangiocyte arterial anatomy between species, blood flow in this plexus appears to be directed from small to larger bile duct segments with occasional anastomotic connections with the portal vein vasculature. In contrast, the portal vein provides the primary source of blood for the hepatic parenchymal cells via the hepatic sinusoids. This structural arrangement is thought to provide for a “cholehepatic” circulation, which may allow solutes that are removed from bile by the biliary epithelium to return to the portal circulation for reuptake by the hepatocyte for metabolic use or reexcretion into bile (149). The hypercholeretic properties of Nor-ursodeoxycholic acid are thought to depend on this cholehepatic circulation (599).

Ekataksin has described the hepatic microcirculatory subunit as a cone-like structure where there are fewer sinusoids and parenchymal cells in the distal pericentral zones of the lobule than in the more proximal periportal regions (149). The functional consequence of this anatomical arrangement would restrict the hepatic distribution of solutes that reach this distal pericentral region of the lobule, so that they are more likely to pass through to the systemic circulation. This arrangement may explain in part why canalicular bile stasis is observed predominantly in pericentral regions of the lobule in cholestatic liver injury (149).



## Components of Bile

A general assessment of the major constituents of bile are listed in Table 1, recognizing that considerable species variations exist. Bile is a highly complex secretion which is aqueous in nature and contains less than 5% solid contents in most species (86). Bile cannot be sampled at its origin for obvious anatomical reasons but is isoosmotic with respect to plasma when collected from the bile ducts. Bile consists of a variety of organic and inorganic solutes. The latter consist primarily of passively secreted ions whose concentrations in bile are similar for the most part to those in plasma.  $\text{HCO}_3^-$  concentrations may be greater in bile than plasma in certain species such as the guinea pig or postprandially as a result of enteric hormones like secretin that stimulate  $\text{HCO}_3^-$  and fluid secretion from the bile duct epithelium.

In contrast, organic anions and cations are highly concentrated in bile by active transport mechanisms at the canalicular membrane. The most prevalent organic solutes in bile in all vertebrates are bile salts. The chemistry of bile salts varies greatly among species. Bile salts are 24 carbon water soluble products of cholesterol metabolism. Two primary bile salts are synthesized in mammalian liver: cholic acid, a trihydroxylated bile salt, and chenodeoxycholic acid (CDCA), a dihydroxy bile salt. Each can be conjugated at the side chain with either taurine or glycine. Conjugation metabolizes bile salts into stronger acids which limits their passive reabsorption as they pass down the biliary tree. Intestinal bacteria then produce “secondary bile acids” by converting cholic acid to deoxycholic acid (two hydroxyl groups), while CDCA is metabolized to lithocholic acid, a monohydroxy bile salt. Most of these conjugated bile salts as well as bile salts that are deconjugated by intestinal bacteria are reabsorbed in the distal intestine and undergo an enterohepatic circulation that maintains the bile acid pool. Thus, at least 12 major conjugated primary and secondary bile salt species are contained in human bile, although primary bile salts are usually predominant (468). Secondary bile salts are characteristic of the bile of rabbits. Murine bile contains significant amounts of the more hydrophilic muricholic acid. See Ref. (233) for more details regarding bile acid metabolism. Some lower vertebrates contain 27 carbon bile alcohols where the side chain is hydroxylated and subsequently sulfated. A more detailed discussion of the evolutionary development of bile acids can be found in Refs. (213–215, 234).

The transport of bile salts from the liver into the bile canaliculus provides the major osmotic driving force for BSDF. Once excreted into the canaliculus they are rapidly incorporated into mixed micelles consisting of bile salts, phospholipid and cholesterol. Phosphatidylcholine (PC) is the major phospholipid in bile while cholesterol is the predominant sterol. Very little neutral lipids (diglycerides, triglycerides), or acidic lipids (fatty acids) are found in bile. Bile is the major route for elimination of cholesterol from the body. The formation of micelles acts to maintain cholesterol in solution while at the same time lowering the free (intermicelle) bile salt concentrations. The ability to form micelles greatly reduces the toxic detergent effects of bile salts on the biliary epithelium. This property is best demonstrated in cases where the liver is injured when PC is absent, as in patients with *MDR3* mutations (131) and in *Mdr2* knockout mice (150). In the intestine, the detergent properties of bile salts facilitate lipid absorption.

Glutathione (GSH) and oxidized GSH (GSSG), as well as GSH conjugates are other prominent organic solutes in bile (43, 49). They are transported into bile by the multidrug resistance protein 2 (MRP2) and are largely responsible for the secretion of canalicular BSIF in most species (47). Bile is the major route of excretion of bilirubin. Bilirubin must first be conjugated with glucuronic acid by uridine glucuronyl transferase in the liver microsomes to form bilirubin mono and diglucuronide conjugates before it can be excreted into the bile by MRP2. Only trace amounts of the highly water insoluble unconjugated bilirubin are present in bile, probably as a result of nonenzymatic hydrolysis of bilirubin monoglucuronide or the result of bacterial glucuronidase enzymes.

Organic cations are also excreted into bile, mostly by the multidrug resistance protein 1 (MDR1) or P-glycoprotein. Many organic cations are drugs and xenobiotics (371, 394). Bile is also the major route of excretion of trace metals, particularly arsenic, copper, manganese, lead, mercury, selenium, silver, and zinc (38, 138, 283). However, for the most part, the mechanisms by which these metals are excreted into bile remain unclear as discussed later (38).

Many proteins are present in bile, but the very high concentrations of interfering substances, particularly lipids and bile salts, and bile's inaccessibility has made proteomic analysis very difficult. This has limited the number of studies and exploration of the bile proteome is still in its infancy. Recent studies have identified over 2500 individual proteins in normal human bile (50, 295). Primary interest in proteomics is related to clinically relevant biomarkers, particularly those important in hepatobiliary cancers (154). Most proteins in the bile appear to come from plasma as emphasized by the relative paucity of proteins in bile of the perfused rat liver. Blood to bile transfer occurs by simple diffusion across the semipermeable tight junction paracellular pathway. The inverse ratio between molecular size of proteins found in bile and their bile-plasma ratio supports these conclusions (316, 451). Four proteins, namely secretory IgA, IgM, hemoglobin, and ceruloplasmin, deviate from this predicted relationship and presumably enter bile as content of intracellular vesicles that fuse with the canalicular membrane. Proteins that are relatively abundant in bile include albumin, hemoglobin/haptoglobin complexes, and apotransferrin. These enter bile via exocytosis of vesicles at the canalicular membrane. IgA is another major protein in bile and contributes to immunological surveillance within the biliary system. Fibroblast growth factor 19 (FGF19), a major inhibitor of bile acid synthesis, is another protein that is present in bile in high concentration compared to plasma. Human gallbladder bile contains ~ 100-fold higher concentrations of FGF19 than plasma and gallbladder epithelia (613). Explants of gallbladder secrete 500× higher amounts of FGF19 compared to ileal explants (613). FGF19 is also synthesized in human liver where it regulates bile salt synthesis. However, the reason for such high concentrations of FGF19 in bile is not clear (613). Many enzymes are also detectable in bile and originate primarily from lysosomes and the canalicular membrane. Lysosomal enzymes enter bile via exocytosis. Canalicular membrane ectoenzymes are also found in bile, presumably related to the detergent effects of bile salts, and represent 2% to 3% of their total content in rat liver (187). Other intracellular enzymes are only detected in bile in trace amounts.



Peptides and amino acids are also found in bile (316). Glycine, cysteine, and glutamic acid are formed in bile from enzymatic breakdown of the tripeptide GSH by  $\gamma$ -glutamyl transpeptidase and dipeptidases located on the luminal membranes of the bile canaliculus and bile duct epithelia (43, 44). These individual amino acids are partially reclaimed from the bile by specific amino acid transporters that also line the luminal membrane of the bile duct epithelium (46, 388). Leukotrienes and their metabolites and other inflammatory cytokines, including tumor necrosis factor (TNF)- $\alpha$  (which is also synthesized in bile duct epithelial cells), appear in bile (398, 453, 464).

Bile also delivers vitamins to the intestine. The vitamin D metabolite, 25-hydroxyvitamin D, is first formed in the hepatocytes by CYP2R1 (112, 113). These metabolites may function in intestinal growth and development in the newborn and in calcium homeostasis in the adult. Folic acid, pyridoxine, and transcobalamin also enter the intestine via the bile. Steroid hormones, estrogens, prolactin, and insulin are other important substances excreted in bile. Bile is also the pathway for excretion of pheromones which play an important role as sexual attractants and suckling in non-human mammals and lower vertebrates, particularly teleost fish and lamprey where bile acid metabolites are thought to account for this function (182, 336). Bile is also the route of excretion of water-insoluble porphyrins, particularly protoporphyrin and coproporphyrin (67, 340). For some solutes, such as glucose and phosphate, efficient transport systems on the bile duct epithelium function to reabsorb these bile components prior to delivery to the intestine. These solutes can be detected in bile when the transport systems are impaired during bile duct injury or when supraphysiologic amounts of the substrate are infused (205, 421).

## Mechanisms of Bile Formation

Bile is formed by filtration in response to osmotic gradients created by the transport of osmotically active solutes into the bile canaliculus lumen. Water and small solutes enter the biliary space passively via solvent drag (514). This process requires energy in the form of ATP, and is not affected by the hydrostatic pressure that comes from blood perfusing the hepatic sinusoids (90).

## Determination of BSDF and BSIF

Figure 4 illustrates the graphic expression of the three major sources of bile. As bile salt excretion increases, bile flow is stimulated. In these types of experiments, BSDF is estimated by extrapolating the linear relationship of bile salt excretion and bile flow to the ordinate since it is not possible to completely deplete bile of bile salts. The magnitude of BSIF is defined where this line intercepts the ordinate while the slope of this line reflects the choleric potential of the bile salt(s). Canaliculus BSIF is estimated by plotting the relationship between bile salt excretion and the clearance of radiolabeled erythritol or mannitol. The linear relationship between bile flow or solute clearance and bile salts will be considerably distorted when choleric agents are infused and bile flow rates are rapidly changing. This is because the collected samples may each have different delays in transit time in the biliary dead space between the bile canaliculus and the end of the biliary cannula where bile salts and the radiolabeled solute are collected. Corrections that estimate the mean transit

times for a given sample of bile are required for these relationships to be more accurately expressed [see Ref. (80) for a more detailed treatment of this issue].

### Pathway for water entry to bile

Water can enter the bile canaliculus through transcellular or paracellular pathways (351). Although it has been difficult to accurately quantitate the contributions of these two sources of biliary fluid, it is likely that the majority of water enters bile across the cells. Both the sinusoidal and canalicular plasma membranes of the hepatocyte are permeable to water presumably through aquaporin (AQP) water channels (4). AQP8, a ~34 kDa protein, has been identified on the canalicular membrane in rat, mouse, and human hepatocytes, while AQP9, a ~32 kDa protein is located at the hepatocyte sinusoidal membrane in rat, mouse, pig, and human (174, 351). While direct evidence for the role of these water channels in the formation of bile remains unclear, AQPs clearly alter water permeability in *in vitro* studies in the liver (174) and are down regulated in animal models of cholestasis (327). Whether they play a role in human cholestatic disorders is not known (174, 475). Although the tight junctions between hepatocytes are classified as “leaky,” their surface area is limited compared to the canalicular membrane. The paracellular pathway is negatively charged and serves primarily for the entry of Na<sup>+</sup> ions and small cations to maintain electroneutrality (89).

A third and highly variable component of bile comes from the bile duct epithelium. In rodents, it comprises less than 10% of total bile production whereas in humans, it may account for as much as a third of daily bile production. Secretion from cholangiocytes is stimulated by hormones released in the small intestine in responses to meals.

### Determination of canalicular bile flow

To determine the fraction of bile that is derived from the hepatocytes, the concept of solute clearance was adopted from renal physiologists whereby biliary clearances could be calculated using nontransported, nonmetabolizable radiolabeled solutes such as erythritol or mannitol (586). This technique assumes that these small molecules gain entrance to bile primarily, if not entirely, at the level of the bile canaliculus. This assumption must be validated by demonstrating that the biliary clearance of a given solute remains unchanged when cholangiocyte bile is stimulated by hormones such as secretin (80, 584, 585). With acceptance of this caveat, estimates of the three components of bile can be made (Table 2).

### Species variations in bile secretion

The total amounts and relative contributions of canalicular BSDF and BSIF and cholangiocyte secretions vary considerably among species (151, 586) (Table 3 and Figure 5). Rats produce ~ 100  $\mu\text{L}/\text{g liver}/\text{h}$  almost entirely from hepatocytes. Humans produce an average of ~17  $\mu\text{L}/\text{g liver}/\text{h}$  of bile each day. In contrast, bile flow in marine elasmobranchs, primitive vertebrates, is 100 $\times$  slower than in rodents when expressed per g of liver (84, 85). The choleric potential of a bile acid is represented by the slope of the BSDF rate in Figures 4 and 5 as microliters of bile produced per  $\mu\text{mole}$  of excreted bile salt (438). As illustrated in Table 3, in the rat taurocholate has a choleric potential of 8 to 15  $\mu\text{L bile}/\mu\text{mole bile salt}$  compared with 30 to 40  $\mu\text{L}/\mu\text{mole}$  for taurodehydrocholate (81) and more than 40  $\mu\text{L}/$

$\mu$ mole for ursodeoxycholic acid (148). Taurodehydrocholate does not form micelles and therefore has a greater osmotic activity per  $\mu$ mole of excreted bile salt than taurocholate, whose critical micellar concentration is about 6 mmol/L. However Nor-ursodeoxycholic acid, a C-23 bile acid has the highest recorded choleric activity based on its ability to undergo cholehepatic recycling (599).

Studies of the source of bile flow in humans are derived from patients in whom indwelling biliary cannulas have permitted estimates (80,447). These studies suggest that canalicular bile production is divided evenly between BSDF ( $\sim$  250 mL/24 h) and BSIF ( $\sim$  250 mL/24 h), whereas another third ( $\sim$  250 mL/24 h) comes from the bile duct epithelium (80). In general, species that are intermittent feeders have a larger fraction of bile secreted from the biliary epithelium than those that feed continuously.

### Nonosmotic choleric effects of bile acids

In addition to their osmotic properties, which are related to the chemical structure of the particular bile salt that determines its critical micellar concentration (204, 599), bile salts also have other biologic effects that can affect BSDF and BSIF: (i) bile salts may affect permeability properties of the canalicular membrane or tight junctions. Experiments in the rat and rhesus monkey show that bile salts can modify the canalicular fraction of BSIF (36). In the partially bile duct ligated rat, the increased flux of bile salts through the unobstructed lobes significantly increases the magnitude of BSIF. In the rhesus monkey, changes in the relative order of different rates of taurocholate infusions affect the magnitude of canalicular BSIF such that high rates of infusion followed by low rates result in a larger BSIF than when the order is reversed. Increases in  $^{14}\text{C}$ -Inulin clearances suggest that permeability in the bile canalicular membrane or paracellular pathway may account for these changes (36, 75). (ii) Acute exposure of rats to taurochenodeoxycholic acid, a dihydroxy, more hydrophobic bile acid, increased recovery of  $^3\text{H}$ -methoxyinulin and the ectoenzymes 5'-nucleotidase and phosphodiesterase in subcellular vesicles from liver tissue and bile, consistent with stimulating the rates of vesicle transport into bile (341). (iii) Bile salts can stimulate the targeting of vesicles and transporters to the canalicular domain (278, 280) and increase the permeability of the tight junction barrier. As the flux of bile salts increases postprandially or with exogenous infusions, hepatocytes are recruited for secretion in more distal regions of the hepatic lobule (320). (iv) Structural changes in the bile canaliculus consisting of increases in canalicular side branching and redundancy of canalicular microvilli have also been observed with freeze fracture replicas after chronic infusion of taurocholate in rats (320). Tubulovesicular transformation can be induced by bile salts in the pericanalicular area. These changes may also provide a partial explanation for bile salt induced changes in the transport maximum for other organic anions including BSP and bilirubin (477). (v) Finally, signal transduction pathways involving calcium signaling induced by bile salts including tauroursodeoxycholate (TUDCA), taurochenodeoxycholate (TCDCA), and tauroolithocholate (TLC) can mobilize intracellular calcium stores and induce calcium oscillations (62). TUDCA induces hepatocellular exocytosis, an effect dependent on calcium (63). Pericanalicular calcium signaling mediated by InsP3R2 plays an important role in maintaining bile salt secretion through posttranslational regulation of the bile salt transporter, Bsep, as observed in rat hepatocyte sandwich cultures (296).

Ursodeoxycholic acid is also “hypercholeric” compared with the more dominant endogenous rat bile salt, taurocholate, presumably because it is a weak acid with a higher pK of about 5 (458). At this pK, ursodeoxycholic acid will be protonated in bile, allowing for back diffusion into the bile duct epithelium (534). That physical property may allow this bile salt to undergo a “cholehepatic” circulation so that a certain fraction recycles to the hepatocyte (599). This effect results in a higher apparent choleric activity than a bile salt such as taurocholate, whose pK is about 2. Very little taurocholate is likely to be protonated at the alkaline pH of bile, minimizing its back diffusion so that the majority of this bile salt is excreted directly. Bile salt uptake and efflux transporters, Asbt and Ost $\alpha$ -Ost $\beta$ , are also expressed on the luminal and basolateral membranes, respectively, in cholangiocytes and gallbladder epithelium so that transporter-mediated shunting may also occur (132, 590). Table 3 compares the choleric potential of several different bile salts in different species.

## Cellular and Molecular Determinants of Bile Formation

In the last two decades, developments in the fields of cell and molecular biology have resulted in major advances in the understanding of the determinants of bile formation. This section discusses the major properties and functional role of each of these transport proteins and enzymes that contribute to this secretion beginning with mechanisms of hepatic uptake (phase 0), followed by a brief description of intracellular events including phase I and phase II metabolism and then focusing on export pumps (phase III) at the canalicular as well as basolateral domain of the hepatocyte.

### The hepatocyte

The major transport systems and enzymes that are expressed in the hepatocyte and contribute to the formation of canalicular bile are listed in Table 4. The location of the membrane transporters is illustrated in Figure 6. Most of these proteins have been characterized at the molecular level from human liver and their functional properties determined in both *in vitro* and *in vivo* preparations (76, 152, 553, 554). These transport proteins and enzymes are commonly divided into four main functional groups or “phases,” designated as Phase 0, I, II, and III (78) (Fig. 7). Phase 0 consists of hepatic uptake mechanisms located on the basolateral membrane of the hepatocyte that determine how organic solutes enter the liver. Phase I functions are carried out primarily by intracellular cytochrome P<sub>450</sub> enzymes that metabolize (hydroxylate) lipid soluble substrates to facilitate phase II reactions. Phase II reactions consist of enzymes that conjugate many organic compounds with sulfate, glucuronides, GSH or acetyl groups to increase their aqueous solubility and enable these substances to be substrates for phase III transporters. Phase III transporters are export pumps that function to extrude these solutes out of the hepatocytes into bile or back into the systemic circulation. For the purpose of this review on bile formation, the focus will be primarily on Phase 0 and Phase III transporters.

### Phase 0 organic solute uptake by basolateral membrane transporters

Transport systems at the basolateral, sinusoidal domain of the hepatocyte facilitate the selective uptake of various albumin-bound organic anions, cations, and neutral compounds that are excreted into bile.

**Sodium-taurocholate cotransporting polypeptide (SLC10A/Slc10a)**—The sodium-taurocholate cotransporting polypeptide is the primary mechanism for the hepatic uptake of conjugated bile salts from sinusoidal blood. Sodium-taurocholate cotransporting polypeptide (NTCP/Ntcp) was the first member of the SLC10 family to be identified at the molecular level using expression cloning technologies with *Xenopus* oocytes (207, 212). NTCP is a sodium coupled transporter that derives its energy from the sodium gradient maintained by the ATP-dependent sodium pump, Na<sup>+</sup>/K<sup>+</sup>-ATPase. The sodium pump is also localized to the basolateral membrane and is expressed throughout the hepatic lobule (66, 318, 541). The sodium pump establishes two important driving forces for hepatic uptake transport mechanisms: (1) an inwardly directed chemical gradient for Na<sup>+</sup>, where 3 Na<sup>+</sup> ions are exchanged for 2 K<sup>+</sup> ions, resulting in intracellular concentrations of these cations of about 15 and 130 mEq per liter, respectively, as well as (2) an intracellular negative electrical gradient of about -35 to -40 meV (197). The outward conductance of K<sup>+</sup> via K<sup>+</sup> channels in the basolateral membrane further contributes to the regulation of this negative membrane potential. Together, the chemical and electrical gradients produced by the sodium pump are able to move solutes into the hepatocyte against their concentration gradients, a process known as secondary active transport.

NTCP is a prototypical secondary active transporter. During fasting, the concentrations of bile salts in sinusoidal blood are approximately 10 to 20 μmol/L, but rise significantly in response to stimulation of the enterohepatic circulation of bile salts by feeding. This transporter has a high affinity for bile salts: the  $K_m$  for human and rat isoforms are ~6 and 25 μmol/L, respectively, as originally expressed in oocytes (212) but vary considerably in subsequent published reports (521). Bile acid uptake by NTCP results in a high first-pass clearance so that little passes into the systemic circulation. However during feeding, the flux of bile salts from the intestine increases in the portal and sinusoidal circulation resulting in recruitment of hepatocytes for bile acid uptake in pericentral as well as periportal zones of the liver lobule and resulting in increases in the concentration of bile salts spilling over into the systemic circulation (101). NTCP/Ntcps belong to the SLC10 family of Na<sup>+</sup>-solute symporters, which also include sodium-dependent bile salt transporters in the ileum (124, 589), renal proximal convoluted tubule (124), and apical membrane of bile duct epithelial cells (321). The human liver NTCP is a 349-amino-acid, 50-kD glycoprotein with seven transmembrane spanning domains, while rat liver Ntcp is a 362-amino-acid, 51-kD glycoprotein. Ntcps are expressed only in mammalian hepatocytes and is first detected in developing rat liver immediately prior to birth (83). Small amounts of mRNA have been detected in rodent kidney, pancreatic acinar cells, and placenta but their functional significance is not clear. Substrate specificity is primarily limited to bile salts, particularly conjugated bile salts, but can accommodate some drugs like the statin, rosuvastatin, as well as estrone-3-SO<sub>4</sub> and bromosulfophathalein (521). Ntcps account for most if not all of the sodium-dependent hepatic uptake of conjugated bile salts. Although microsomal epoxide hydrolase has been proposed as an alternative sodium dependent bile acid uptake transporter (570) the elimination of uptake of bile salts in liver mRNA injected *Xenopus* oocytes by antisense oligonucleotides to Ntcp suggests otherwise (211).

NTCP expression is regulated in several physiologic conditions including pregnancy (33) and cholestasis (175). Retinoids stimulate expression by binding to retinoid-X nuclear receptor/retinoic acid receptor (RXR/RAR) in the promoter region of the rodent liver *Ntcp* gene that also contains a TATA element, a hepatocyte nuclear factor-binding site (HNF1), and a response element for cytokines (264). Bile salts regulate the expression of *Ntcp* indirectly via the nuclear receptor farnesoid X-receptor (Fxr) which in turn stimulates expression of the short heterodimer protein, Shp, which in turn blocks the effects of RXR/RAR binding to the *Ntcp* promoter (137). Bile salts may also affect the function of HNF1 (255). *Ntcp* expression can be rapidly upregulated posttranscriptionally by cyclic adenosine monophosphate (cAMP) (392) which stimulates insertion of vesicles containing *Ntcp* into the plasma membrane (27, 28). *Ntcp* is also downregulated by a number of other conditions including estrogens (499), bile duct ligation (175), endotoxin (200, 389, 587), and pregnancy (1, 33). Prolactin, by contrast, upregulates *Ntcp* transcription by a Stat-5-dependent tyrosine phosphorylation signal transduction pathway (172, 173), despite its downregulation during pregnancy. Endotoxin downregulates transcription by activating cytokine production which diminishes the activity of several other transcription factors including HNF1 and the RXR and its heterodimeric partner, RAR (136). For more details on transcriptional and posttranscriptional regulation of NTCP see Ref. (13).

**Organic anion transporting polypeptides (superfamily SLCO) (formerly SLC21A, the OATP/Oatps)**—Unlike conjugated bile salts, unconjugated bile salts are taken up by the liver at the hepatocyte basolateral sinusoidal membrane by sodium-independent mechanisms on carriers from the SLCO superfamily, originally known as organic anion transporting polypeptides or organic anion transporting polypeptides (OATPs) (418, 469). OATP/Oatps have broad substrate specificity and facilitate sodium-independent hepatic uptake of many different organic amphipathic compounds without respect to structure or electrical charge that are primarily large hydrophilic organic anions (206, 208, 209). The OATP/Oatps are predicted to have 12 transmembrane domains with intracellular amino and carboxy termini. OATP/Oatps function as electroneutral anion exchangers and are thought to transport bile salts and other solutes in exchange for intracellular anions such as GSH (333, 334) and bicarbonate (479), although more study is needed to confirm these data. There are eleven human OATPs that are divided into six families (OATP1–OATP6). Four OATPs have been functionally identified in human liver and many are also expressed in other tissues. The four OATPs that have been functionally identified in human liver include OATP1A2, formerly OATPA, gene symbol *SLCO1A2*; OATP1B1, formerly OATPC, gene symbol *SLCO1B1*; OATP1B3, formerly OATP8, gene symbol *SLCO1B3*; and OATP2B1, formerly OATPB, gene symbol *SLCO2B1*. OATP1B1 and 1B3 are hepatocyte specific and functionally the most important (210, 300, 418). OATP1B1 is expressed on the basolateral membrane throughout the hepatic lobule in human liver while OATP1B3 is most strongly expressed in pericentral regions of the hepatic lobule. In addition to endogenous substrates (examples include bile salts, thyroid hormones, conjugated steroid hormones, prostaglandins, and bilirubin, as well as cyclic and linear peptides) the OATP/Oatps transport a wide variety of xenobiotics and environmental toxins. Most OATP/Oatp substrates are organic anions with molecular weights greater than 300, but OATP/Oatps also transport cations and neutral compounds. Polymorphisms in OATP1B1 account for



differences in clearance of drugs, and mutations in this OATP have been described in patients with pravastatin induced rhabdomyolysis (347, 377). Polymorphisms in OATP1A2 have been associated with severe reactions to methotrexate (35) while common polymorphisms of OATP1B1 can affect the disposition of irinotecan metabolites resulting in adverse gastrointestinal effects of this drug (415).

In human liver, OATP1A2 is expressed only in cholangiocytes. Its membrane localization remains unclear but it is thought to function in the reabsorption of xenobiotics excreted into bile (326). Table 5 lists some of the more common substrates transported by these OATPs (418). Also see several recent reviews for a more comprehensive discussion of OATP1B1 (418, 540).

OATP1B3 transports unconjugated bilirubin from the blood to the liver and variants have been described in patients with neonatal jaundice (242). Conjugated bilirubin is extruded into bile but also back into the sinusoids by MRP3 (564).

*OATP/Oatps* can be regulated by transcription factors and their expression in rodents is reduced in HNF1 $\alpha$  and HNF4 $\alpha$  knockout mice (226, 493). Human *OATP1B1* is also transactivated by HNF1 (254) while *OATP1B3* is downregulated by HNF3 $\beta$  (569) and positively regulated by FXR which binds to its promoter (256). OATPs are differentially regulated in human cholestasis: *OATP1B1* is down regulated in Sclerosing cholangitis (424). OATP1B3 is upregulated by bile salts (210, 256), while *OATP1A2* is unchanged (424). [see Ref. (540) for more details on regulation of OATP/Oatps].

**Organic anion transporters OAT/Oat (SLC22 superfamily)**—In contrast to the OATPs, OATs (*SLC22A*) transport smaller and usually hydrophilic organic anions. Like OATPs, OATs are 12 membrane spanning domain proteins that are widely expressed. Oat2 is most highly expressed in liver based on rodent data (469). OAT2 is exclusively expressed in the liver on the basolateral membrane where it exchanges short chain fatty acids for sulfated conjugates (495). Human OAT2 transcription is dependent on HNF-4 $\alpha$  and is downregulated by bile salts via the FXR, short heterodimer partner (SHP) inhibitory pathway (445).

**Organic cation transporters OCT1/Oct1; (SLC22A1/Slc22a1) and OCT3/Oct3 (SLC22A3/Slc22a3)**—The OCTs are 12 membrane spanning domain electrogenic cation transporters that transport a wide variety of endogenous and exogenous structurally unrelated small organic cations by bidirectional passive facilitated diffusion via their electrochemical gradients (288, 469). OCT1 and OCT3 are expressed in human liver on the hepatocyte basolateral membrane. OCT1 was originally cloned from rat kidney, but was found to be expressed primarily in hepatocytes (100, 202). OCT1 is a 556-amino-acid, 95-kD protein that facilitates the hepatic uptake of small (type I) organic cations including tetraethylammonium, choline, *N*-methyl-4,5-phenylpyridine and a number of cationic drugs, including antiviral drugs and metformin [see Ref. for a list of substrates (409)]. The expression of OCT1 and 3 are highly variable. Human OCT1 expression is ~15-fold greater than the expression of OCT3 (410). Their substrates largely overlap although endogenous substrates that are specific for OCT3 include adrenalin, noradrenalin and histamine. *OCT1*

transcription is activated by HNF-4 $\alpha$  and can be inhibited by SHP (476). OCT proteins contain phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC) but how this affects expression in the liver is not known. Genetic variants of *OCT1* (R61C, G401S, 420del, or G465R) delay the uptake of metformin (498). Variants of *OCT3* influence oxaliplatin and lamivudine clearance (257, 381). Cholestasis markedly down regulates both human and rodent Oct1 and 3 expression (135, 410).

### Transcellular bile acid and organic solute transport and metabolism

Following uptake into the hepatocyte, organic solutes that ultimately are excreted into bile must move across the cell to the apical membrane. This can occur by rapid diffusion in association with cytosolic proteins or in association with intracellular membranes (particularly the ER) or by transcytosis in membrane vesicles associated with microtubules. The hydrophobicity of the compound influences which pathway the solute will take. The more hydrophobic the solute the more it will associate with intracellular membranes rather than the cytosolic proteins (30, 225).

After transport into the hepatocyte, bile salts and other hydrophilic anionic substances bind to cytosolic proteins, such as 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD) (525, 526), GSH *S*-transferase (539), and liver fatty acid binding protein (L-FABP) (358) and then rapidly diffuse to the canalicular membrane for excretion. 3 $\alpha$ -HSD is the most important cytosolic binding protein for bile salts. Indomethacin competes with bile salts for binding to HSD causing bile salts to efflux from hepatocytes (526). In contrast, more lipophilic substrates may associate with intracellular membranes and undergo phase II metabolism before excretion, while organic cations can become sequestered within negatively charged lysosomal structures thus delaying their excretion into bile (225). HNF1 regulates both 3 $\alpha$ -HSD and L-FABP expression (493) and both of these proteins are induced by bile salts via FXR (500).

Unconjugated bile salts such as cholic acid first shuttle through peroxisomes where conjugation takes place with the amino acids taurine and glycine (450). A second, more difficult to establish mechanism is diffusion within the lipid bilayer of intracellular membranes and/or vesicles. This presumably occurs primarily in the ER or endosomal membrane compartments. Highly hydrophobic solutes, such as bilirubin, likely follow this pathway prior to conjugation with glucuronic acid which occurs enzymatically within the cisterna of the ER (611, 612). A third mechanism is microtubule-dependent vesicular transcytosis (126, 127) which is how bulky organic solutes (e.g., class II cations) and proteins such as transferrin, asialoglycoproteins, and polymeric immune globulin A (pIgA) move across the cell to the apical membrane (512). The role of intracellular vesicle transport of bile salts remains controversial, as does a 35 kD bile salt-binding protein identified from human liver (368, 525).

IgA is a well-studied example of a protein that crosses the cell (hepatocytes in the rat and bile duct epithelia in the human) via microtubular-dependent vesicular transcytosis (236, 543). In rodents, pIgA binds to its receptor, secretory component, and is taken up by endocytosis at the basolateral domain of the hepatocyte. Vesicles containing the receptor and the bound ligand then fuse with the early endosomal compartment before budding off in

transcytotic vesicles attached to the positive ends of microtubules in association with the molecular motor, dynein, a myosin-like ATPase. The IgA containing vesicle then moves toward the negative end of the microtubules to the subapical region of the hepatocyte where it fuses with the subapical endosomal compartment. Here the vesicle is associated with a small guanosine-5'-triphosphate (GTP)-binding protein, Rab3D, that regulates specific steps in intracellular trafficking (315, 349). pIgA is then sorted to the canalicular membrane where a protease cleaves secretory component into two smaller fragments (509). The larger 80-kd fragment enters bile together with pIgA. The process of vesicular transcytosis, fusion, and exocytosis is highly regulated by second messengers and by a family of small molecular weight, Ras-like GTP-binding proteins and other members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein complex that determine the specificity of vesicle budding and sorting in cells (470, 537). The molecular details of these processes still remain unclear in hepatocytes but are likely to be similar to those in yeast and nerve synaptic terminals (243, 401).

Canalicular membrane proteins that are glycosylphosphatidylinositol (GPI)-anchored proteins (GPI linkages) are also targeted to the canalicular domain by transcytotic vesicle transport mechanisms (51, 240, 559). These proteins are sorted in vesicles via an indirect route to the basolateral domain following synthesis in the ER; they are then resorted to the canalicular membrane via the microtubule-dependent transcytotic pathway, which is regulated by various protein kinases and phosphatases (558, 559). During cholestatic liver injury, vesicles containing canalicular transport proteins accumulate within the pericanalicular cytoplasm, reflecting the diminished bile excretory capacity (51, 314). In contrast, ABC bile export pumps traffic to the canalicular membrane from the Golgi through direct pathways via an apical submembranous, late endosomal compartment associated with Rab11a (573).

### **Intracellular metabolism of bile salts and other organic anions**

As illustrated in Figure 7, bile salts, particularly hydrophobic potentially toxic secondary bile salts (lithocholate and deoxycholate), and other lipid soluble organic anions like bilirubin and drugs may undergo phase I (Cytochrome P450 3A1) and or phase II (Uridine glucuronyl transferase and sulfatase) metabolism to form water soluble glucuronide and sulfate conjugates. These important metabolic processes enable these compounds to be more readily transported out of the hepatocyte by phase III (MRP2, 3, and 4) export pumps.

Unconjugated bile salts are processed in peroxisomes where they are conjugated with taurine or glycine through the action of the enzyme, bile acid acyl co-A transferase. See reference (160) for details of the role of peroxisomes in bile salt metabolism. For a detailed account of the enzymes, regulation, and genetics of bile acid synthesis, see reference (473).

### **Canalicular membrane transporters**

The major determinants of bile flow and bile composition are the transport proteins localized at the apical canalicular domain of hepatocytes (Fig. 6). Most of these canalicular membrane transporters belong to the ABC superfamily (31) and excrete organic solutes into bile against large concentration gradients of ~1:100 to 1:1000 compared with their concentration in

plasma, a process requiring the expenditure of ATP (407). They consist of MDR1 (ABCB1), also known as P-glycoprotein (20,189), which transports organic cations; MDR3 (ABCB4), a floppase that translocates PC to the outer domain of the canalicular membrane (506); the MRP2 [multispecific organic anion transporter (ABCC2)] that transports a variety of drug and other organic conjugates including bilirubin digucuronide (275,289); the bile salt export pump, (BSEP, ABCB11) which transport bile salts (181,412,523); the breast cancer resistance protein (BCRP, ABCG2), which transports substrates similar to MRP2 (216, 376), and the sterolins 1 and 2, (ABCG5 and ABCG8) which are heteromeric transporters that excrete cholesterol and plant sterols (195, 227, 601). Other apical membrane transporters include familial intra-hepatic cholestasis 1, (FIC1, *ATP8B1*) (96, 105, 286) which is an ATPase that functions as a flippase by transferring phosphatidylserine to the inner cytoplasmic face of the canalicular membrane which together with MDR3 maintains apical membrane lipid asymmetry (201) and the multidrug and toxin extrusion transporter, (MATE1, *SLC47A1*) that uses the proton gradient to extrude cationic substrates (387).

Mutations in many of these apical transporters result in cholestatic liver disease and serve as definitive proof of their critical roles in bile formation and so will also be discussed in this section (407, 554).

**MDR1/mdr1a+b P-glycoprotein (ABCB1, Abcb1)**—MDR1, originally called P-glycoprotein, and its murine homologues, Mdr1a and Mdr1b, were first described in colchicine-resistant Chinese hamster ovary cells (253), where they conferred drug resistance by exporting anticancer drugs out of the cell (484). ABCB1 is expressed on a number of luminal membranes such as the intestine and choroid plexus where it serves a “barrier” function by extruding various drugs and toxins from these cells. Mdr1 and MDR1 were the first ABC transporters to be localized to the canalicular excretory membrane of the hepatocyte (547) yet its endogenous substrates are still uncertain and no mutations have been described that produce human disease. Nevertheless, studies in knockout mice have demonstrated the importance of this transporter in the biliary excretion of a variety of large hydrophobic compounds, many of which are organic cations, including exogenous and endogenous metabolites, drugs and toxins, hydrophobic peptides, glycolipids, and steroid hormones (482–484). Known as “hydrophobic vacuum cleaners,” many substrates of MDR1/Mdr1a and b are amphipathic cationic drugs (type II) such as quinidine, verapamil, and daunorubicin. MDR1 is a 1280-amino-acid, 170-kDa protein. Mdr1a and Mdr1b are 150-kDa mouse homologues of the human MDR1. The Mdr1b appears to be the major canalicular transporter for bulky amphipathic organic cations in mice based on double knockout studies of *Mdr1a* (–/–) and *Mdr1b* null mice (483), whereas Mdr1a is also expressed in cholangiocytes (184). Recent studies in canalicular membrane vesicles indicate that MDR1 is capable of transporting bile salts but with fivefold lower affinity than the bile salt export pump (309). Unlike most other canalicular membrane transporters, Mdr1a and Mdr1b are usually upregulated in several different forms of liver injury including *Spgp* (*Bsep*) null mice (577), bile duct ligation, alpha-1-naphthylisothiocyanate administration, and partial hepatectomy (2, 489) suggesting that Mdr1 may facilitate bile excretion as part of the adaptive response to cholestasis, at least in mice. MDR1 expression in human liver is quite

variable. NF $\kappa$ B affects Mdr1b transcription in insulin treated rat hepatoma cells (608) and rodent Mdr1a is inducible by PXR (519).

**The multidrug resistance protein 3 (ABCB4/Abcb4)**—Despite close homology to MDR1, MDR3/Mdr2 does not confer drug resistance but is a floppase that maintains PC on the external face of the canalicular membrane bilayer (228, 426). By doing so, this phospholipid becomes available for excretion into bile and participates in the formation of mixed micelles along with bile salts and cholesterol (504). This important function of MDR3 was established after discovering that deletion of the gene *Mdr2* in the mouse homolog resulted in the absence of PC in bile, despite normal biliary bile salt transport capacity (425, 505). Over the time, the inability to extrude phospholipids into bile in *Mdr2*<sup>-/-</sup> mice results in bile duct proliferation, and progression to biliary cirrhosis, and even the development of liver tumors (361). *Mdr2*<sup>-/-</sup> mice are now recognized as a good animal model for sclerosing cholangitis in humans (161). In the absence of phospholipid in bile, the continuous exposure to hydrophobic bile salts is thought to exert direct toxic effects on the apical epithelial membrane of the cholangiocyte, resulting in liver injury. Mdr2 is a 150-kD protein in mice and rats and a 170-kD protein in humans where it is highly expressed on the canalicular membrane. Bile salts are thought to stimulate the excretion of PC in bile by extracting this phospholipid from areas in the outer lipid layer of the canalicular apical membrane where MDR3 has created outpouchings into the bile canaliculus (427, 471) (see Fig. 8). Homozygote deficiency of MDR3 would be predicted to produce significant liver disease in humans whereas MDR3 heterozygotes might require a second hit to result in a clinical phenotype. Indeed, homozygous deficiency of MDR3 results in progressive familial intrahepatic cholestasis type 3 (PFIC-3) (131,134) while heterozygosity predisposes women to cholestasis of pregnancy and others to low phospholipid-associated cholelithiasis, a form of cholesterol gallstone disease (129) or drug-induced cholestasis (600). However, other acquired cholestatic liver diseases like primary biliary cirrhosis or sclerosing cholangitis have not been associated with MDR3 mutations. (430). Several families of adult patients have been described with MDR3 mutations who present either with progressive idiopathic cholestasis, gallstones, or cholestasis of pregnancy (247, 342, 467). PFIC3 is distinguished from PFIC1 and 2 by high levels of gamma-glutamyl transferase in the serum as well as the complete absence of phospholipid in bile.

A number of mutations have been described in patients with PFIC3 as well as genetic variants in patients with cholestasis of pregnancy and acquired cholestatic liver disease. However little mechanistic information has been published, although defects in protein trafficking (142) and temperature-sensitive folding defects have been reported (133). Functional studies of MDR3 mutations and single nucleotide polymorphisms (SNPs) have been limited because of the difficulty in measuring phospholipid transport from the inner to the outer leaflet of the plasma membrane and apparent toxicity of PC in transfected cells (407).

MDR3 is a regulated transporter and its expression is upregulated via bile acid activation of FXR (238).

**Familial intrahepatic cholestasis 1 (ATP8B1/Atp8b1)**—Familial intrahepatic cholestasis 1 (FIC1), also resides on the canalicular membrane and is a flippase that is responsible for maintaining aminophospholipids on the inner bilayer of the canalicular membrane. It is a type IV P-type ATPase, rather than an ABC transporter and counter balances the canalicular lipid membrane asymmetry established by MDR3 (201). Using positional cloning, Laura Bull first identified a mutant *ATP8B1* gene on chromosome 18q21 in patients with PFIC1 and benign recurrent intrahepatic cholestasis 1 (BRIC1) (97). ATP8B1 functions as a complex in association with the accessory protein CDC50 (434). In the absence of this flippase, membrane lipid asymmetry is disrupted and the function of BSEP is impaired leading to cholestasis (105, 435). PFIC1 was first described in an Amish family cohort in Pennsylvania whose founder name was Joseph Byler (120). Electron microscopic studies in patients with PFIC1 and *in vitro* models of FIC1 deficiency demonstrate disruption of the canalicular membrane bilayer (96, 105) and luminal accumulation of intracellular membranous material known as Byler's bile (120). Patients with "Byler's disease" have now been described in many countries including clusters of disease in Eastern Greenland Inuits (25).

ATP8B1 is predicted to have ten transmembrane domains and is expressed in a number of tissues in addition to the liver, including small intestine, uterus and pancreas (97,437). Mutations in *ATP8B1* produce PFIC1, with low  $\gamma$ -GT levels, BRIC1, and rare cases of intrahepatic cholestasis of pregnancy (ICP). Presumably, the different clinical phenotypes result from different mutations but this has not been clearly established. However, *in vitro* expression of constructs with PFIC1, BRIC1, and ICP mutations in Chinese Hamster cells suggest that some PFIC1 mutations fail to interact with CDC50, and all PFIC1 mutations result in complete absence of protein on the plasma membrane. In contrast residual canalicular protein is seen with BRIC1 and ICP mutations (164) and may be partially restored by treatment with 4-phenyl butyrate (566).

Because ATP8B1 is highly expressed in the small intestine, cochlear hair cells, and pancreas, extrahepatic systemic symptoms of diarrhea (346) are often present, as well as sensorineural hearing loss (518) and occasionally pancreatitis (560). Patients undergoing liver transplantation for cirrhosis and end stage liver failure which often develops in the second decade of life in patients with PFIC1, are usually left with these extrahepatic manifestations of PFIC1 deficiency after liver transplantation (346). Unlike PFIC2 where mutations that produce severe disease present with advanced liver disease, the extrahepatic diseases are the more prominent manifestations of severe PFIC1 mutations (439).

**Multidrug resistant associated protein 2, previously known as the multispecific organic anion transporter**—MRP2/Mrp2 is the major ABC conjugate export pump in the canalicular membrane, the primary determinant of BSIF, and the gene, when mutated in humans, results in the Dubin-Johnson syndrome. The Eisai hyperbilirubinemic rat (EHBR) and the Groninger Yellow/transporter deficient rat (TR<sup>-</sup>) strains (237, 250) are animal models of Mrp2 deficiency and have provided much information about the function of this important ABC transporter. Studies in *Mrp2*<sup>-/-</sup> mice give similar results (119, 297). MRP/Mrp2 functions to transport a large number of different amphipathic, usually multivalent organic anion conjugates from the liver into bile (69, 273,



274, 297, 408). The major endogenous substrates for this transporter include bilirubin diglucuronides, GSH conjugates, GSSG, leukotrienes, heavy metals, sulfated and glucuronidated substrates, including bile salts, as well as a variety of different drug conjugates, antibiotics, and a number of other exogenous compounds [see Table 2 in ref (273) and Table 1 in Ref. (408) for a more complete list of substrates].

In rat strains deficient in Mrp2, point mutations lead to a stop codon and inhibition of expression of the Mrp2 protein. Similar mutations in the MRP2 gene are found in the Dubin-Johnson syndrome (265, 436). Some mutations in human MRP2 result in retention in the ER and failure of the protein to traffic to the canalicular membrane (269). MRP2 mutants have significant reductions in BSIF as a result of the inability to excrete GSH, a low-affinity substrate (40, 250, 281, 432).

In hepatocytes, Mrp2 is also localized to intracellular vesicles that are associated with microtubules (512). This intracellular pool of transporters cycles to and from the canalicular membrane in response to both choleretic and cholestatic stimuli, respectively. Dibutyl cAMP increases bile salt independent bile flow (219) and insertion of Mrp2 protein into the canalicular membrane, increasing the canalicular transport capacity for Mrp2 substrates (461–463). This process can be inhibited by depolymerizing microtubules (461). Recent studies suggest that cAMP-induced MRP2 translocation in hepatic cells is mediated via phosphatidylinositol 3-kinase (PI3K)-independent and mitogen-activated protein kinase kinase 3 (MKK3) mediated activation of p38alpha mitogen-activated protein kinase (MAPK) (488).

Mrp2 contains Discs-large homologous regions (PDZ binding domains) in its C-terminus that bind to Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor 1 (NHERF-1), a PDZ protein that cross links proteins to actin filaments. Nherf-1 knockout mice have reduced expression of Mrp2 at the canalicular membrane and a 50% reduction in GSH excretion and BSIF, while maintaining normal bile salt excretion (335). Radixin, an apically located Ezrin-radixin-moesin protein (166) is also required for the insertion of Mrp2, as well as other ABC transporters, in the canalicular membrane in rat liver, but does not appear to bind directly to Mrp2 (276, 578). Radixin and ezrin are both independently required for apical expression of human MRP2 in human intestinal Caco-2 cells (596).

In contrast, protein kinase C agonists counterregulate the activity of cAMP (463) and reduce bile formation in isolated perfused rat livers (122) and the canalicular insertion of membrane proteins in isolated rat hepatocyte couplets (56). Cholestatic liver injury in rats rapidly diminishes Mrp2 protein at the canalicular membrane by retrieval of the transporter into submembranous vesicles induced by bile duct ligation (431), TLC (61, 379), estrogens, (390), endotoxin (143,299), or oxidative stress (462). Estrogen-induced internalization of Mrp2 is independent of microtubule function while its reinsertion is not (391). Many human cholestatic liver diseases also result in internalization of MRP2 as well as other canalicular export pumps emphasizing the importance of this mechanism for maintaining the bile secretory process. (462). Inhibition of transcription of Mrp2 mRNA during cholestasis also results in diminished levels of expression of this transporter, but this is a delayed response compared to its retrieval from the apical membrane (325, 552).

**Bile salt export pump (ABCB11/Abcb11)**—Prior to the molecular discovery that the sister of P-glycoprotein was the key canalicular membrane bile salt transporter, bile salt transport had been functionally characterized as an ATP-dependent transport system in isolated canalicular membrane vesicles initially by Nishida (411) and subsequently by three other groups (3, 393, 524). Originally cloned as a close relative of P-glycoprotein, and thus named the sister of P-glycoprotein (115), the full length rat homolog *Abcb11* was ultimately found to function as a bile salt export pump when expressed in an Sf9 insect cell expression system, and thereafter was renamed the bile salt export pump (Bsep) (181). Northern analysis demonstrated that Bsep mRNA was expressed primarily in the liver and subsequently the full length human ortholog of ABCB11 was obtained by positional cloning (181, 412) and validated when a series of mutations were associated with patients with PFIC2 (528). Small amounts of RNA have been identified in other tissues but lack confirmation with protein so that the functional significance of these extrahepatic BSEP mRNAs is not certain (521).

The *BSEP* gene is located on chromosome 2q23–31 and encodes 1321 amino acids. Rodent Bseps are ~80% identical with their human orthologue. Human BSEP is a glycoprotein containing four putative glycosylation sites and has a MW of ~140 to 170 kDa (102,181,412). It is predicted to have two transmembrane domains, each with six transmembrane spanning helices [see Ref. (521) for more details].

Interestingly, *Bsep* knockout mice develop a nonprogressive form of cholestasis unless infused with cholic acid, probably because they have a relatively hydrophilic bile salt pool and form tetrahydroxy bile salts and  $\beta$ -muricholic acid which can be transported into bile (577). Upregulation of *Mdr1* (*Abc1a*) may also play a role in protecting these genetic Bsep knockout mice (309). BSEP is highly expressed in liver and is present throughout the lobule at the canalicular membrane where it has been localized to the microvilli by immunogold labeling (181) and thought to reside in caveolin enriched lipid microdomains resistant to extraction by Lubrol WX (244). Overexpression of caveolins increase bile salt secretion in the mouse (385), suggesting that caveolins may alter the cholesterol environment in the membrane. Canalicular membrane cholesterol is known to affect Bsep activity (433).

BSEP is present in lower vertebrates and is 69% identical in the marine skate *Leucoraja erinacea*, indicating considerable conservation of this transporter during vertebrate evolution (104). BSEP is first expressed in human liver at midgestation but is considerably below adult levels at birth (110), probably contributing to the transient elevation in serum bile salts observed at birth (536).

Substrate specificity for BSEP/Bsep is predominantly restricted to bile salts with good correlations between transport properties for different bile salts among species. See Ref. (521) for details of the specific bile acid substrates that have been tested for Bsep in various species. Pravastatin is the only nonbile salt solute yet confirmed as a substrate for BSEP (230).

BSEP/Bsep is a major target for drug-induced cholestatic liver injury (428). Based largely on studies in Sf9 vesicles, cholestatic drugs or their metabolites can either inhibit the

transporter directly from the cytoplasmic side; examples are bosantin, glibenclamide, rifampicin, and cyclosporine (155, 522), or indirectly after excretion into the canaliculus by MRP2 as shown for estradiol-17 $\beta$ -glucuronide (522). The list of drugs that can inhibit Bsep continues to grow [see Table 4 in Ref. (521) for the most complete recent list].

BSEP/Bsep is highly regulated both at the transcriptional and posttranscriptional levels. Studies in rodents suggest that Bsep exists in a recycling pool for rapid mobilization and insertion into the plasma membrane (280). Bile salt secretion is stimulated by cAMP in isolated perfused rat livers and is sensitive to microtubule inhibitors (219, 221). Various cholestatic insults in rats, such as estradiol-17 $\beta$ -glucuronide, lithocholate, lipopolysaccharide, and hypoxia among others, lead to internalization of Bsep into sub apical membrane vesicles and spotty staining of the protein at the canalicular membrane. In contrast, cAMP, tauroursodeoxycholic acid, and taurocholic acid all stimulate insertion of Bsep into the canalicular membrane (521). Altogether these observations suggest that the functional expression of Bsep is rapidly up-regulated in response to increases in its substrate as well as downregulated when cholestatic injury occurs, the latter perhaps serving as a cytoprotective mechanism. Bseps half life in the apical membrane is ~4 to 6 days and the protein constitutively recycles from the apical membrane to Rab11 positive submembranous endosomes, requiring myosin Vb (279, 280, 572, 574). Mechanisms of BSEP retrieval from the apical membrane are still not well known but *in vitro* studies suggest that clathrin-dependent components, including Hax-1, cortactin, and EPS15 may be involved (422). Recent studies with human BSEP suggest that constitutive internalization of BSEP is dependent on a tyrosine-based endocytic motif at the C-terminal end of BSEP (310) that may interact with an AP2 adapter complex (222), findings consistent with clathrin-dependent endocytosis. Additional studies beyond the scope of this review suggest that posttranscriptional regulation of BSEP trafficking and localization can also be affected by glycosylation, phosphorylation and ubiquitylation. See Ref. (418) for more details.

Human BSEP expression is also highly regulated by transcriptional mechanisms and its expression is highly variable between individuals (232, 549). BSEP expression is sensitive to bile acid flux through the liver which activates the nuclear receptor FXR. FXR transactivates the proximal promoter of BSEP in humans (22) and in rodents. The critical role of FXR in BSEP expression is demonstrated by its low basal expression in *Fxr*<sup>-/-</sup> mice and the lack of an induced response to bile salts (263). CDCA is the major endogenous ligand for FXR. The BSEP promoter is also induced by the hepatocyte-specific liver receptor homolog-1 (LRH-1, NR5A2) (510) while 1,25-dihydroxyvitamin D3 suppresses CDCA-FXR transactivation (235). The BSEP promoter is also positively regulated by the nuclear erythroid 2 p45-regulated factor 2 (NRF2) that plays a significant role in responses to oxidative stress. NRF2 binds to response elements that regulate many hepatic phase I and II enzymes and efflux transporters such as MRP3 and MRP4 (582). Altogether these transcriptional and posttranscriptional effects indicate that the expression of BSEP is regulated by multiple physiological and pathological stimuli.

The discovery that mutations in the bile salt export pump were the cause of PFIC type 2 (PFIC2) not only provided final confirmation that this transporter was the determinant of BSDF, but led to the clarification of the mechanisms of a number of other cholestatic

disorders, including certain cases of cholestasis of pregnancy, BRIC2, and drug-induced cholestasis (308, 521). Currently, more than 100 different *BSEP* variants have been described comprising missense, nonsense, deletions and insertions, and splice-site mutations (249; 272, 306, 480, 528, 529, 568). Patients with PFIC2 may even develop hepatocellular carcinoma (128) or antibody-mediated recurrent disease following liver transplantation (251, 266).

A common result of many of these mutations is a reduction or loss of expression of the BSEP protein at the canalicular membrane (529). Some of these mutations or polymorphisms can lead to aberrant pre-mRNA splicing or exon skipping and reduced levels of mRNA resulting in variable levels of expression of the transporter. This may explain why some common mutations (p.D482G) result in variable clinical phenotypes (103, 232).

A large number of polymorphisms in BSEP have also been described (232,312,313) with considerable variation between different ethnic groups. Most are rare events, however two common nonsynonymous SNPs, c.1331T>C (p.V444A) and c.2029A>G (p.M677V), are consistently seen in different populations. Lower levels of BSEP expression are usually seen in patients with the c.1331T allele (232, 312). This V444A variant is associated with ICP and drug-induced cholestasis, despite retaining functional activity with *in vitro* assays (141, 232, 272, 312, 370).

To understand the phenotypic expression of some of these mutations and SNPS in the *BSEP* gene, *in vitro* expression studies have been performed in various mammalian cell lines. When BSEP mutations from patients with PFIC2 (D482G, E297G) were expressed in MDCK, HEK293, or HepG2 cells, the protein failed to reach or be stabilized at the plasma membrane (259, 307, 443, 576). When these mutations for PFIC2 were compared with those from BRIC2 (A590T, R1050C) and ICP (N591S), the amount of protein expressed on the cell surface tended to correlate inversely with the severity of the clinical disease (307, 308). Transport activity for taurocholate is significantly reduced in PFIC2 mutants while partial or normal activity is retained in BRIC and ICP mutations, respectively (307). The majority of the mutations in BSEP lead to retention in the ER and failure to target to the apical plasma membrane as determined by immunostaining in heterologous expression systems and liver biopsies from patients. ER-associated degradation probably accounts for the removal of misfolded proteins in the ER. Ubiquitinylation with E3 ubiquitin ligases shortens the already short half life of the PFIC2 mutant D482G, although small amounts of the mutant protein still reach the plasma membrane (575). This and other studies indicate that the residence time on the cell surface of D482G and E297G mutant proteins is greatly reduced due to accelerated internalization, reduced recycling, or targeting of the endocytosed protein for degradation and have led to attempts to “rescue” the mutant proteins with small molecules (224, 259). *In vitro* studies have shown that treatment with 4-phenylbutyrate (4-PBA) can increase the cell surface expression of PFIC2 mutant proteins D482G and E297G and stimulate bile secretion and Bsep expression in rats (223). These exciting results have now led to the successful treatment of a patient with PFIC2 with 4-PBA (188).

**Breast cancer resistance protein (ABCC2/Abcc2)**—This member of the ABC family of membrane transporters has broad substrate specificity for a number of drugs, environmental

agents, and endogenous substrates (294,444). The endogenous solute, protoporphyrin, is specifically transported by BCRP, as is the drug nitrofurantoin. Other endogenous substrates include dehydroepiandrosterone sulfate, estrogen, and folic acid. BCRP expression in stem cells accounts for the “side population” characterized by dye extrusion with flow cytometry. BCRP is expressed on the apical membrane of numerous cell types and functions to protect these cells from toxic compounds. BCRP functions in a somewhat similar manner to MRP2 and other organic anion transporters and substrate specificities often overlap. However, BCRP protein expression in human liver is considerably less than the expression of MRP2 (557). Controversy exists as to whether BCRP transports bile salts. Studies in bile duct ligated mice and *Bcrp* null mice suggest that *Bcrp* does not have a significant role in the adaptive response to cholestasis in the liver but may have a more important role for solute excretion in the kidney and intestine during cholestasis (375). *Bcrp* deficiency impairs the biliary secretion of pitavastatin and some fluoroquinilones (305).

BCRP expression in hepatocytes can be induced by xenobiotics including phenobarbital. Studies of the BCRP promoter reveal estrogen receptor-binding elements, PPAR $\gamma$  binding sites, and aryl hydrocarbon receptor (AHR) and constitutive androstane receptor (CAR) response elements (58).

**Sterolin 1 and Sterolin 2 (ABCG5/G8/Abcg5/g8)**—The biliary excretion of cholesterol is an important mechanism for regulating cholesterol homeostasis and is mediated in large part by the heteromeric transporters, ABCG5 and ABCG8 known as sterolin 1 and 2, respectively (195). These transporters also regulate the excretion of plant and animal sterols and mutations in the human genes result in  $\beta$ -sitosterolemia (59, 239). Together these seven membrane spanning domain “half” ABC transporters combine at the canalicular membrane to facilitate this process (194). Mice with genetic deficiency of *Abcg5* and *Abcg8* have a marked reduction in biliary cholesterol excretion (602). ABCG5/G8 functions together with Nieman Pick C1-like 1 protein (NPC1L1) which in humans is believed to reabsorb cholesterol from hepatic bile (14, 546). Ezetimibe is an inhibitor of NPC1L1 which is also expressed in the intestine. In contrast Nieman-Pick C2 (NPC2) is a cholesterol binding protein that is excreted in bile and helps to stimulate cholesterol excretion (594) together with ABCG5/G8. Human *ABCG5/G8* and *NPC1L1* are positively and negatively regulated by cholesterol, respectively, via LXR (59), reflecting the tight regulation that is exerted over cholesterol excretion (245). *ABCG5* and *ABCG8* are also positively and negatively regulated by bile salts and phospholipids, respectively, when overexpressed in Caco-2 cells (593). A large number of mutations and SNPs have been identified in both heteromers although their clinical significance is mostly unknown (227).

**Multidrug and toxin extrusion transporters (SLC47A1/Slc47a1)**—While certain organic cations enter the liver via OCT1, they exit the cell into bile via a putative 13 membrane domain transporter, MATE1. This canalicular membrane transporter utilizes the proton gradient to mediate the efflux of cation drugs such as tetramethylammonium, cimetidine, metformin, and procainamide via a proton coupled electroneutral exchange mechanism (284). The functional importance of MATE in the normal mechanisms of bile formation is not clear. However *Mate1*<sup>-/-</sup> mice develop lactic acidosis when fed metformin

in their drinking water (550). Neither Ahr, Car, Pxr, Ppar, or Nrf2 affect the expression of mouse *Mate1*. Polymorphisms in human *MATE1* may influence cationic drug pharmacokinetics and toxicity (598).

**Concentrative and equilibrative nucleoside transporters CNT1 and 2 and ENT1 (SLC28A1 and 2 and SLC29A1)**—Nucleoside transporters contain 13 putative transmembrane domains and transport the nucleosides adenosine, cytosine, guanosine, thymidine, uridine, and inosine. They also transport nucleoside analogues which are drugs developed to treat viral infections and cancer, such as didanosine, fialuridine, 5-fluorouridine, and ribivirin. CNTs transport nucleosides from bile back into hepatocytes and are an important mechanism for salvaging nucleosides for DNA and RNA synthesis. ENTs function as bidirectional transporters and facilitate transport of these substrates down their concentration gradients (191,284). In human hepatocytes all of these transporters are expressed on the canalicular membrane as well as the basolateral membrane and in the cytoplasm (190).

**Chloride/bicarbonate exchanger, AE2/Ae2 (SLC4A2/Slca2)**—A second determinant of canalicular bile salt independent bile flow is the chloride/bicarbonate exchanger ( $\text{Cl}^-/\text{HCO}_3^-$  exchanger). This transporter has been characterized functionally in canalicular membrane vesicle systems in the rat and in isolated hepatocyte systems (55, 366). Immunohistochemical studies localize the AE2 isoform specifically to the apical membrane in human hepatocytes, as well as to the luminal surface of the bile duct epithelium (359). This  $\text{Cl}^-/\text{HCO}_3^-$  exchanger functions as an acid-loading mechanism to protect the hepatocyte against increases in intracellular pH when intracellular pH rises above its set point (530), as well as a mechanism to stimulate the secretion of an alkaline bile (530). This  $\text{Cl}^-/\text{HCO}_3^-$  exchanger is also present on intracellular pericanalicular membrane vesicles that are associated with microtubules and translocates to the apical membrane when stimulated by increases in cell pH (91), cell swelling (92), or cAMP (57). The latter effect is opposed by PKC. Canalicular bicarbonate secretion is also regulated by glucagon which stimulates a bicarbonate rich choleresis (330). Glucagon acts through the cAMP-dependent protein kinase A pathway, which also stimulates AQP 8 insertion into the canalicular membrane in rat hepatocytes via microtubular-dependent intracellular membrane vesicles (17, 174, 193).

The  $\text{Cl}^-/\text{HCO}_3^-$  exchanger is also thought to work in concert with an apical membrane chloride channel. The presence of outwardly rectifying chloride channels has previously been demonstrated in rat canalicular membrane vesicles (491). Two isoforms of the CIC-3 chloride channel have subsequently been identified at the molecular level in rat canalicular membrane and intracellular sites that are consistent with this finding (494). These anion channels allow for electrogenic chloride efflux to compensate for chloride influx via the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. Hepatocyte cell swelling, such as occurs after meals with the influx of amino acids, results in activation of this chloride channel to maintain cell volume homeostasis (339). Substrates of Mrp2 have also been shown to activate chloride channels in hepatocytes which does not occur in  $\text{TR}^-$  rats that are deficient in Mrp2. This suggests that Mrp2 is capable of accelerating this volume-activated response (337).



**Sodium proton exchanger 3 (SLC9A3 /Slc9a3)**—Although the sodium proton exchanger 3 (NHE3) isoform of the sodium hydrogen exchanger has been localized to the canalicular membrane in rat hepatocytes, its functional role at the canalicular domain remains unclear. In contrast, this isoform plays an important role in the regulation of cholangiocyte fluid reabsorption (373).

**Aquaporins: AQP0, AQP8, and AQP9**—AQPs are small (25–34 kD) 6-transmembrane domain proteins that function as water channels in membranes. Some AQPs also transport glycerol and urea. There are three AQPs that have been described in rat hepatocytes (AQP0, AQP8, and AQP9). Studies of hydraulic water permeability across the basolateral and apical membranes of isolated rat hepatocyte couplets and the paracellular pathway demonstrate higher values,  $\sim 3 \times 10^{-4} \text{ cm} \times \text{s}^{-1} \times (\text{osmol/kg})^{-1}$ , than can be accounted for by diffusional water permeability measured by tracer flux of  $^3\text{H}_2\text{O}$  (7). These findings support a functional role for water channels in hepatocytes membranes and the formation of bile (252).

AQ8 is localized to intracellular vesicles in the hepatocyte but can redistribute to the canalicular membrane in response to choleric stimuli such as dibutyl cAMP or glucagon which increases the apical membrane water permeability. AQP9 is an aquaglycerol AQP and is located at the basolateral membrane of hepatocytes. Although AQP8 knockout mice do not have a defect in bile secretion (595), the rapid insertion of water channels into the canalicular membrane would provide a molecular mechanism for efficient coupling of osmotically active substrates and the production of canalicular bile (355). Several animal models of cholestasis including estrogen administration, bile duct ligation, and sepsis-induced cholestasis have been associated with downregulation of AQP8 protein suggesting that a decrease in water permeability at the canalicular membrane might also contribute to cholestasis (351).

### Phase III basolateral membrane transporters

These export pumps function primarily to protect the hepatocytes from the accumulation of toxic compounds but they can influence the overall clearance of bile salts and other organic anions excreted into bile by limiting the concentrations of these substances within individual hepatocytes.

**Multidrug resistance associated protein 3 (ABCC3, Abcc3)**—MRP3/Mrp3 is a member of the ABC superfamily expressed in the liver and is localized to the basolateral membrane of hepatocytes and cholangiocytes. MRP3/Mrp3 has two 6-membrane spanning domains and an extra 4-membrane spanning domain at its N-terminus similar to MRP2/Mrp2. MRP3 transports a wide range of substrates consisting primarily of solutes conjugated with glucuronides and GSH (603, 607). MRP3/Mrp3 excretes bile salts conjugated with sulfate, glycine or taurine, bilirubin glucuronides,  $17\alpha$ -glucuronosyl estradiol, leukotriene, and a number of drugs (5, 231, 290). However, human MRP3 has a lower affinity for bile salts compared to Mrp3 in the rat.

One of its main physiologic functions may be to protect the hepatocyte in the proximal portion of the hepatic lobule from an overload of substrates such as bilirubin by extruding these solutes back into the blood sinusoids so that they are distributed to more distal

hepatocytes along the hepatic lobule (564, 565). MRP3/Mrp3 expression in the liver is normally low but it is upregulated in cholestatic liver injury in rodents (144, 511) and humans (106, 114, 267, 290, 609) as part of the adaptive protective response. MRP3/Mrp3 may also function to extrude GSH conjugates into the blood during oxidative stress and when MRP2/ Mrp2 expression is deficient in the conjugated hyperbilirubinemic Dubin-Johnson syndrome in humans and the TR<sup>-</sup>/Groningen/EHBR mutant rat models (95, 265, 362, 436).

MRP3/Mrp3 expression is transcriptionally regulated. Bile duct ligation in mice results in upregulation and binding of the nuclear receptor Lrh-1 to the *Mrp3* promoter which increases Mrp3 expression, a process that is dependent on TNF- $\alpha$  signaling pathways (68). This appears to be a protective adaptation since *Tnf* receptor knockout mice are more susceptible to liver injury following bile duct ligation (68). Both MRP3 expression and TNF- $\alpha$  expression are also increased in obstructive cholestasis in humans in association with increased SP1 and LRH-1 expression and binding to the *MRP3* promoter (106). This is associated with activation of the stress-activated protein kinase/c-Jun NH2-terminal kinase pathway (JNK/SAPK) which is known to activate SP1. SP1 is a known positive activator of both rat and human MRP3 (542,561) and binds to its proximal promoter. RXR $\alpha$ :RAR $\alpha$  compete with SP1 for binding to the human *MRP3* promoter (111). Since RXR $\alpha$ :RAR $\alpha$  expression is diminished by cholestatic liver injury, loss of RXR $\alpha$ :RAR $\alpha$  may contribute to the upregulation of MRP3/Mrp3 expression in these disorders (111). Nrf2 may also upregulate Mrp2 in rodents (503). Dioxin response elements have been identified in the human *MRP3* promoter where Ahr/Arnt (the aryl hydrocarbon receptor /aryl hydrocarbon nuclear translocator) heterodimers may bind (542), thus MRP3 may also be upregulated by Ahr. Mrp3 can also be acutely regulated by protein kinase A and C pathways (108).

**Multidrug resistance associated protein 4 (ABCC4/Abcc4)**—MRP4/Mrp4 is also expressed on the basolateral membrane of the hepatocyte and is one of the short forms of the MRP ABC transporters as it lacks the N-terminus 4 membrane spanning domain seen in MRP2 and 3. MRP4/Mrp4 transports sulfated organic conjugates such as estrone-S04, estradiol 17-beta-D-glucuronide, dehydroepiandrosterone 3-sulphate (DHEAS), sulfated and glucuronidated bile salts, and nucleoside analogues (472; 490; 588, 603, 606). MRP4 also transports urate, the end product of purine metabolism in man (563), prostaglandins (449), and GSH (455). In the presence of GSH, human MRP4 also has high affinity for glycine and taurine conjugates and therefore may compete with BSEP for export of bile salts under cholestatic conditions (454).

MRP4 expression in human liver is highly variable and this difference in expression levels is not explained by genetic variations (192). However, cholestatic liver injury is a major stimulus for upregulation of this efflux transporter both in animal models and in patients with clinical cholestatic disorders (107, 192, 610). Studies in *Mrp4* null mice (374), unlike *Mrp3* null mice (53, 607), indicate that MRP4/Mrp4 plays a major role in the adaptive protective response to cholestatic liver injury. Oxidative stress also stimulates increases in Mrp4 expression in animal studies (348, 419). The AhR and NF-E2-related factor 2 (Nrf2) play important roles in protecting cells from oxidative stress and agonists of these receptors upregulate human MRP4 expression, suggesting that agents that enhance their activity may

be of therapeutic benefit for cholestasis (591). Activators of the nuclear receptor CAR induce the expression of MRP4 in primary human hepatocytes and HepG2 cells; however, CAR response elements have been difficult to identify in the human *MRP4* promoter (591). Recent studies suggest that FXR response elements are embedded in CAR response elements in the human *MRP4* promoter and that FXR agonists may impair CAR-mediated activation of MRP4. *Mrp4* mRNA is also increased in *Fxr* knockout mice (500).

**Organic solute transporter alpha and beta**—Organic solute transporter alpha and beta ( $OST\alpha$ - $OST\beta$ ) is a unique heteromeric sterol transporter that is located on the basolateral membrane of hepatocytes, cholangiocytes and gallbladder, the renal proximal tubule, and the terminal ileum (39, 130).  $OST\alpha$ - $OST\beta$  is responsible for the transfer of bile salts from these tissues into the venous blood and is a major link in the enterohepatic, cholehepatic, and renal-hepatic circulation for bile salts. It is also expressed in tissues that transport sterols such as the adrenal gland, ovaries and testes.  $OST\alpha$  has 7 membrane spanning domains and a molecular weight of 360 daltons while  $OST\beta$  has a single membrane spanning domain and is 128 amino acids in size. There are no known homologs in the human or mouse genomes. Both subunits are required for the functional expression of this transporter. The beta subunit is required for formation of the heterodimer as well its trafficking to the surface membrane (562).  $OST\alpha$ - $OST\beta$  is a facilitated transporter and bile salts and other sterols can be driven bidirectionally across the membrane of the cell according to the electrical chemical gradient. In addition to bile salts,  $OST\alpha$ - $OST\beta$  transports sterols conjugated with sulphate or glucuronic acid, including DHEAS, estrone-3-sulfate, digoxin, and prostaglandin E2 (45). Unconjugated sterols do not seem to be substrates (inhibitors) for  $OST\alpha$ - $OST\beta$  (153). Both subunits of  $OST\alpha$ - $OST\beta$  are highly regulated by bile salts via FXR response elements. Two FXR response elements have been identified in the promoter of  $OST\alpha$  and one in  $OST\beta$  (311). Under cholestatic conditions,  $OST\alpha$ - $OST\beta$  can efflux bile salts from the liver into the systemic circulation as part of the adaptive response to increases in concentration of bile salts in the liver (88).

## Bile Duct Cells (Cholangiocytes)

The primary function of cholangiocytes is to fluidize and alkalinize canalicular bile, a process that involves a number of secretory and absorptive functions of these cells. Cholangiocytes form the epithelium of the intrahepatic biliary tree. The biliary tree is a complex heterogenous network of ducts that begins when bile flows from the bile canaliculi into the Canals of Hering (< 15  $\mu\text{m}$  in diameter) (also the site of progenitor cells), and leads progressively to interlobular (15–100  $\mu\text{mol/L}$ ), septal (100–300  $\mu\text{m}$ ), area ducts (300–400  $\mu\text{m}$ ), segmental ducts (400–800  $\mu\text{m}$ ), hepatic ducts (>800  $\mu\text{m}$ ), and major ducts as originally defined by Ludwig (214) (Fig. 9). Ultimately, these conduits deliver bile to the gallbladder and intestine. Three-dimensional reconstruction of the biliary system from human liver provides estimates of its volume (mean 20.4  $\mu\text{m}^3$ ) and surface area (398  $\text{cm}^2$ ) (343). Microvilli that line the biliary tree magnify this surface area approximately fivefold which increases geometrically from segment to segment as bile proceeds down this conduit (343).

The cholangiocytes that line the biliary tree also vary in structure, with the canals of Hering being lined in part by hepatocytes and in part by cholangiocytes (466). The cholangioles are

cuboidal in shape and lead to the bile ductules. The shape of the cholangiocytes progressively enlarge and become more columnar the more distal their location in the biliary tree. All cholangiocytes contain primary cilia in their apical membrane that regulate cell differentiation, proliferation, and secretion (317). Cilia activate intracellular signaling pathways which sense changes in bile flow rates and osmolarity, as well as other molecular constituents (317). Cholangiocyte cilia express specific proteins, including polycystin-1, polycystin-2, fibrocystin, TRPV4, P2Y12, and AC6, that account for ciliary mechano-, osmo-, and chemo-sensory functions (360).

Cholangiocyte secretory and absorptive functions provide a variable component to the final volume of bile in different species but can significantly modify the final secretion that is delivered to the duodenum (74, 261, 350). Although comprising only 3% to 5% of the total population of liver cells, the biliary epithelium accounts for ~30% of daily bile production in humans and thus is an important component of BSIF (80, 447). In contrast, it accounts for less than 10% of basal bile flow in the rat (545).

To carry out these secretory and absorptive functions, cholangiocytes contain a number of specific transport systems that are highly regulated by meal-induced hormone release and their specific receptors (183). Since the major function of cholangiocytes is to alkalinize the bile, many of these mechanisms involve transporters that regulate intracellular pH. By increasing bile luminal pH, lipophilic weak acids are prevented from being protonized and reabsorbed by diffusion across the biliary epithelium. In addition, the alkaline bile helps to neutralize the acidic pH of gastric secretions. Although all bile duct epithelial cells express the bile duct-specific markers cytokeratin 19 and  $\gamma$ -glutamyl transpeptidase (except in mice), only medium and large bile duct cells express the secretin receptor, cystic fibrosis transmembrane regulator (CFTR), and the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger as described for rats and mice (12, 262, 263, 185) or show a secretory or pH response to secretin or cAMP (8, 261) (Fig. 9). In human liver CFTR has also been localized to or near the apical membrane of cholangiocytes but not hepatocytes (121) and secretin receptors have been identified at the molecular level in normal bile duct and ductules (291). Immunohistochemical studies have identified AE2 ( $\text{Cl}^-/\text{HCO}_3^-$  exchanger) on the canalicular membrane and the apical membrane of small and medium bile ducts (359).

Secretion of fluid and bicarbonate anions from bile duct cells occurs as a physiologic response to meals. The major transporters and receptors involved in fluid secretion in cholangiocytes are illustrated in Figure 10. Specifically, acidic pH, and to a lesser extent fatty acids and bile salts, stimulate the duodenum to release secretin (277) and cholecystokinin into the portal circulation. Secretin stimulates fluid and bicarbonate secretion while cholecystokinin stimulates gallbladder contraction (338). Secretin is a 27-amino acid peptide that is produced from S cells in the mucosa of the proximal intestine. After release to the circulation, it binds to heteromeric G protein-coupled basolateral receptors on the basolateral surface of large bile duct cells (8), which then leads to activation of adenylyl cyclase and production of cAMP (329). PKA is then activated, leading to phosphorylation and activation of CFTR (19, 246), a chloride channel present on the luminal membrane of bile duct cells which is required to facilitate bicarbonate extrusion (356, 531). Another  $\text{Cl}^-$  channel, which is sensitive to  $\text{Ca}^{2+}_i$  rather than cAMP, also is expressed

apically (158, 163), and may provide an alternative mechanism for chloride secretion. As chloride is secreted, the bile duct cell is depolarized which activates basolateral  $\text{Na}^+/\text{Cl}^-/\text{HCO}_3^-$  or  $\text{Na}^+/\text{HCO}_3^-$  cotransport (in humans or rodents, respectively), resulting in increased cellular levels of  $\text{HCO}_3^-$  anions (532). The changing gradients for  $\text{HCO}_3^-$  and  $\text{Cl}^-$  drive apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange. The net result is  $\text{HCO}_3^-$  secretion (16,74).  $\text{Na}^+, \text{K}^+, -2\text{Cl}^-$  cotransporters are also expressed on the basolateral membrane of cholangiocytes, as in other chloride secreting epithelia, and contribute to chloride entry into the cell, thereby facilitating conductive chloride excretion into the biliary lumen (501). At the same time, cAMP stimulates the insertion of water channels (AQPs) into the cell membrane, which increases the water permeability of this epithelium and thus further facilitates fluid movement (352–354). Once secretin is removed, protein phosphatases 1 and/or 2A inactivate the process (19) probably by inducing conformational changes in the R domain of CFTR which is a critical determinant of the chloride conduction pathway (332).

NHE3 is also localized to the apical membrane of cholangiocytes in rodents where it functions to reabsorb fluid from the biliary tree in the resting state (373). cAMP and protein kinase A may inhibit this activity. Fluid reabsorption fails to occur from IBUDs following forskolin stimulated secretion in *Nhe3* knockout mice or with NHE3 inhibitors in the rat (373). Thus inhibition of fluid reabsorption during meal-induced elevations in cAMP may also contribute to the secretion of biliary fluids, possibly by recycling NHE3 between subapical compartments and the apical membrane of cholangiocytes as described in other epithelia (6, 302).

A variety of other neural and humoral factors also influence bicarbonate secretion from cholangiocytes (183). Acetylcholine, a parasympathetic neurostimulator binds to  $\text{M}_3$  receptors on cholangiocytes and potentiates secretin's stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchange (15). VIP and bombesin, two neuropeptides, also stimulate cholangiocyte bicarbonate secretion via specific receptors but by cAMP-independent pathways (116, 118). In contrast, somatostatin, gastrin, and insulin have inhibitory effects on cholangiocyte secretion. Somatostatin binds to SSTR2 subtype receptors in large bile ducts and inhibits secretin induced cAMP levels (548). Gastrin inhibits secretin-induced secretion by binding to CCK-B/gastrin receptors which inhibit secretin receptor expression via  $\text{Ca}^{2+}$ -dependent  $\text{PKC}\alpha$  mechanisms (186). Insulin works through a similar mechanism (331). Finally, glucocorticoids activate glucocorticoid receptors in rodent cholangiocytes and enhance the activity and protein expression of apical  $\text{Cl}^-/\text{HCO}_3^-$  exchangers and basolateral  $\text{Na}^+/\text{H}^+$  exchange (18).

Cholangiocytes also contain other transporters on the apical luminal membrane that function to recover certain substances that are excreted in the bile (205). SGLT1, a  $\text{Na}^+/\text{glucose}$  cotransporter, is responsible for uptake of glucose from bile (322). Bile duct cells express another glucose transporter, GLUT1, on their basolateral domain (322). Together these transporters permit reabsorption of glucose from bile to blood. The tripeptides glutathione and GSSH are excreted into bile in mM amounts as major determinants of BSIF (48). Canalicular and ductular  $\gamma$ -glutamyltransferase metabolizes GSH to glutamic acid plus cysteinylglycine, while a dipeptidase metabolizes the latter to cysteine and glycine. These GSH constituents are reabsorbed, in turn, by specific dipeptide and aminoacid transporters

on the luminal membrane (44, 46). Biliary epithelia cells also express the intestinal-type H<sup>+</sup>-peptide cotransporter PEPT1 in their apical membrane which should function to recover peptides from bile (287).

The apical sodium-dependent bile salt transporter ASBT (*SLC10A*) is also localized to the luminal membrane of large but not small bile duct cells as well as the gallbladder (8, 132, 321). Together with the organic solute transporter OST $\alpha$ -OST $\beta$ , bile salts can recycle back to the liver from the bile in a manner similar to the terminal ileum and proximal renal tubule (39, 41). This process probably occurs normally in the gallbladder during periods of fasting, although it is unclear to what extent cholehepatic recycling takes place in cholangiocytes since the relatively high  $K_m$  of Asbt in bile duct epithelium suggests that only small amounts of bile salt would be taken up by this route (10). However, Asbt may function to reabsorb bile salts from the biliary tree during obstructive cholestasis. TGR5, a G-coupled protein bile acid receptor is also expressed on the luminal surface and cilia of cholangiocytes and gallbladder epithelium (268, 271). Bile salts stimulate bile duct proliferation via this receptor since bile duct ligation in *Tgr5* knockout mice prevent this proliferation (268). Bile duct proliferation is a characteristic response to obstructive cholestasis and Asbt continues to be expressed on the luminal membrane of proliferating cholangiocytes (9, 324). Bile salt stimulation of TGR5 results in increases in cAMP and upregulation of secretin receptors in human gallbladder epithelium and an increase in biliary bicarbonate secretion (268, 456). This process is presumably regulated by secretin-induced recruitment of intracellular subapical membrane vesicles containing the needed transporters (11, 94).

## Signal Transduction Pathways in the Regulation of Bile Secretion

Multiple posttranscriptional regulatory pathways exist in both hepatocytes and cholangiocytes for modulating the final bile secretory product. These consist of signal transduction second messenger pathways that are modulated in large part by hormones via hormone receptors, calcium via Inositol triphosphate receptors, nucleotides via purigenic receptors, and cytokines via cytokine receptors. In general, hormone receptor-mediated increases in adenylyl cyclase activity and cAMP production result in a net increase in bile secretion while receptor-mediated mobilization of Ca<sup>2+</sup><sub>i</sub> and activation of PKC tend to decrease bile flow (122, 220, 399, 400).

Hormones that increase intracellular calcium in hepatocytes, such as vasopressin, noradrenaline, or angiotensin, activate PKC by hydrolysis of PIP<sub>2</sub>, leading to formation of InsP3 and diacylglycerol, and result in a decrease in bile flow (400). In contrast, glucagon also increases Ca<sup>2+</sup><sub>i</sub> but activates adenylyl cyclase, resulting in an increase in bile flow, possibly because glucagon activates both calcium and chloride channels as noted in rat hepatocytes (32).

Hepatocytes functionally express P1 and P2 nucleotide (purigenic) receptors on the plasma membrane (157). By activating these receptors, extracellular nucleotides and nucleosides regulate a number of functions in the hepatocyte, including bile secretion. Ectonucleotidases (NTPDases, ecto 5' nucleotidase) are also expressed on the cell surface where they regulate the concentration of extracellular nucleotides including those in bile (597). NTPDase8 is



restricted to the bile canaliculus and converts ATP to AMP, which, in turn, is broken down to adenosine by ecto 5'-nucleotidase (156). Hepatocytes secrete sufficient amounts of ATP, ADP, and AMP in bile [ $\sim 5 \mu\text{mol/L}$  (109)] to stimulate these receptors, which result in either increases in intracellular calcium or cAMP (157).  $P_2Y$  receptors are also expressed on cholangiocytes and can respond to ATP secreted in bile. Hepatocytes can secrete ATP in sufficient amounts to stimulate  $P_2Y$  receptors (146,485) and can stimulate increases in intracellular calcium and alkalization of bile (146). cAMP-induced ductular bicarbonate secretion also depends in part on an autocrine signaling pathway that involves CFTR, apical release of ATP, stimulation of apical nucleotide receptors, and subsequent activation of apical, type III InsP3Rs. Thus, another role of CFTR in cholangiocyte secretion may be to regulate secretion of ATP in addition to secretion of chloride and/or bicarbonate (380).

Tauroursodeoxycholic acid and ursodeoxycholic acid, but not taurocholic acid, can also promote bile flow by inducing hepatocytes to release ATP into bile, which then stimulates fluid and electrolyte secretion by bile duct epithelia downstream via changes in cytosolic  $\text{Ca}^{2+}$  (402). Thus, biliary secretion of ATP by hepatocytes provides a paracrine mechanism for regulation of secretion in bile ducts via  $\text{Ca}^{2+}_i$  signaling (159) (Fig. 11).

### Cytokine and other receptors

Proinflammatory cytokines, IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , all inhibit the formation of bile secretion by reducing the expression of both basolateral and canalicular transport proteins, as well as by altering cytoskeletal proteins and the permeability of the tight junction (180, 200, 389, 446, 554). Administration of either IL-1 $\beta$  or TNF- $\alpha$  decreases bile flow by decreasing expression and activity of *Ntcp* at the transcriptional level (136, 264, 551) as a result of decreased binding of the nuclear receptor Hnf1  $\alpha$  to the *Ntcp* promoter. *Oatps* are also downregulated (179, 325). Expression of apical transporters, *Mrp2* and *Bsep*, is also impaired (325) as a result of decreased expression of nuclear hormone receptors, *Fxr*, *Pxr*, and *Car* (136, 179). Cytokine-induced inflammation also increases circulating and hepatic nitric oxide (NO), alters the cytoskeleton and increases tight junction permeability (99). In contrast, exogenous sources of NO in isolated perfused rat livers increase BSIF by stimulating GSH disulfide excretion, effects that are independent of cyclic guanosine monophosphate (cGMP) (556). On the other hand, endotoxin impairs the biliary excretion of GSH and blocks the choleric effects of NO (555). Proinflammatory cytokines also inhibit secretion from the bile duct epithelium (516).

### Second messengers

**Cyclic adenosine monophosphate**—cAMP is a primary agonist for stimulation of bile secretion from both hepatocytes and cholangiocytes. cAMP stimulates vesicular transcytosis (219), targeting of vesicles to the canalicular membrane, (87, 461) exocytosis, and BSIF (219) and also decreases the intracellular transit time it takes for bile salts to stimulate BSDF (87). cAMP stimulates taurocholate uptake via *Ntcp* but not *Oatp* (203). This is potentiated by  $\text{Ca}^{2+}_i$ /calmodulin, although increases in  $\text{Ca}^{2+}_i$  alone do not stimulate *Ntcp*. cAMP increases bile acid uptake by translocating *Ntcp* to the plasma membrane (392) and increasing the transport maximum. The PI3 kinase/PKB signaling pathway also seems to be

involved (581). However, cAMP-mediated phosphoinositide-3-kinase-independent activation of Rab4 facilitates Ntcp translocation in HuH-Ntcp cells (487).

cAMP stimulates organic anion excretion into bile by increased targeting of Mrp2 to the canalicular membrane (461) by PI3K-independent and MKK3-mediated activation of p38 $\alpha$  MAPK (488). cAMP also increases lipid (sphingomyelin) transport to the canalicular membrane, a process that is opposed by PKC in HepG2 cells (604). cAMP has no effect on paracellular permeability in hepatocytes (405).

In bile duct cells, cAMP also stimulates secretion through multiple mechanisms, as briefly reviewed in the section on cholangiocyte secretion. Forskolin administration to rats *in vivo* stimulated secretion by cAMP/PKA/Src/MEK/ERK1/2 pathway, presumably at the level of cholangiocytes (167).

**Ca<sup>2+</sup><sub>i</sub> and protein kinase C**—Ca<sup>2+</sup><sub>i</sub> and PKC have variable effects on bile secretion from hepatocytes although in general they are opposite to the effects of cAMP. Ca<sup>2+</sup><sub>i</sub> and PKC exert several different effects that stimulate secretion. First, Ca<sup>2+</sup> induces contractions of the bile canaliculus which promotes biliary peristalsis and pulsatile increases in flow of bile (403, 405, 579, 580). Second, activation of PKC by phorbol dibutyrate stimulates apical exocytosis in the isolated perfused rat liver (93). Third, activators of PKC stimulate biliary excretion of Mrp2 substrates (460). Tauroursodeoxycholic acid stimulates canalicular exocytosis in association with mobilization of extracellular calcium (63), presumably by activating  $\alpha$ PKC (64) and inserting Mrp2 into the canalicular membrane (61). Thus, PKC seems to be intimately involved in biliary exocytosis. In contrast, Ca<sup>2+</sup><sub>i</sub> and PKC also exert inhibitory effects on bile flow. First, Ca<sup>2+</sup><sub>i</sub> increases tight junction permeability, which is dependent on activation of PKC (405). Second, PKC opposes the effects of cAMP by inhibiting the transfer of lipid (sphingomyelin) to the canalicular membrane, as shown in HepG2 cells (604). Finally, both Ca<sup>2+</sup><sub>i</sub> agonists and PKC activators decrease BSIF in the perfused rat liver (122, 404).

In contrast to hepatocytes, increases in Ca<sup>2+</sup><sub>i</sub> promote secretion in bile duct cells as noted previously (159). Increases in Ca<sup>2+</sup><sub>i</sub> also potentiate secretin-induced cAMP production and secretin's stimulatory effect on bile duct secretion (15). In cholangiocytes, PKC is thought to prime a pool of intracellular vesicles for exocytosis induced by cell swelling. This process is dependent on both activation of PKC and PI-3 kinases (176).

**PI-protein kinases**—Regulation of canalicular exocytosis and targeting of vesicles to the canalicular domain appear to involve PI-3-kinases (303, 382) although the precise mechanisms remain complex and it is difficult to provide an overall view (218, 558). Trafficking of Bsep to the canalicular domain in response to taurocholate seems to be dependent on PI3K activity, in contrast to cAMP stimulation of other ABC transporters which are PI3K independent (382, 383). Rather, in hepatocytes, cAMP stimulates Mrp2 translocation by activating p38 $\alpha$ MAPK (488). p38(MAPK) has been shown to regulate BSEP trafficking from the Golgi to the canalicular membrane in HepG2 cells and in rat hepatocytes (298). Tauroursodeoxycholic acid-induced stimulation of canalicular

taurocholate excretion also involves a MAP kinase-dependent translocation of subcanalicular Bsep to the canalicular membrane (304).

Recent studies suggest that a series of kinases are necessary for the development of the canalicular and bile duct systems. The master kinase LKB1 when knocked out in mice results in a cholestatic phenotype with failure to insert Bsep and radixin into the apical domain and defective canalicular and bile duct structural development. Other studies show that AMPK and its upstream kinase, LKB1, regulate canalicular network formation, and maintenance in rat hepatocyte sandwich cultures (168). Further studies indicate that bile salts stimulate canalicular network formation via this cAMP-Epac-MEK-LKB1-AMPK pathway (169).

**Prostaglandins**—Early studies on the role of prostaglandins in bile formation reported stimulatory effects on bile flow (260, 319). Later studies examined the effects of prostaglandins F2 alpha, D2, and E2 and found inhibitor effects on bile flow and bile salt secretion that were independent of hemodynamic changes (52). When prostaglandin synthesis was suppressed, bile flow was stimulated (52). Prostaglandins function by binding to specific receptors that exert multiple metabolic functions via G protein-coupled receptors. PGF<sub>2α</sub> and PGE<sub>2</sub> induce Ins(1, 4, 5) P<sub>3</sub>-mediated increases in intracellular Ca<sup>2+</sup> which regulate many cell functions, including bile secretion (448). Other studies suggest that PGF<sub>2α</sub> can enhance or diminish propagation of Ca<sup>2+</sup> signals, depending on the time of exposure, and thus may play a moderating role on other determinants of the bile secretory process, for example by inhibiting gap junction permeability (292, 406).

**Nitric oxide**—NO is a gaseous substance that mediates many biologic functions including bile formation. NO increases BSIF at the level of the hepatocyte by oxidation of GSH to GSSG (556) which is excreted into bile by the canalicular transporter Mrp2. NO also activates guanylyl cyclase, which increases cGMP (556). cGMP can also stimulate hepatocyte bicarbonate secretion and bile flow (397), but this effect of NO appears to be minor (556). Bile salts can also activate nitric oxide synthase in endothelial cells in the liver (eNos) presumably via TGR5, the G protein-coupled bile acid receptor which is expressed on endothelial cells but not hepatocytes (270).

NO can also directly activate PKC, which, in turn, increases tight junction permeability in hepatocytes (99), as well as inhibiting canalicular contractions (147), effects that would be expected to decrease bile flow. Chronic elevations of NO in circumstances of infection might contribute to the cholestasis of inflammation by this mechanism. NO can also inhibit bile salt uptake in hepatocytes by a process of S-nitrosylation of NTCP (486).

NO has no acute effects on cholangiocyte secretion when assessed in IBDUs (556). However, NO has a very short half life of approximately a millisecond or less, but its effect can be prolonged by nitrosylation of cysteinyl residues of proteins such as albumin and GSH (517). These nitrosylthiols then can act outside the site of NO synthesis. An example of this is the stimulation of formation of S-nitrosoglutathione in hepatocytes by UDCA but not TUDCA or cholic acid infusions. This is followed by excretion of S-nitrosoglutathione into bile via Mrp2 where it subsequently acts on protein kinase B in cholangiocytes leading to

enhancement of UDCA-stimulated ATP release and bile duct secretion. Presumably, this mechanism contributes to the hypercholeretic effect of UDCA in the intact animal (459).

In cholangiocytes, stimulation of nitric oxide production by cytokines leads to inhibition of adenylyl cyclase and cAMP-dependent secretion, possibly contributing to the cholestasis of inflammation (515).

**Carbon monoxide and H<sub>2</sub>S**—While CO is another diffusible messenger molecule that shares many properties with NO, it can regulate bile secretion by independent mechanisms. CO is endogenously produced during heme metabolism by heme oxygenase. Early studies suggested that inhibition of CO production is associated with decreased hepatic cGMP and leads to increased BSDF and bile salt output in isolated perfused rat liver, despite increases in microvascular resistance secondary to constriction of sinusoids (478). Increases in bile flow appear to be mediated by increases in both canalicular contractile frequency and intracellular Ca<sup>2+</sup> concentrations that are attenuated by the addition of CO in isolated hepatocyte couplets (496).

In addition to CO, heme oxygenase also produces bilirubin IX<sub>α</sub>. When perfused rat livers are administered concentrations of CO in the 4 to 6 μmol/L range, a choleresis is induced in which increased biliary excretion of bilirubin IX<sub>α</sub>, GSH, and bicarbonate are seen. These effects are dependent on the function of Mrp2 and tetraethylammonium (TEA) sensitive K<sup>+</sup> channels but are independent from cGMP-mediated secretion (414). This effect of CO may be a beneficial response to “stress” related events such as hypoxia and oxidative stress.

CO binds to the prosthetic heme of cystathionine betasynthase (CBS), the rate-limiting enzyme in transsulfuration pathways and H<sub>2</sub>S generation, suggesting that CBS serves as a CO-sensitive modulator of H<sub>2</sub>S production (497). H<sub>2</sub>S generated through the enzymatic action of cystathionine gammalyase (CSE) modulates biliary bicarbonate excretion and thus is another determinant of bile salt-independent bile formation in rat liver (171). H<sub>2</sub>S generated by CSE also affects hepatic perfusion and loss of these functions may reduce sinusoidal perfusion (162) and affect bile flow.

**Epigenetic regulation of bile acid metabolism and transport**—In addition to transcriptional and posttranscriptional regulation of the determinants of bile formation reviewed in previous sections, recent studies have revealed that the activity of enzymes and transporters that determine bile acid synthesis, metabolism and transport can be regulated by posttranscriptional reversible epigenetic modifications of histones and chromatin (507). There are two classes of factors that modify chromatin. The first class are histone modifying transcriptional cofactors that catalyze enzymatic posttranscriptional modifications of core histones by acetylation and methylation. These reactions modify the chromatin structure allowing access of transcription factors and other transcriptional machinery that reversibly increase or decrease gene transcription. Histone acetylation is catalyzed by histone acetyltransferases and counteracted by histone deacetylases (HDACs). These two enzymes add or remove acetyl groups on lysine residues of core histones which generally activate and repress genes, respectively. Histone methyltransferases and demethylases add or remove methyl groups to lysine or arginine residues in histones which either activate or repress gene

function depending on the amino acid residues that are methylated and whether they form mono-, di-, or trimethylated substrates, The second class of transcriptional cofactors are ATP-dependent remodelers of chromatin. Examples include Swi/Snf complexes that contain the ATPases Brm or Brg-1. For a much more detailed summary of this field see Ref. (507).

Examples of epigenomic transcriptional cofactors that regulate bile acid metabolism and transport include:

1. SHP, the nuclear receptor the short heterodimer partner, while not directly modifying histones, coordinates the sequential recruitment of chromatin modifying cofactors to the promoter of *Cyp7A1*, the rate limiting enzyme in forming bile salts from cholesterol, resulting in inhibition of bile salt synthesis. These cofactors include HDACs, G9a, and Brm-Swi/Snf (378).
2. The histone lysine methyltransferase MLL3 has been identified in histone H3K4 tri-methylation and is important in Fxr activation of bile salt transporter genes, including *SHP*, *BSEP*, *MRP2*, and *NTCP*. MLL3 methyltransferase is downregulated in bile duct ligated mice. MLL3 occupancy of the (ASC-2)-containing coactivator complex in *Bsep*, *Mrp2*, and *Ntcp* promoters was reduced in these cholestatic mice (24, 282).
3. The coactivator-associated arginine methyltransferase1 (CARM1) is an epigenomic regulator of the BSEP gene initiated by the nuclear receptor, FXR. Occupancy of CARM1 and FXR on the BSEP promoter increases in response to FXR agonists (23). Thus, FXR potentiates the expression of BSEP by recruiting this chromatin modifier.
4. The protein arginine methyltransferase also functions as a FXR coactivator, increasing the activation of *Bsep* and *Shp* genes in response to 6-EDCA (457), a potent FXR ligand.
5. Set7/9, a lysine methyltransferase, has also been determined to methylate FXR and contribute to the transcriptional activation of FXR-target genes (37).

These few examples of this emerging field illustrate how epigenomic modifications of chromatin regulate key determinants of bile secretory function.

## Transport and Excretion of Specific Substances

### Lipid Secretion

The major lipid constituents of bile are PC and cholesterol. Their concentrations range from 140 to 810mg/dL and 97 to 320 mg/dL, respectively (73). PC accounts for nearly all biliary phospholipids in bile even though the canalicular membrane also contains sphingomyelin, phosphatidylethanolamine, and phosphatidylserine (427). Excretion of PC requires a canalicular floppase (*MDR3* in humans or the mouse homologue, *Mdr2*) and is dependent on the output of bile salts (425,505) (see section on *MDR3/Mdr2*). PC is absent from bile in *Mdr2* null mice (505) irrespective of bile salt output (425). There are two proposed mechanisms to explain how PC is excreted into bile (Fig. 8). First PC is delivered to the canalicular membrane via either a cytosolic transport protein or within vesicles, and then is

flopped to the outer leaflet via MDR3/Mdr2. The resultant accumulation of PC on the outer leaflet of the canalicular membrane is inherently unstable, thus bile salts excreted into the canalicular lumen might directly facilitate PC extraction (427). An alternative hypothesis suggests that bile salts destabilize microdomains of PC that have accumulated on the outer leaflet, leading to vesicles that first bud, then pinch off into bile (125). Both hypotheses account for the observations that MDR3/Mdr2 and bile salts are required for phospholipid excretion.

As discussed earlier [see section on sterolin 1 and 2 (*ABCG5/G8*)] most cholesterol is excreted into bile by these heteromeric ABC transporters. Phospholipid excretion via MDR3/Mdr2 may play some role in cholesterol excretion as well, since mice lacking Mdr2 have little to no cholesterol in bile (425, 505), although administration of bile salts to *Mdr2* null mice stimulates excretion of cholesterol, but not phospholipid (425). While the mechanism by which *ABCG5/G8* results in extrusion of cholesterol is still unclear, evidence indicates that this process requires micelle forming bile salts rather than other cholesterol acceptors such as APA1 or high-density lipoprotein (HDL) (571). Cholesterol excretion in bile is counteracted by Nieman-Pick C1-like 1 (*NPC1L1*) protein on the canalicular membrane while NPC2, a cholesterol-binding protein secreted by the biliary system, is a positive regulator of biliary cholesterol secretion by stimulating *ABCG5/G8*- mediated cholesterol transport (594). Hepatic *NPC1L1* may control cholesterol homeostasis via the downregulation of NPC2 (592).

Cholesterol associated with HDL is taken up by the receptor SR-B1 at the basolateral membrane of hepatocytes and directly excreted into bile (452). The mechanism by which cholesterol reaches the canalicular membrane is not entirely clear, although it has been suggested that the cytosolic protein sterol carrier protein-2 binds cholesterol to aid in its delivery to the canaliculus (21, 170). Overexpression of the HDL receptor SR-B1 increases biliary cholesterol while decreasing serum cholesterol (293). Since SR-B1 is also expressed on the canalicular membrane it presumably plays a role in facilitating cholesterol excretion in bile. See ref (140) for more details regarding the multiple factors involved with cholesterol excretion into bile.

### Proteins in bile

See earlier discussion.

### Amino acids and peptides

Rat bile contains significantly more acidic amino acids (aspartic and glutamic) and sulfur containing amino acids (cystine and methionine) than in serum and much lower amounts of basic amino acids (lysine and ornithine) (165). Acidic and sulfur-containing amino acids are present in mmol/L amounts in bile (73). The tripeptide GSH is a major biliary peptide whose secretion by Mrp2 represents the principle determinant of canalicular BSIF (48). The enzymatic activity of  $\gamma$ -glutamyltransferase in the luminal membranes of the bile canaliculus and bile ducts metabolizes GSH to glutamic acid plus cysteinylglycine, while a dipeptidase metabolizes the latter to cysteine and glycine. These amino acid and peptide constituents are then reabsorbed from bile by specific transport systems (44, 48) discussed previously.



## Metals

Bile is the major route of excretion of divalent heavy metal cations, including copper, iron, zinc, manganese, mercury, lead, silver, and cadmium (123, 283). The precise mechanisms by which most of these metals are excreted remains unclear. (38). Metals are normally complexed with amino acids, peptides, proteins, and other tissue constituents and usually do not exist in free solution. A few metals like  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  which are similar in concentration in blood plasma and bile, may enter bile by a paracellular pathway by diffusion across the tight junctions (73). A P-type ATPase, ATP7B, is required for the biliary excretion of copper and is mutated in Wilson's disease (98, 217, 345, 465, 544). This protein is localized to the *trans*-Golgi apparatus and nearby vesicles (241). ATP7B traffics in vesicles where it fuses either to the canalicular membrane or to pericanalicular lysosomes so that copper is excreted into bile either directly across the canalicular membrane or by exocytosis from lysosomes (139). Some paracellular excretion may also occur (229). Manganese may also enter bile after transport into vesicles at the basolateral and sinusoidal pole of the hepatocyte. In rat hepatocytes and WIF-B cells (328) this is mediated by secretory pathway  $\text{Ca}^{2+}$ -ATPase isoform 1 (SPCA1). Accumulation of manganese in cholestatic liver disease can lead to neurotoxicity resembling Parkinsonism and may play a role in hepatic encephalopathy in chronic liver injury. The ABC canalicular transporter, Mrp2 (ABCC2), mediates the transport of a number of metals that can complex with high affinity to the cysteine moiety of GSH including arsenic, lead, gold, zinc, cisplatin, possibly copper, cadmium, silver, and mercury (38). Mutant rats that lack Mrp2 are not able to excrete zinc, copper, silver, cadmium, and methyl mercury into bile, whereas copper excretion is unaffected (42,538).

The role of other canalicular transporters in metal transport including MDR1, MDR3, and MATE remains unclear (38). Some metals can be reabsorbed from bile by amino acid transporters, presumably as cysteine complexes.

## Vitamins

Both water-soluble and fat-soluble vitamins are found in bile although little is known about their specific mechanisms of excretion. Many reports have established that radiolabeled retinol, retinyl acetate, or retinoic acid or their metabolites are rapidly excreted into bile following administration to rodents (413,420). Vitamin A metabolites in bile reflect hepatic levels and increase when liver levels are high (395). Vitamin E (tocopherol) and its oxidative metabolites are excreted in bile and its biliary excretion can be blocked by verapamil in mice and rats, so presumably ABC transporters are involved (395,396). Vitamin D is 25-hydroxylated in the liver, excreted in bile, absorbed in the intestine, and then 1,25-hydroxylated in the kidney. This metabolite is the most bioactive form of vitamin D, and up to 25% of circulating 1,25-dihydroxy vitamin D may be recirculated via the bile each day (301). Studies in man suggest that glucuronide metabolites are the predominant form (323). Vitamin B2 (Riboflavin) is excreted into bile and undergoes a limited enterohepatic recirculation (258). Dietary folate is first transported into the liver, reduced to tetrahydrofolic acid, then methylated. Most of the methylated tetrahydrofolic acid is excreted into bile, then reabsorbed by the intestine. Methylated tetrahydrofolic acid is less avidly taken up by hepatocytes but then is also largely excreted into bile (364,520). Since

Mrps and BCRPs can transport naturally occurring folates, it is likely that these ABC transporters are also important in their biliary excretion as well (34). Vitamin B<sub>12</sub> (cyanocobalmin) is largely stored in the liver. Approximately 5 µg are excreted in bile each day in man and primates (198, 199). Most is reabsorbed together with intrinsic factor. This steady biliary excretion of cyanocobalamin contributes to the development of pernicious anemia in settings where production or absorption of intrinsic factor is defective (364). Studies with radiolabeled vitamin B6 (pyridoxine) in rats suggest that biliary excretion and enterohepatic circulation play a minor role in the economy of this vitamin (344).

## Conclusions

Bile formation and secretion is a unique and vital function of the liver. Although a “hidden secretion” whose primary source is secreted into minute 1 µm diameter canaliculi, the application of cellular and molecular techniques to the study of bile formation in the last two to three decades has resulted in considerable expansion of knowledge in this field. The historical development of these important concepts is detailed in the beginning of this review. Most of the major transporters and enzymes that determine the mechanisms of bile formation have now been characterized at the molecular level in animals and in man. Mutations and polymorphisms in some of these determinants have been discovered that have served to illuminate the specific function of a given transporter or enzyme. Details of these important mechanisms form the body of this text. Much has been learned about the determinants of both BSDF and BSIF and about the specific mechanisms of hepatocyte vs. cholangiocyte secretion. These secretory processes are highly regulated both at transcriptional and posttranscriptional levels with the latter involving complex receptor mediated signal transduction pathways. This knowledge now offers the opportunity to regulate this secretory process therapeutically and future research should offer new insights into these mechanisms which should be beneficial to patients where bile formation is impaired from cholestatic liver disease. The number of different solutes that are excreted into bile is large and in many cases their mechanism of excretion still remains to be studied, particularly for minor constituents such as metals, vitamins and proteins. Although bile is a “hidden secretion” its major determinants are now much better understood.

## Acknowledgments

I am greatly indebted to the many postdoctoral fellows and colleagues whose work has done much to advance information in this field during the past 40 years, since I first became fascinated to understand the mechanisms of bile formation. In many instances, they have become major leaders in the field in their own right. To those whose important work I have omitted or incorrectly cited, please accept my apologies. I welcome your comments for improvement of this on-line overview. I also thank my colleague Carol Soroka for her careful review of the article and for help with illustrations. Publications cited in this review from my laboratory have been continuously supported by NIH USPHS Grant DK25636 since 1972 and P30-34989 since 1984.

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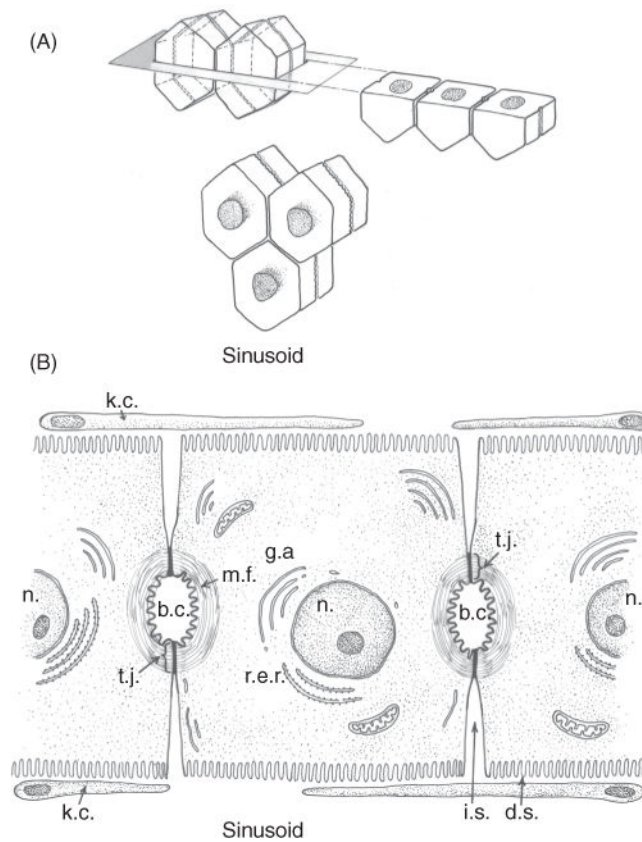
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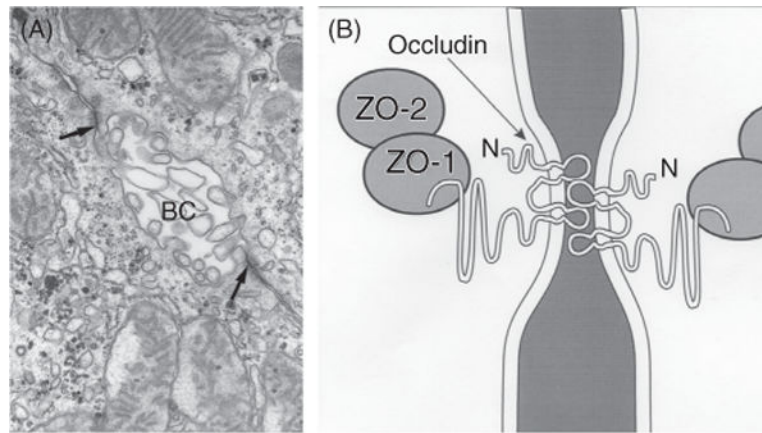




**Figure 1.**

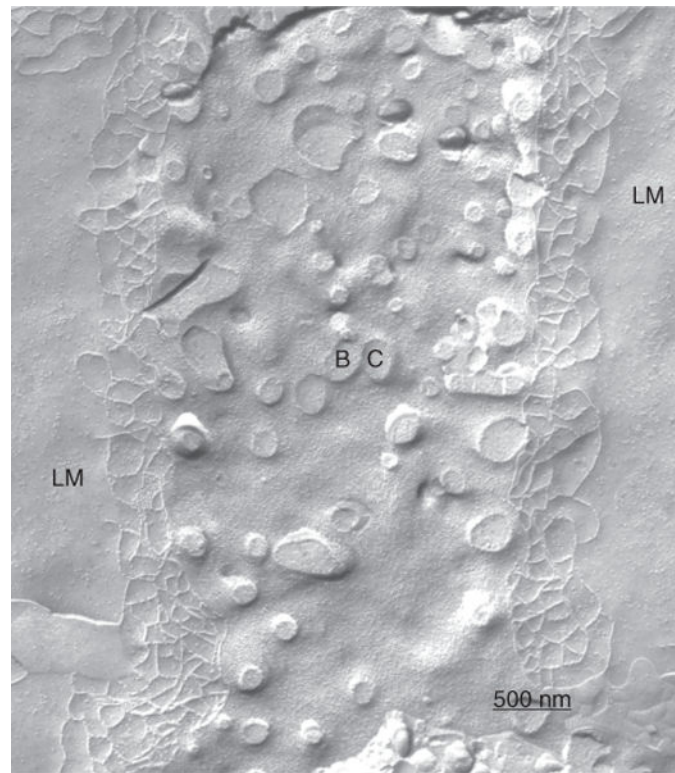
(A) Three-dimensional projection of arrangement of hexagonal hepatocytes in liver plates illustrating the position of bile canaliculi which form a "chicken wire" mesh of interconnecting conduits of the primary secretion of bile. (B) Adjoining hepatocytes illustrating the location of the bile canaliculus (b.c.), intercellular space (i.c.), Disse's space (d.s.), and fenestrated endothelial lining cells and Kupffer cells (k.c.). Tight junctions seal the lumen of the bile canaliculus (t.j.) whose luminal membrane is surrounded by microfilaments (m.f.) and other cytoskeletal elements that provide a contractile mechanism for canalicular peristalsis. Golgi apparatus (g.a.) and rough endoplasmic reticulum (r.e.r.) are also illustrated. Reprinted, with permission, from Ref. (71).



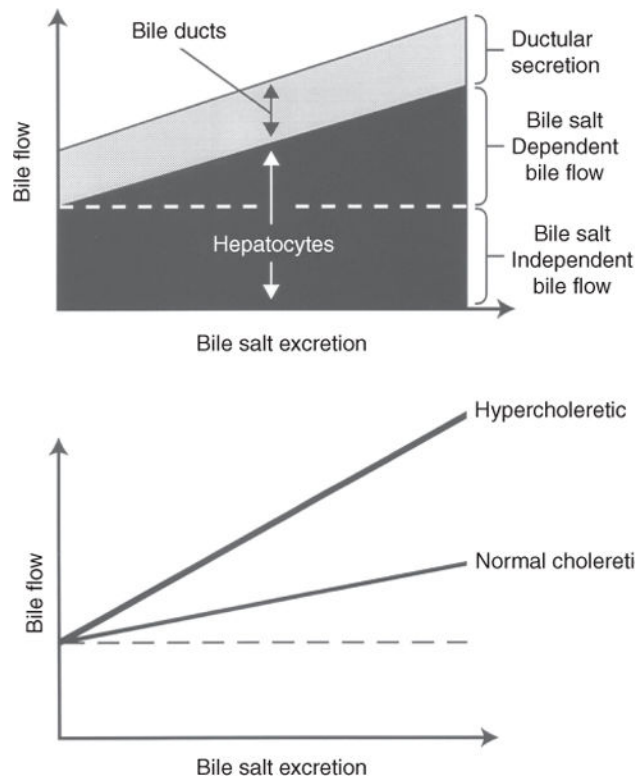


**Figure 2.**

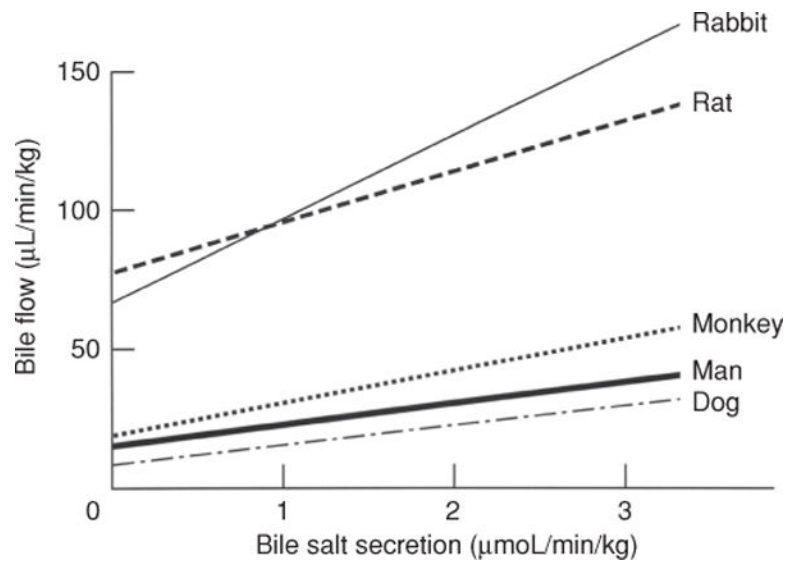
The hepatocyte tight junction complex. (A) Electron micrograph of the bile canaliculus formed between two adjacent hepatocytes and whose lumen is filled with microvilli and sealed by the tight junctions (arrows). (B) Schematic of the tight junction complex showing that occludins and claudins are transmembrane proteins forming the junction seal, whereas zonula occludens proteins 1 and 2 (*ZO1* and *ZO2*) are cytoplasmic proteins that may serve as anchors for occludins. The latter protein forms the interconnecting strands illustrated in the freeze fracture in Figure 3. See Ref. (384) for more details of tight junction anatomy. Reprinted, with permission, from Ref. (76).



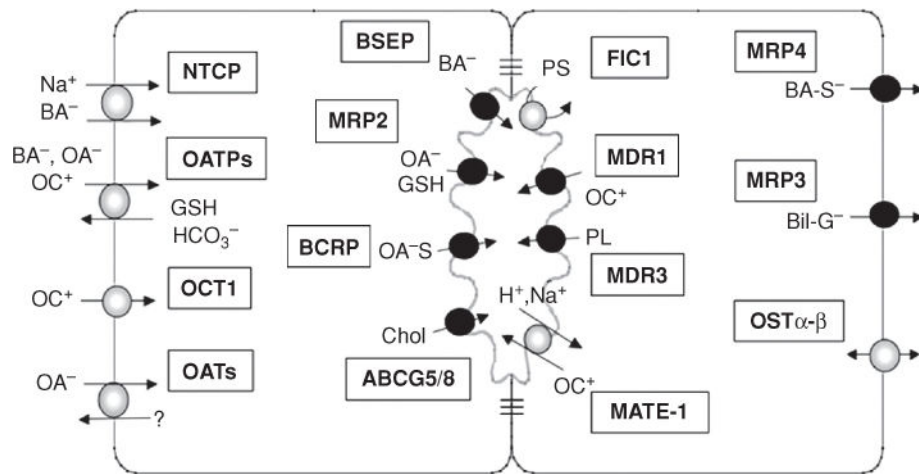
**Figure 3.** Freeze fracture replica of the bile canaliculus (BC). The tight junction elements represent the only anatomical barrier between bile and the intercellular space lined by the lateral membrane (LM) of the hepatocyte. Magnification  $\times 39,186$ . Reprinted, with permission, from Ref. (72).



**Figure 4.** Graphic representation of determinants of bile flow. Top: canalicular bile flow consists of a bile salt-dependent (BSDF) and a bile salt-independent (BSIF) fraction. The BSDF increases linearly as a function of bile salt excretion. Both canalicular bile flow and secretion from the bile ducts contribute to total bile flow. Bottom: choleretic potential varies among bile salts. Bile flow is linearly related to bile salt excretion, but hypercholeretic bile salts increase bile flow more rapidly than do bile salts with normal choleretic potential. Reprinted, with permission, from Ref. (76).

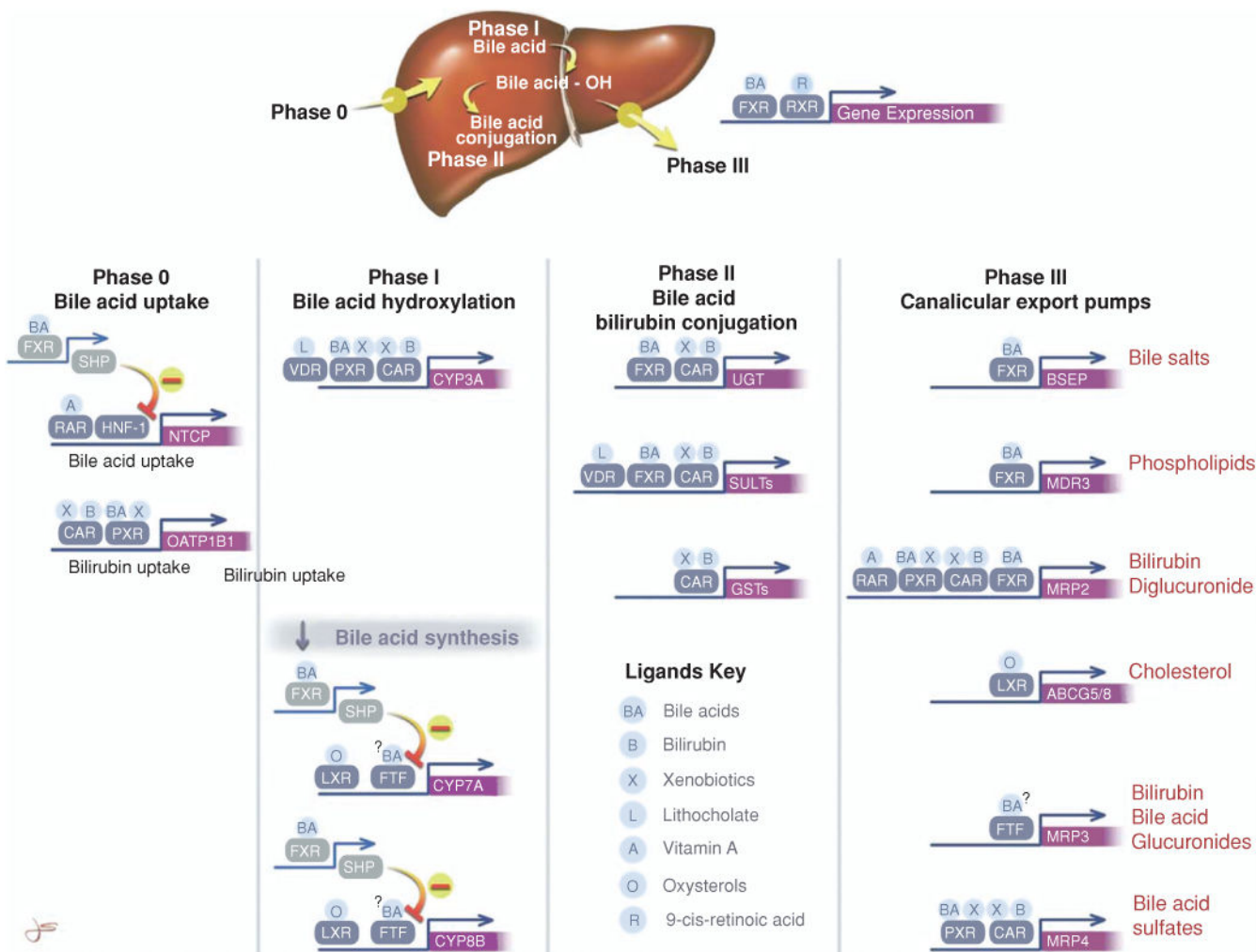


**Figure 5.** Relationship between bile salt concentration and bile flow in various animal species. Note that extrapolation to zero bile salt excretion yields a positive intercept for BSIF that is greater in rats and rabbits than in the three other species. Reprinted, with permission, from Ref. (73)



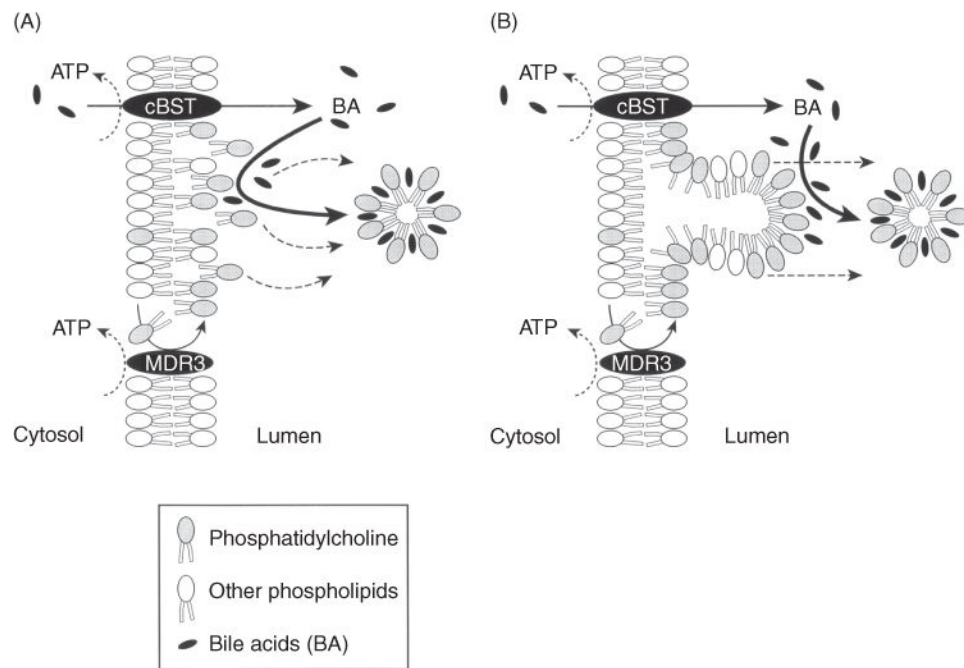
**Figure 6.**

Membrane transporters that determine the uptake and excretion of bile salts and other organic solutes in hepatocytes. (Na<sup>+</sup>, sodium; BA<sup>-</sup>, bile salts; OA<sup>-</sup>, organic anions; OC<sup>+</sup>, organic cations; GSH, glutathione; HCO<sub>3</sub><sup>-</sup>, bicarbonate; OA-S, sulfated organic anions; Chol, cholesterol; H<sup>+</sup>, proton; PL, phospholipid; PS, phosphatidyl serine; BA-S<sup>-</sup>, sulfated bile salts; Bil-G, bilirubin glucuronide). Also see Table 4 for full terminology and function for these and other transporter determinants of bile secretion. Reprinted, with permission, from Ref. (79).

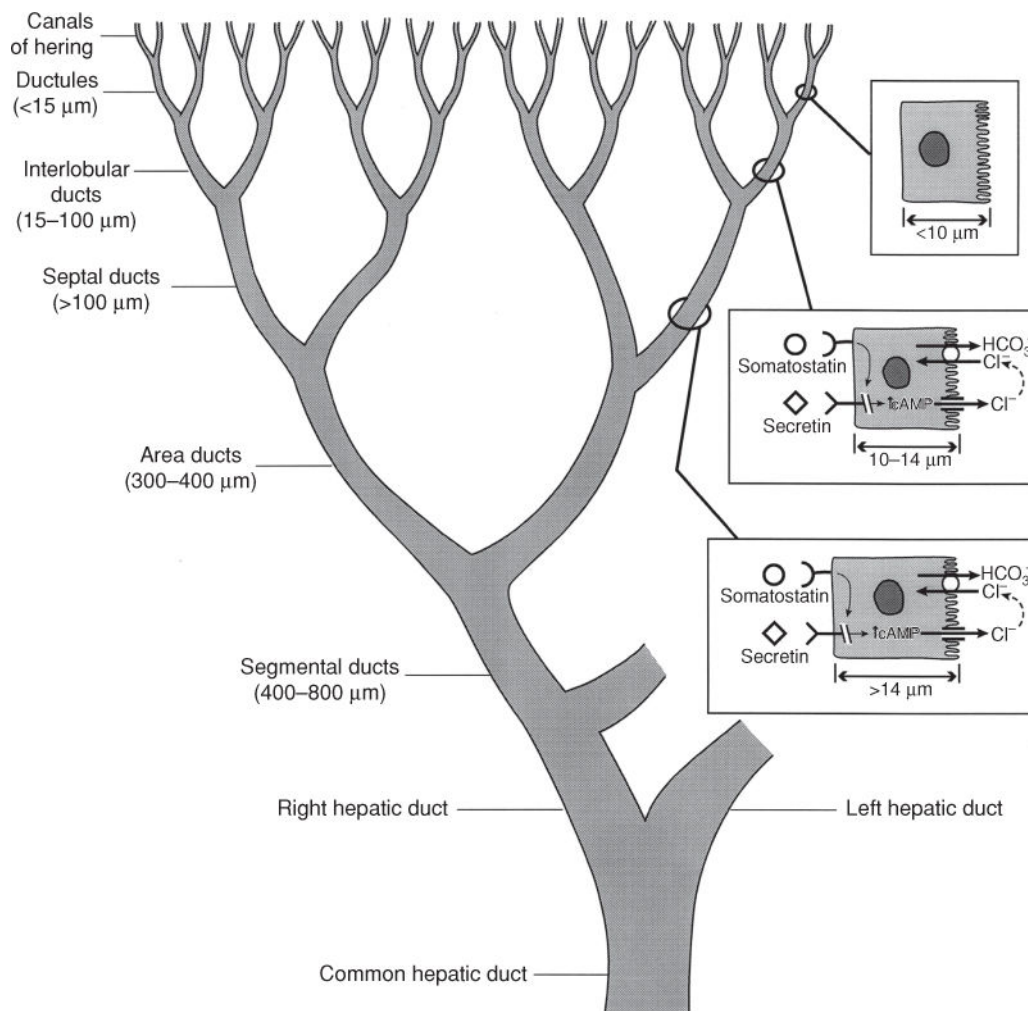


**Figure 7.** The hepatic clearance of bile salts and other organic solutes is determined by four steps or phases; Phase 0, hepatic uptake; Phase I, hydroxylation by cytochrome 3A and other CYP450s; Phase II, conjugation reactions with glucuronides, glutathione, sulfates, or acetates; and Phase III, export from the liver by adenosine triphosphate-dependent ATP-binding cassette (ABC) transporters. The figure also shows the coordinated ligand-activated regulation of gene expression that determines the hepatic clearance of bile salts, bilirubin, and xenobiotics. Some of the major nuclear receptors that regulate the expression of these key genes are shown. Unless otherwise indicated by ↓ or – symbols, these ligands stimulate gene expression. Normally, many of these nuclear receptors form heterodimeric complexes with the retinoid X receptor (RXR). This complex then binds to specific response elements in the gene promoter. Other nuclear receptors such as short heterodimeric protein-1, fetal transcription factor (FTF), and hepatocyte nuclear factor 1 (HNF-1) do not form heterodimers with RXR and do not have specific ligands. Reprinted, with permission, from Ref. (78).

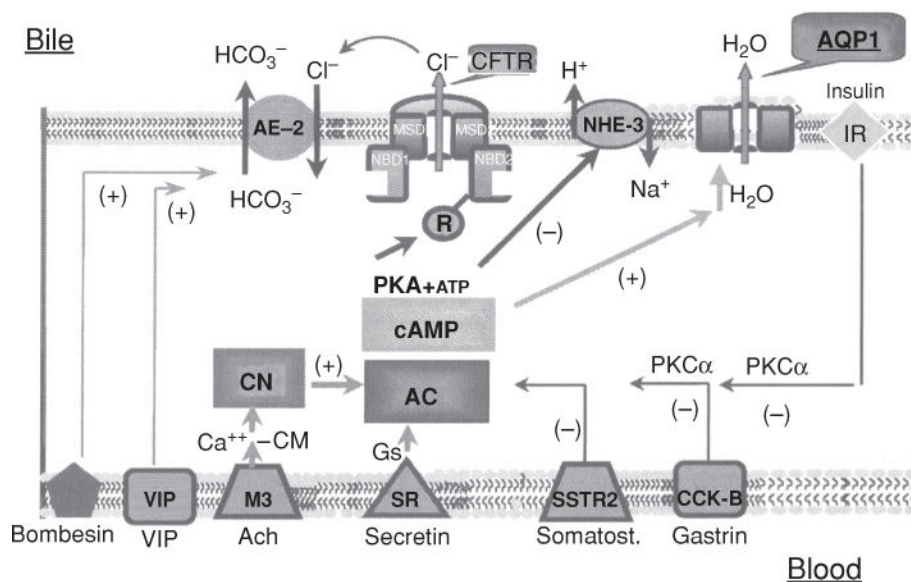




**Figure 8.** Two alternative mechanisms for the mechanism of phosphatidylcholine (PC) excretion into bile. (A) Bile salts are transported into the canalicular lumen by the canalicular bile salt transporter (cBST) (now termed BSEP), and PC accumulates on the luminal side of the canalicular membrane by the action of MDR3. Luminal bile salts then extract the phospholipid from the membrane into micelles. (B) MDR3 flops PC to the external domains of the canalicular membrane bilayer which extrude into the bile lumen and are destabilized by bile salts which pinch off the membrane. Reprinted, with permission, from Ref. (76) as modified from Oude Elferink RPJ, Tytgat GNJ, Groen AK. *Faseb J*, 11: 19, 1997(427).

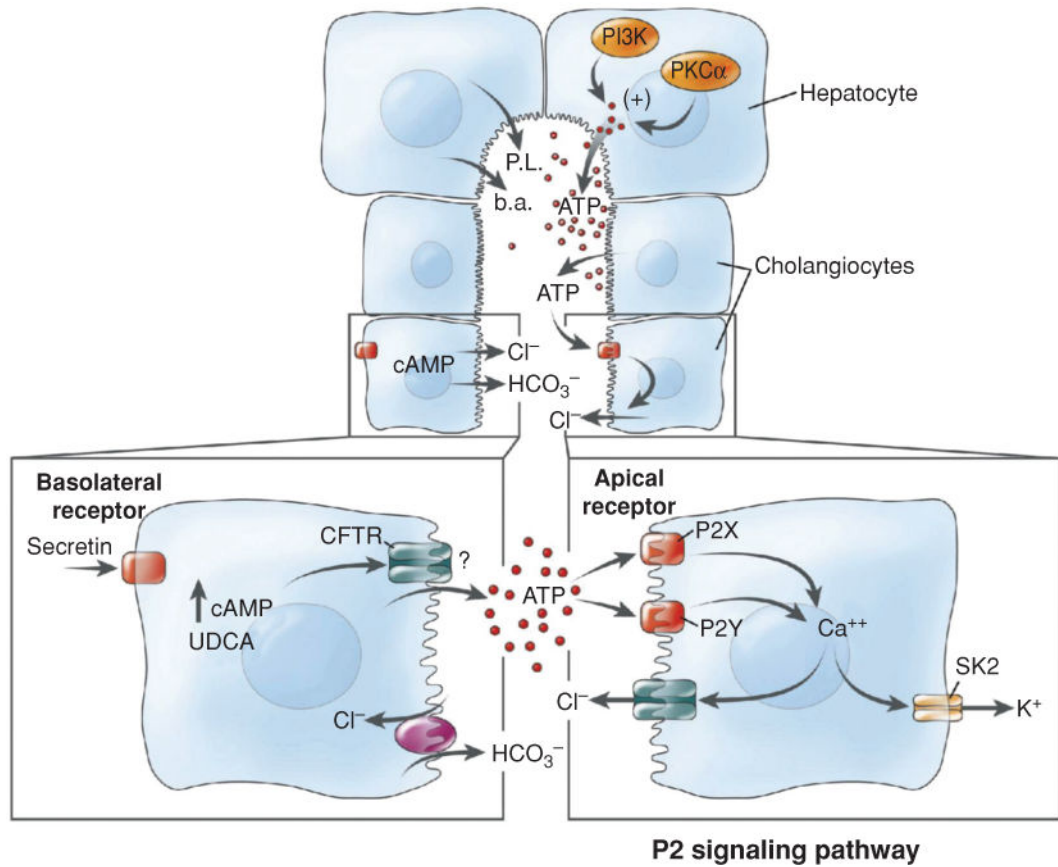


**Figure 9.** The figure illustrates the heterogeneity of the structure and function of the biliary tree and bile duct epithelial cells. Canalicular bile secreted by hepatocytes enters the biliary tree by joining upstream with the canals of Hering. As branches of the biliary tree join, the luminal diameter increases (values in parentheses) and the bile duct epithelial cells become larger. The range of receptors and transporters on medium and large bile duct cells is similar although secretin receptor expression and  $\text{Cl}^-/\text{HCO}_3^-$  exchange activity is greater in the median and large size bile duct cells. Reprinted, with permission, from Ref. (76).



**Figure 10.**

Hormonal regulation of cholangiocyte  $\text{HCO}_3^-$  excretion based on studies in rodents. Secretin induces ductular bicarbonate-rich choleresis by activation of apical  $\text{Cl}^-/\text{HCO}_3^-$  exchanger via a cyclic adenosine monophosphate (cAMP) and PKA-dependent pathway; acetylcholine, by activation of calcineurin, induces a “sensitization” of adenylcyclase to secretin leading to a maximal stimulation of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. Vasoactive intestinal peptide (VIP) and bombesin stimulate cholangiocyte bicarbonate secretion via a cAMP and cyclic guanosine monophosphate (cGMP)-independent pathway. Somatostatin, gastrin, and insulin inhibit both basal and hormonal induced bicarbonate cholangiocyte secretion via a PKC- $\alpha$ -dependent pathway. Ach, acetylcholine; M3, muscarinic receptor 3; SR, secretin receptor; CM, calmodulin; AC, adenyl cyclase; PKA, protein kinase A; PKC $\alpha$ , protein kinase c alpha; AE-2,  $\text{Cl}^-/\text{HCO}_3^-$  exchanger; CFTR, cystic fibrosis transmembrane conductance regulator; NHE-3, sodium hydrogen exchanger isoform 3; AQP1, aquaporin 1; IR, insulin receptor. Reprinted, with permission, from Ref. (183).



**Figure 11.**

The role of extracellular adenosine triphosphate (ATP) in bile formation. Proposed model of P2 signaling. Bile formation begins via transport of bile salts, phospholipids, and ATP from the hepatocyte canalicular membrane. Hepatocyte ATP release is positively regulated by phosphatidylinositol 3-kinase (PI3K) and protein kinase C. Secretin stimulates increases in cholangiocyte cyclic adenosine monophosphate (cAMP) levels via stimulation of basolateral receptors resulting in Cl<sup>-</sup> efflux through CFTR and an increase in Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange. Increases in cAMP, as well as exposure to the bile salt ursodeoxycholate (UDCA), may also increase ATP release through a CFTR-dependent mechanism. BA, bile acids; PL, phospholipids; SK2, Ca<sup>2+</sup>-activated K<sup>+</sup> channel; CFTR, cystic fibrosis transmembrane conductance regulator. Reprinted, with permission, from Ref. (159).

Table 1

Composition of Bile<sup>a</sup>

Water (~95%)		Bile/plasma ratio
Electrolytes Na K		
Na	141–165 mEq/L	~1
K	2.7–6.7 mEq/L	~1
Cl	77–117 mEq/L	~1
HCO <sub>3</sub>	12–55 mEq/L	~1
Ca	2.5–6.4 mEq/L	~1
Mg	1.5–3 mEq/L	~1
SO <sub>4</sub>	4–5 mEq/L	
PO <sub>4</sub>	1–2 mEq/L	
Organic anions		
Bile salts	3–45 mmol/L	>1
Bilirubin	1–2 mmol/L	>1
Lipids		
Cholesterol	97–310 mg/dL	<1
PC	140–810 mg/dL	<1
Steroid hormones, estrogen		
Proteins	<10 mg/mL	<1
Plasma proteins		
Albumin		<1
Haptoglobin		
IgA		>1
Apo-transferrin		>1
Pheromones		
Prolactin		
Insulin		
Hepatocyte proteins		
Alkaline phosphatase		<1
Acid phosphatase		<1
<i>N</i> -acetyl- $\beta$ -glucosaminidase		<1
$\beta$ -glucuronidase		
$\beta$ -galactosidase		<1
5'-nucleotidase		<1
80-kd secretory component of pIgA receptor	>1	
Peptides and amino acids		
GSH	3–5 mmol/L	>1
GSSG	0–5 mmol/L	>1
Cystinyl glycine		>1
Glutamic acid	0.8–2.5 mmol/L	>1
Cysteine		

Water (~95%)		Bile/plasma ratio
FGF19		>1
Aspartic acid	0.4–1.1 mmol/L	>1
Glycine	0.6–2.6 mmol/L	>1
Nucleotides		
ATP	0.1–6 $\mu$ mol/L	
ADP	0.1–5 $\mu$ mol/L	
AMP	0.06–5 $\mu$ mol/L	
Heavy metals		
Cu	2.8 mg/L	>1
Mn	0.2 mg/L	>1
Fe	< 1mg/L	>1
Zn	0.2–0.3 mg/L	>1
Vitamins		
25-OH vitamin D		
Cyanocobalamin	15–200 $\mu$ g/L	
Riboflavin		
Folate	4–60 $\mu$ g/L	

<sup>a</sup>Values are from measurements in human or rodent bile.

NOTE: Modified from Ref. (73) with permission. (Boyer JL. Mechanisms of bile secretion and hepatic transport. In: Andreoli TE, Hoffman JF, Fanestil DD, Schultz SG, eds. Physiology of membrane disorders. New York: Plenum Publishing, 1986:609–636, with permission.)



Table 2

Components of Bile Flow<sup>a</sup> [adapted, with permission, from Ref. (73)]

	Rhesus				
	Man	Dog	Monkey	Rat	Rabbit
Total flow ( $\mu\text{L}/\text{min}/\text{Kg}$ b.w.)	5	10	15	70	90
Bile salt-stimulated flow ( $\mu\text{L}/\mu\text{mole}$ bile salt)	7	8	16	15	70
Bile salt-independent flow ( $\mu\text{L}/\text{min}/\text{Kg}$ b.w.)	2	5	7	40	60
Bile duct flow ( $\mu\text{L}/\text{min}/\text{Kg}$ b.w.)	2	— <sup>b</sup>	— <sup>b</sup>	NS	NS

<sup>a</sup> Representative values from the literature.

<sup>b</sup> Ducts reabsorb canalicular flow following cholecystectomy and cannula insertion.

Abbreviation: NS, not significant.

**Table 3**

Influence of Bile Salt Structure [micelle vs. nonmicelle (TDHC); conjugated (TC) vs. unconjugated (NC) on Their Choleric Potential ( $\mu\text{L}$  of bile per  $\mu\text{mole}$  of bile salt)]

Species	TC	NC	UDC	Nor-UDC	TDHC
Rat	8.4–15 (285,438)	10 (416)	~40 (282)	91 (416)	30–40 (81)
Rabbit	9 (474)	–	–	–	–
Guinea Pig	20–30 (599)	–	~22 (599)	~31 (599)	–
Hamster	~ 10 (204)	–	~23 (599)	~ 67 (599)	–
Dog	8 (417,586)	–	–	15 (417)	–
Cat	7.9 (492)	–	–	–	–
Rhesus monkey	13–20(36,145)	–	–	–	60–78 (36)
Baboon	10–22 (527)	–	–	–	–
Human	6.7 (80)	–	–	–	17.3 (80)

NOTE: Numbers in parentheses are references.

Data are estimated from studies in different species (Modified from (76) with permission).

Abbreviations: TC, taurocholate; NC, nor-cholate; UDC, ursodeoxycholate; Nor-UDC, nor-ursodeoxycholate; TDHC, taurodehydrocholate.

**Table 4**

Nomenclature, Location, and Function of the Major Hepatocyte and Cholangiocyte Membrane Transporters Involved in Bile Secretion

Name	Abbreviation (gene)	Phase	Location	Function
Sodium-taurocholate cotransporter	NTCP (SLC10A1)	0	Basolateral membrane of hepatocytes	Primary carrier for conjugated bile-salt uptake from portal blood
Organic-anion-transporting polypeptides	OATPs (SLCO1B1 and 1B3)	0	Basolateral membrane of hepatocytes	Broad substrate carriers for sodium-independent uptake of bile salts, organic anions, and other amphipathic organic solutes from portal blood
Organic solute transporter alpha/beta	OST $\alpha/\beta$	III	Basolateral membrane of hepatocytes, cholangiocytes, ileum, and proximal tubule of kidney	Heteromeric solute carrier for facilitated transport of bile salts across basolateral membrane of ileum. Expression induced in liver in cholestasis
Organic cation transporter-1	OCT-1 (SLC22A1)	0	Basolateral Membrane of hepatocytes	Facilitates sodium-independent hepatic uptake of small organic cations
Organic anion transporter-2	OAT-2 (SLC22A7)	0	Basolateral membrane of hepatocytes	Facilitates sodium-independent hepatic uptake of drugs and prostaglandins
Multidrug-resistance-1 P-glycoprotein*	MDR1 (ABCB1)	III	Canalicular and cholangiocyte apical membrane	ATP-dependent excretion of various organic cations, xenobiotics, and cytotoxins into bile; barrier function in cholangiocytes
Multidrug-resistance-3 P-glycoprotein (phospholipid transporter)*	MDR3 (ABCB4)	III	Canalicular membrane	ATP-dependent translocation of PC from inner to outer leaflet of membrane bilayer
Bile salt export pump*	BSEP (ABCB11)	III	Canalicular membrane	ATP-dependent bile-salt transport into bile; stimulates BSDF
Multidrug-resistance-associated protein 2 (canalicular multispecific organic-anion transporter)*	MRP2 (ABCC2)	III	Canalicular membrane	Mediates ATP-dependent multispecific organic-anion transport (e.g., bilirubin diglucuronide) into bile; contributes to bile salt-independent bile flow by GSH transport
Multidrug-resistance-associated protein 3*	MRP3 (ABCC3)	III	Basolateral membrane of hepatocytes and cholangiocytes	Expression induced in cholestasis. Transports bilirubin and bile acid glucuronide conjugates
Multidrug-resistance-associated protein 4*	MRP4 (ABCC4)	III	Basolateral membrane of hepatocyte; apical membrane of proximal tubule of kidney	Expression induced in cholestasis transports sulfated bile acid conjugates and cyclic nucleotides
Multidrug-resistance-associated protein-6*	MRP6 (ABCC6)	III	Basolateral membrane of hepatocyte	ATP-dependent transport of organic anions and small peptides. Mutations of MRP6 gene

Name	Abbreviation (gene)	Phase	Location	Function
Breast cancer resistance protein *	BCRP (ABCG2)	III	Canalicular membrane, proximal tubule of kidney	result in pseudoxanthoma elasticum ATP-dependent multispecific drug transporter, particularly sulfate cong; protoporphyrins are endogenous substrate. Substrate overlap with MRP2
Sterolin-1 and 2 *	ABCG5/G8	III	Canalicular membrane and apical membrane of intestine	Heteromeric ATP-dependent transporter for cholesterol and plant sterols (e.g. sitosterol)
Multidrug and toxin extrusion protein 1	MATE-1 (SLC47A1)	III	Canalicular membrane and brush border of kidney	Organic cation/H <sup>+</sup> exchanger extrudes cationic xenobiotics

\* These transporters are members of the ABC superfamily. Table reproduced, with permission, from Ref. (79).

**Table 5**

## OATP Substrates

<b>Table 2 substrates transported by the deferent human OATPs</b>			
<b>OATP1A2</b>	<b>OATP1B1</b>	<b>OATP1B3</b>	<b>OATP1CJ</b>
Hormones and conjugates	Hormones and conjugates	Hormones and conjugates	Hormones and conjugates
Estradiol-17 P-glucuronide	Estradiol-17 (3-glucuronide)	Estradiol-17 (3-glucuronide)	Estradiol-17 (3-glucuronide)
Estrone-3-sulfate	Estrone-3-sulfate	Estrone-3-sulfate	Estrone-3-sulfate
DHEA-S	Thyroxine (T4)	DHEA-S	Thyroxine (T4)
Reverse triiodothyronine (TT)	Triiodothyronine (T3)	Testosterone	Triiodothyronine (T3)
Thyroxine (T4)	DHEA-S	Drugs	Reverse triiodothyronine (rT3)
Triiodothyronine (T3)	Prostaglandins	Atrasentan, Bosentan	Thyroxine sulfate (T4S)
Prostaglandins	Prostaglandin Ei	Cefadroxil, Cefazolin	Others
Prostaglandin £2	Bile acids	Cephalexin, Digoxin	BSP
Bile acids	Cholate	Enalapril, Fexofenadine	
Cholate	Taurocholate	Fluvastatin, Gimitecan	
Taurocholate	TUDCA	Lopinavir, Methotrexate	
Glycocholate	Drugs	Demethylphalloin	
TCDCA	Atorvastatin, Olmesartan	Paclitaxel, Docetaxel	
TUDCA	Bosentan, Phalloidin	Methotrexate, Imatinib	
Others	Caspofungin, Pitavastatin	Olmesartan, Phalloidin	
DPDPE	Cefazolin, Pravastatin	Pitavastatin, Telmisartan	
Drugs	Cerivastatin, Rapamycin	Rapamycin, Rifampicin	
Acebutolol, Rosuvastatin	Darunavir, Rifampicin	Rosuvastatin, Valsartan	
Atenolol, Pitavastatin	Enalapril, Rosuvastatin	SN-38	
Sotalol, Ouabain, Labetalol	Ezetimibe, Saquinavir		
Deltorphan n, Nadolol	Flavopiridol, SN-38		
Ciprofloxacin, Talinolol	Fluvastatin, Temocapril		
Fexofenadine, Saquinavir	Gimatecan, Troglitazone		
Gatifloxacin, Darunavir	Lopinavir, Valsartan		
Levofloxacin	Methotrexate		
Imatinib, Methotrexate			
<b>OATP2A1</b>	<b>OATP2B1</b>	<b>OATP3A1</b>	<b>OATP4A1</b>
Prostaglandins	Hormones and conjugates	Hormones and conjugates	Hormones and conjugates
Prostaglandin Ei	Estrone-3-sulfate	Thyroxine (T4)	Estradiol-17 (3-glucuronide)
Prostaglandin E2	DHEA-S	Estrone-3 -sulfate	Estrone-3-sulfate
Prostaglandin Fj,,	Thyroxine (T4)	Prostaglandins	Thyroxine (T4)
Prostaglandin Hi	Prostaglandins	Prostaglandin Ei	Triiodothyronine (T3)
Prostaglandin D2	Prostaglandin E2	Prostaglandin E>	Reverse triiodothyronine (rT3)
8-Iso-prostaglandin F2 <<	Drugs	Prostaglandin F2a	Bile acids
Others	Atorvastatin	Drugs	Taurocholate
Thromboxane B2	Bosentan	Deltorphan BQ-123	Prostaglandins
Drugs	Ezetimibe	Benzyl penicillin	Prostaglandin E >

**Table 2 substrates transported by the deferent human OATPs**

OATP1A2	OATP1B1	OATP1B3	OATP1CJ
Latanoprost	Fluvastatin	Others	Drugs
	Glibenclamide	Vasopressin	Benzylpenicillin
	Pitavastatin	Arachidonic acid	Unoprostone metabolite
	Pitavastatin		
	Montelukast		
	Rosuvastatin		
	Talinolol		

Abbreviations: BSP, bromosulphthalein; DHEA-S, dehydroepiandrosterone sulfate; DPDPE, [D-penicillamine 2,5] enkephalin Reprinted, with permission, from Ref. (418).