History of hepatic bile formation: old problems, new approaches

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Javitt NB. History of hepatic bile formation: old problems, new approaches. Adv Physiol Educ 38: 279–285, 2014; doi:10.1152/advan.00076.2014.—Studies of hepatic bile formation reported in 1958 established that it was an osmotically generated water flow. Intravenous infusion of sodium taurocholate established a high correlation between hepatic bile flow and bile acid excretion. Secretin, a hormone that stimulates bicarbonate secretion, was also found to increase hepatic bile flow. The sources of the water entering the biliary system with these two stimuli were differentiated by the use of mannitol. An increase in its excretion parallels the increase in bile flow in response to bile acids but not secretin, which led to a quantitative distinction between canalicular and ductular water flow. The finding of aquaglyceroporin-9 in the basolateral surface of the hepatocyte accounted for the rapid entry of mannitol into hepatocytes and its exclusion from water movement in the ductules where aquaporin-1 is present. Electron microscopy demonstrated that bile acids generate the formation of vesicles that contain lecithin and cholesterol after their receptor-mediated canalicular transport. Biophysical studies established that the osmotic effect of bile acids varies with their concentration and also with the proportion of mono-, di-, and trihydroxy bile acids and provides a basis for understanding their physiological effects. Because of the varying osmotic effect of bile acids, it is difficult to quantify bile acid independent flow generated by other solutes, such as glutathione, which enters the biliary system. Monohydroxy bile acids, by markedly increasing aggregation number, severely reduce water flow. Developing biomarkers for the noninvasive assessment of normal hepatic bile flow remains an elusive goal that merits further study.

bile flow; bile acids; osmosis; canaliculi; aquaporins

Delineating the basic mechanisms that determine hepatic bile flow is a challenging problem to investigators and demands solution because their disturbances are a pathogenic basis for the development of cirrhosis. In humans, the cirrhosis that occurs when initiated by disturbances in bile flow is usually very slowly progressive. In an experimental model, complete bile duct obstruction (ligation) activates hepatic stellate cells and portal fibroblasts within 24 h (56). In progressive disease, variations in the rate of intracellular accumulation of bile acids probably accounts for the degree of injury and rate of progression to cirrhosis (35).

To develop minimally invasive techniques that will quantify hepatic bile flow, it is useful to review how the initial concepts developed and the problems that arose. This limited review focuses on this early history and how the answers to many of the problems that were identified had to await basic discoveries, such as aquaporins, before further understanding could be obtained.

Chen and Chen, in their book Understanding the Liver: a History (13), write that the earliest reference to bile can be found in the Ebers papyrus (16th century BCE) and that Galen (2nd century CE) described yellow bile as a specific secretion of the liver. Since these ancient times, there have been many studies of bile composition and the effect of biliary constituents on bile flow. A more recent historical review, in 2005, focused on the enterohepatic circulation (58) and credits Moritz Schiff (1823–1896) with the first systematic study of “the biliary cycle.”

As far as I am aware, Ralph W. Brauer (1921–2000) was the first individual to raise the question as to the source of the energy for the water flow that comprises >95% of hepatic bile. He obtained a doctoral degree in both Biochemistry and Physical Chemistry from the University of Rochester. It was this perspective that focused his attention on the quantitative aspects of liver function (9) and the sources of energy for generating water flow.

Brauer used a straightforward experimental design that yielded unequivocal results (10). The study was done after it had been well established that the energy for urine formation was the hydrostatic pressure generated by cardiac muscle contraction and transmitted through renal arteries to generate a flow of water across the glomerular membrane. Perhaps Brauer fully expected that hydrostatic pressure might be the energy for hepatic bile formation, but his experimental design yielded a different result. The isolated liver was perfused at different known pressures. The flow of bile into a cannula inserted in the bile duct and held in a vertical position indicated that the bile always rose to a higher level than the perfusion pressures that were used. Hydrostatic pressure could not account for this finding, and he therefore concluded that “bile formation requires the application of metabolic energy to perform osmotic work” (10). By metabolic energy, he specified that it “must involve a process of active secretion.” However, perhaps because Brauer was focused on the type of energy required to generate hepatic bile flow, he didn’t specify the solutes in hepatic bile that are actively secreted.

The specific focus on bile acids as the predominant solutes to generate water flow came several years later, in 1959, in a review (67) by Ivar Sperber (1914–2006), Professor of Anatomy and Physiology at the Royal College of Agriculture in Uppsala, Sweden. Being cognizant of Brauer’s studies, he stated that “it appears quite possible to assume osmotic filtration as a factor in bile formation. The primary event in bile formation would be the active transfer (from the cells or through the cells) of bile acids (and possibly other, less quantitatively important compounds) into bile capillaries. The osmotic effect of these would result in a flow of water and dissolved molecules and ions into bile capillaries.” As part of his review, there were also experimental data indicating a positive relationship between bile acid excretion and bile flow.

Attempts to quantify hepatic bile formation started in the laboratory of Stanley Bradley (1913–1999) at Columbia University College of Physicians and Surgeons, where, in addition

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to establishing a research laboratory, he chaired the Department of Medicine from 1959 to 1970. He spent 2 yr (1940–1942) in the laboratory of Homer Smith (1895–1962) at New York University School of Medicine. In a personal note (7), Bradley summarizes the life of Homer Smith, their initial meeting, and the many investigators who trained with him that developed indirect methods for quantifying renal plasma flow and glomerular filtration rate using what today are referred to generically as biomarkers. Extensive studies of the biological properties of many nontoxic compounds led this group to identify certain molecules with biological properties that established their usefulness (8). Thus, when it was found that nonmetabolized mannitol, a naturally occurring sugar alcohol with a molecular weight of 182, and inulin, a naturally occurring polysaccharide with a molecular weight of 5,100, had almost identical urine-to-plasma concentration ratios (mannitol: 129 and inulin: 130), it was reasoned that what they had in common was their ultrafiltration at the glomerulus and the lack of reabsorption in the nephron as the filtered water was reabsorbed. Later, polyethylene glycol, with a molecular weight of 1,000 (4), was also found to yield the same urine-to-plasma concentration ratio as inulin.

In 1968, Wheeler et al. (73) summarized their findings using inulin and mannitol for the study of hepatic bile formation. Compared with urine formation, the results were quite different. They obtained bile-to-plasma concentration ratios of ~0.1 for inulin; for mannitol, the values ranged from 1.2 to 3. It was also found that when a naturally occurring bile acid, sodium taurocholate (72), was infused intravenously, both bile flow and mannitol excretion increased, but when the hormone secretin was given only bile, flow increased and mannitol excretion remained relatively constant. Knowing that secretin is a hormone that stimulates the secretion of a solution of bicarbonate from the pancreas, it was reasoned that the sources of the increased water flow in response to bile acids and secretin were different. Water containing mannitol in response to bile acid infusions is derived from canaliculi, and water not containing mannitol is derived from ductules. These findings, in four dogs, were essentially the same as those previously reported in studies by Forker (23) and Chenderovitch et al. (14) using guinea pigs, with the exception that in the steady state, the bile-to-plasma concentration ratio for mannitol was much less in guinea pigs.

Other previous studies, not using biomarkers, published in 1928 (47) were those of John Mellanby (1879–1939). He found that the effect of secretin on bile flow was different from that of bile acids. The introduction of biomarkers, if they could be further validated, provided a quantitative approach to distinguishing the two sites, canaliculi and ductules.

Based on the analogy to the nephron, where mannitol once filtered at the glomerulus diffuses little during water reabsorption and therefore yields a high urine-to-plasma concentration ratio, the low bile-to-plasma concentration ratio was interpreted as evidence that there is very little reabsorption of the water entering the biliary system at the level of the canaliculus. However, when it was found that polyethylene glycol-900 had a much higher bile-to-plasma concentration ratio than mannitol (24, 25, 30), this interpretation became untenable. Thus, other than distinguishing between water entry at both canaliculi and ductules, the biomarkers developed so successfully for quantifying water filtration and reabsorption in the nephron were not applicable to hepatic bile formation.

Further insights on the relationship of inulin and mannitol to the liver can be obtained from the study of Schanker and Hogben (60) published in 1961. The volume of distribution of inulin in the liver indicated that it did not enter the hepatocyte and remained extracellular. Thus, the very low bile-to-plasma concentration ratio of ~0.1 was consonant with the knowledge that it was absent from water moving from the hepatocyte into the canalculus but that it might enter via a paracellular site, the tight junction.

The presence of a protein, claudin-2, with a pore size of 6.5 Å (water: 2.8 Å) in the tight junction that seals the canalculus and the finding of reduced hepatic bile flow with an increase in bile acid concentration in mutant mice lacking this protein implied that some water rapidly equilibrates through the tight junction in response to bile acid transport (46).

With regard to mannitol, which is a well-established biomarker of extracellular volume distributing in ~20% of total body water, it was surprising that in the liver, mannitol rapidly distributed into a space equivalent to 72% of tissue weight, indicating rapid entry into the hepatocyte. This difference between inulin and mannitol with respect to the hepatocyte paralleled the findings with respect to bile-to-plasma concentration ratios, although the anomalous behavior of mannitol with respect to the hepatocyte compared with other tissues remained a puzzle.

The discovery of aquaporins by Agre and coworkers (1) in 1993 provided a much better understanding of water movements at the molecular level. Of the different aquaporins that have been recognized thus far, the properties of aquaporin-1 (68) and aquaporin-9 (71) have been particularly well defined. Aquaporin-1 only permits the movement of solute-free water rapidly across the cell membrane. As might be expected, it is found in the membrane of cells along the length of the nephron (51) and accounts for the reabsorption of water without inulin and other biomarkers of glomerular filtration rate. In contrast, the aquaporin found in the basolateral surface of the hepatocyte is aquaporin-9 (21, 52). When the properties of this aquaporin were studied, it was found to also allow the movement of glycerol, thus changing its designation to aquaglyceroporin (11). On further study, it was found to also allow the movement of mannitol (70), which has an equatorial diameter similar to glycerol. Thus, the mechanism for the rapid entry of mannitol into hepatocytes at the molecular level now became known.

Indirect studies indicating the movement of polyethylene glycol-900 through membranes along its vertical axis (42) supported its entry into hepatocytes via aquaglyceroporin-9, since its equatorial diameter is also similar to mannitol and glycerol.

Thus, the discovery of aquaporins accounted for the presence of these biomarkers in the hepatocyte.

However, there are currently no identifiable aquaglyceroporins in the canicular surface of the hepatocyte, and, therefore, they cannot account for the entry of mannitol or polyethylene glycol-900 into canicular water. The major aquaporin in the canicular surface of the hepatocyte is aquaporin-8 (29), which is not known to allow for the movement of glycerol.

The probable mechanism for the entry of mannitol and polyethylene glycol-900 into canicular water relates to the effect of the naturally occurring bile acids after their transport into the canaliculus. Normally, naturally occurring bile acids,
on entering the canaliculus, generate the formation of vesicles containing phospholipids (lecithin) and cholesterol (15). These vesicles also contain water derived from the hepatocyte and therefore also contain mannitol and polyethylene glycol-900. The entry of these molecules via vesicle formation could be tested directly by the infusion of micellar (vesicle) and non-micellar forming bile acids, since the latter would not be expected to increase mannitol excretion rate. Evidence can also be obtained from review of early data indicating that although sodium taurocholate increased both bile flow and mannitol excretion, the bile-to-plasma concentration ratio of mannitol decreased, implying that only some of the canicular water contained mannitol (73).

With the identification of aquaporins (Fig. 1) and recognition of bile acid-related vesicle formation, water entry into the hepatocyte and canaliculus and its relationship to potential biomarkers such as mannitol and polyethylene glycol have been defined more precisely, but more needs to be learned about events happening downstream of canaliculi.

If we jump to the lower end of the system, the bile ductules, then we encounter aquaporin-1, which only permits water movement (45), and it becomes obvious why secretin stimulates the flow of water that is free of mannitol.

The problem is what is happening in between the canaliculus and ducts. There are a variety of other aquaporins (44, 57) listed as being expressed along the biliary system whose properties have not been fully defined. The canals of Hering, which are very important to bile formation, have no identified aquaporins. Thus, we again must await further elucidation of the properties of these determinants of water movement. In lieu of more precise data, the discrepancy between the bile-to-plasma concentration ratio of mannitol (1.2–3) and polyethylene glycol (~30) probably signifies considerable reabsorption of canicular water with less diffusion of the latter compared with the former.

In contrast to the persistent problems in attempting to define quantitative water movements, molecular biology has greatly advanced our knowledge of solute transport.

Clues to what would be found at the molecular level appeared in 1954 when two pathologists, Dubin and Johnson, at the Armed forces Institute of Pathology reported a new clinical-pathologic entity, “Chronic idiopathic jaundice with unidentified pigment in the liver” (20), that in 1956 was reported to be familial in occurrence (36). Functional analysis of Dubin-Johnson syndrome indicated that although they all had in common conjugated hyperbilirubinemia, the serum bile acids were normal in 12 of 13 patients (32). It was therefore suspected that when the molecular basis for these transport processes became known that they would be different. Indeed, the major canicular transporter for bilirubin glucuronides, multidrug resistance-associated protein 2 [MRP2; also called ATP-binding cassette (ABC) subfamily C member 2] (34), is different from the canicular transporter for bile acids, bile salt export pump (BSEP; also called ABC subfamily B member 1) (19), and mutations in these transporters result in entirely different clinical problems. Mutations in MRP2 (55) result in the accumulation of bilirubin glucuronides in the hepatocyte and an increase in their level in plasma. Although the liver appears dark, there is no inflammation or evidence for cirrhosis. The transporter is not specific for bilirubin conjugates; thus, many other organic anions, particularly xenobiotics (19), and their metabolites accumulate. Therefore, the half-life of many medications is prolonged, which increases the potential for drug-related liver injury if the dose of medication is not adjusted accordingly.

In contrast, mutations in BSEP (41), which reduce canicular transport, always cause a slow progression to cirrhosis. This normal canicular transporter, which is essential for maintaining the secretion of bile acids and can total 12 g per 24 h in an individual eating 3 meals/day, was identified in 1991 by two groups (50, 53). Both identified it as one of the large group of ATP-dependent transporters, and, in 1998, BSEP was further recognized as one of a large group of ABC-type proteins (26).

Although polymorphisms exist in the Na\(^{+}\)-K\(^{+}\)-ATPase transporter in the basolateral membrane that generates an electrochemical gradient for the efficient uptake of bile acids into hepatocytes (16), nonfunctional mutations have not been recognized. From what is known, one might suspect that when imbalances occur between the activities of the basolateral and canicular transporters, they favor the accumulation of bile acids intracellularly to the detriment of the hepatocyte.

Hepatocyte injury and the progression to fibrosis and cirrhosis have been attributed to many different biological effects of these acidic sterols when they accumulate intracellularly (64). It is of considerable interest that mutations in the gene determining the expression of cytochrome P-450 family 27, subfamily A, polypeptide 1, known clinically as cerebrotendinous xanthomatosis, that prevent bile acid synthesis result in the hepatocellular accumulation of large amounts of the triol in-
termed in bile acid synthesis with no evidence of liver injury (5).

Another form of liver injury that is related to canalicular bile acid transport is mutation in ABC subfamily B member 4 (ABCB4; also called multidrug resistance protein 3) (69), the canalicular transporter for phospholipids. The formation of a mixed micelle in bile consisting of phospholipid (lecithin), cholesterol, and conjugated bile acid requires three canalicular transporters: ABCB4 (27), ABC subfamily G member 5/8, and BSEP. The importance of lecithin in modulating the surface-active properties of conjugated bile acids became dramatically apparent when a mutation in ABCB4 (17) was found to be the molecular basis of one type of progressive familial intrahepatic cholestasis: progressive familial intrahepatic cholestasis type 3. Since this review focuses on the history of hepatic bile formation, it does not dwell in depth on the more recent contributions of molecular biology to identifying and characterizing basolateral influx and efflux transporters and other canalicular transporters.

Nor does this review focus on a very new area of organic solute transport that merits full exploration. Until relatively recently, the focus has been almost exclusively on basolateral and canalicular transporters, with little attention on how organic solutes move from one surface of the cell to the other. It is not free flight, as in a baseball game when the ball travels from the outfielder to the catcher, but rather a very tightly controlled process involving the Golgi apparatus (40). Elucidation of this process is likely to identify other molecular bases for disturbances in hepatic bile formation.

We return to the relationship of solute transport to water flow and the early studies that differentiated bile acid-dependent and -independent flow. In 1968 (73), a proposal was made that canalicular water flow can be partitioned between a bile acid-dependent fraction and a bile acid-independent fraction. In one sense, if one accepts the concept initially proposed by Brauer et al. (10) in 1954 and amplified by Sperber (67) in 1959 that it is an osmotically determined flow, then the proposal needs no proof, since, by definition, any solute that enters the canalicular system is potentially capable of generating water flow.

However, attempting to quantify a bile acid-independent fraction by extending the line to a theoretical zero bile acid excretion has problems (Fig. 2). By infusing sodium taurocholate intravenously, which greatly augments the endogenous excretion rate, it was possible to establish a relationship between hepatic bile flow and bile acid excretion that, when graphed, appears to be linear.

By extending this line to the y-axis, equivalent to zero bile acid excretion, a positive intercept can be obtained, which led to the suggestion that “the most probable explanation for the positive zero intercept is that some process other than conjugated bile acid excretion is responsible for the production of canalicular bile at a rate of ~0.1 ml/min” (73). Unfortunately, this interpretation rapidly developed into a paradigm that has become widely adopted and has obscured one of the important physiological properties of conjugated bile acids.

In retrospect, the underlying assumption in extending the line in a linear mode beyond experimentally determined values between bile acid excretion and bile flow is that the osmotic activity of conjugated bile acids is constant. Data bearing on the osmotic activity of sodium taurocholate appeared in 1979 (12). It was found by Carpenter and Lindenbaum (12) at the Department of Pharmaceutical Chemistry at the University of Kansas that the osmotic activity of sodium taurocholate and other bile salts that were studied is much lower at the high concentrations that occur during its infusion and increased as the bile acid concentration falls when the bile acid pool is depleted (Fig. 3). Thus, the osmotic activity of sodium taurocholate at 28 mM is 1.25 and falls to 0.66 at 149 mM (12). The curve of osmotic activity versus concentration (Fig. 3) mimics results from animal studies. Thus, at high concentrations, the osmotic activity appears to plateau, accounting for the apparent linear relationship between bile flow and bile acid excretion. As the bile acid concentration falls, osmotic activity increases, thus accounting for the preservation of flow and positive y-intercept.

The physiological importance of this relationship becomes apparent when we consider the episodic movement of bile acids via the enterohepatic circulation. Thus, the emptying of the gallbladder after meals results in large amounts of conjugated bile acids entering the intestines and the load being rapidly reabsorbed when it reaches the ileum, where they are rapidly absorbed and transported to the liver for rapid uptake and excretion with >90% efficiency. If their osmotic activity remained constant, then bile acid-dependent bile flow would fluctuate similarly. However, the lower osmotic activity at high rates of excretion and increase in osmotic activity as bile acid excretion diminishes maintain the relative constancy of hepatic bile flow.

Since there is no experimental evidence that draining bile from an animal will result in a bile that contains zero bile acids, bile acid-dependent bile flow is always operative but does not exclude a contribution by other solutes. The most studied
Bile acid-independent hepatic bile flow as a concept is in keeping with an osmotically determined flow generated by solute transport. However, clinical relevance may relate more to events in intrahepatic bile ducts. The severe cholestasis that occasionally occurs in neonates with cystic fibrosis places a focus on the CFTR gene normally expressed in intrahepatic bile ducts (39). Expression of this ABC transporter class ion channel promotes Cl− transport followed by Na+ and then water to satisfy the osmotic gradient. Absence of this type of flow is apparently sufficiently severe to occasionally cause cholestasis in the newborn infant.

Although it was known that the relationship of bile flow to bile acid excretion varied with the type of bile acid, the total cessation of bile flow induced by naturally occurring monohydroxy bile acids (31) provided a new tool for understanding the pathogenesis of cholestatic syndromes. It is reasonable, in the absence of direct experimental evidence, that the reduction in bile flow caused by the excretion of monohydroxy bile acids is related to the biophysical properties of bile acids in forming aggregates or micelles.

Thus, the greater rate of bile flow that occurs when sodium taurodehydrocholate is infused (54) compared with sodium taurocholate (Fig. 4) is attributed to the lack of micelle formation with the former compared with the latter. This view is in keeping with the osmotic activity of solutes, which is considered a colligative property that directly relates to the number of molecules in solution rather than their molecular size. When taurolithocholate, a monohydroxy bile acid, is added to a taurocholate infusion (38), the slope of the line decreases, the opposite of that occurring with a mixture of taurodehydrocholate and taurocholate (65). The assumption was that the monohydroxy bile acid increased micelle size, thus lowering osmotic activity and bile flow. Direct proof was not obtained, although electron microscope studies (6, 48) after the infusion of monohydroxy bile acid that reduced bile flow to zero indicated the existence of electron-dense material consistent with aggregates. More direct evidence was recently obtained when a biophysical study (61) of different bile acids established that the addition of monohydroxy bile acids to mixtures of di- and trihydroxy bile acids increased their aggregation number and, depending on the amount added, could result in the complex coming out of solution, a complete loss of osmotic activity. For completeness, it should be noted, as discussed in detail by Dietzsch and Moore (49), that either an increase in micelle size and/or a change in the activity of Na+ will lower osmotic activity. Thus, at low concentrations, sodium taurocholate can be considered to be completely ionized, having an osmotic effect of two ions in solution. However, as aggregates develop, the ionization diminishes, leading to a reduction in somatic activity. These in vitro studies now provide a biophysical basis for understanding naturally occurring events and provide a tool for determining the potential osmotic effects of other organic anions and cations excreted in bile. To further bridge the gap between the in vitro certainty and in vivo uncertainty, the development of microscopic techniques for establishing the aggregate status of

**OSMOTIC EFFECTS OF BILE ACIDS**

![Fig. 4. Osmotic effects of bile acids. Compared with sodium taurocholate, a naturally occurring conjugated bile acid, taurodehydrocholate, a triketo compound that does not form micelles (aggregates), generates a much higher rate of bile flow (65). In contrast, sodium taurolithocholate, a monohydroxy bile acid that may be found in bile in small amounts, is known to diminish (38) and stop bile flow (31) Although precipitates caused by this bile acid could be seen by electron microscopy (6, 48), the occurrence of aggregates was established recently by an in vitro study (61). Based on the in vitro data, it is now possible to attribute the cholestatic effect of monohydroxy bile acids to a progressively increasing micelle size, which lowers osmotic activity until the aggregates precipitate and no longer have an osmotic effect.](http://advan.physiology.org/)

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**Sodium taurocholate* Osmotic coefficient* vs millimolar concentration**

![Fig. 3. Osmotic activity of sodium taurocholate. As bile acid concentration decreases, the increase in the osmotic coefficient indicates that water flow is maintained as excretion rate decreases. At high excretion rates, the slope of the line relating osmotic activity to concentration is much less and gives the appearance of a linear relationship. [Figure taken from Carpenter and Lindenbaum (12) with permission.](http://advan.physiology.org/)
bile acids in the biliary system will clarify pathogenic mechanisms underlying cholestatic syndromes.

How to recognize when cholestasis begins remains an unsolved problem. Biomarkers for diminished canalicular flow could be as clinically helpful, such as plasma cystatin C, which detects diminished glomerular filtration rate (62). Large-molecular-weight biomarkers for cholestasis, such as S'-nucleotidase, alkaline phosphatase, and γ-glutamyl transferase, are not always reliable (66), and reduced canalicular transport of small-molecular-weight biomarkers, such as bilirubin glucuronide or bile acids, is followed by an increase in their renal excretion before their plasma levels increase significantly. The initial interest in serum bile acids (37) occurred at a time when simple accurate methods for their determination did not exist and large population studies with appropriate statistical analyses could not be made. Later, when sensitive, rapid, inexpensive enzymatic recycling methods became available for total serum bile acids (74) or for total urine bile acid sulfates (28), interest had waned. A recent report (63) showing that total serum bile acids correlates very well with fibroscan data in the less expensive parameter.

It is axiomatic that the earlier a disease is recognized, the greater the opportunity to modify its course. The experience with ursodeoxycholic acid in the treatment of primary biliary cirrhosis (59) provides motivation for seeking earlier reliable biomarkers of cholestatic liver disease.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.B.J. conception and design of research; N.B.J. performed experiments; N.B.J. analyzed data; N.B.J. interpreted results of experiments; N.B.J. prepared figures; N.B.J. drafted manuscript; N.B.J. edited and revised manuscript; N.B.J. approved final version of manuscript.

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HEPATIC BILE FORMATION


