Regulation of biliary lipid secretion
Frijters, C.M.G.

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LIVER ANATOMY

The liver lies in the abdominal cavity, in contact with the diaphragm; its mass is divided into several lobes, the number and size of which vary among species. In most mammals, a greenish pear shaped sac (the gall bladder) is seen attached to the liver and careful examination will reveal the common bile duct, which delivers bile from the liver and gall bladder into the duodenum. More than 500 different functions are ascribed to the liver. Most of these critical roles are fulfilled by the parenchymal cells or hepatocytes and roughly can be divided in three types of functions. For instance, the liver serves as an exocrine gland, secreting bile into the small intestine to help solubilise fats for digestion and absorption and to eliminate waste products through the faeces. The liver also serves as an important storage organ. The hepatocyte cytoplasm contains granules of glycogen, a polysaccharide storage form of glucose, and lipid droplets, depots of triglycerides. Finally, being an important endocrine gland the liver secretes many important substances into the blood, like blood clotting proteins such as fibrinogen, transport proteins such as retinol binding protein (carries vitamin A), lipoproteins (carry lipids), and transferrin (carries iron) and albumin. All these functions can be regarded as separate events but in combined action they make the liver a metabolic control unit responsible for maintaining pH homeostasis and a constant blood composition. For instance, the liver is the organ with primary responsibility for maintaining a constant blood glucose level. Excess glucose is taken up and stored as glycogen. Glucose is produced by hydrolysis of glycogen, glycogenolysis, and by metabolism of amino acids, gluconeogenesis. Keeping the blood glucose level as constant as possible is especially critical for normal brain function.

To perform these tasks optimally, the liver serves as an interface between blood returning from the digestive tract (the portal venous system) and the rest of the bloodstream (via the hepatic venous system). Roughly 75% of the blood entering the liver is venous blood from the portal vein. Importantly, all of the venous blood returning from the small intestine, stomach, pancreas and spleen converges into the portal vein. As a consequence the liver gets "firstpickings" of everything absorbed in the small intestine. Nutrients absorbed from the intestine and carried in the portal blood are processed and stored in the liver and then mobilised into the hepatic blood when needed by other organs. In addition, many toxic substances absorbed from the intestine can be inactivated and eliminated from the body by the liver before they enter the main circulation. To supply the liver with oxygen, the remaining 25% of the blood supply to the liver is arterial blood from the hepatic
artery. Terminal branches of the hepatic portal vein and hepatic artery merge as they enter sinusoids in the liver.

The liver sinusoids are unusual capillaries composed of a discontinuous layer of endothelial cells with large, irregular openings or fenestrations, about 100 nm in diameter. The basement membrane (basal lamina) under these endothelial cells is also incomplete. Between the sinusoids and the hepatocytes is a subendothelial space called the space of Disse. Microvilli in the basal membrane of the hepatocytes protrude into this space. As blood flows through the sinusoids, a considerable amount of plasma is filtered into the space of Disse and comes into intimate contact with the hepatocytes, thereby facilitating exchange. On the lumenal surface of the endothelial cells, the sinusoids also contain scattered phagocytic Kupffer cells. These are macrophages that ingest and degrade senescent old erythrocytes, particles and lipopolysaccharides from the blood. Breakdown of haemoglobin in these macrophages produces bilirubin, a yellow, hydrophobic and toxic compound. Bilirubin is transported to the hepatocytes and is conjugated to render it more soluble and then excreted into the bile. When bilirubin excretion is impaired or production exceeds the excretion capacity of the liver, it accumulates in blood and peripheral tissues, causing jaundice. The sinusoids conduct the blood along single sheets of hepatocytes and finally drain into a central vein. Finally blood from these central veins is collected in hepatic veins and enters the main circulation through the vena cava inferior.

Hepatocytes are arranged like folded sheets with their basal membrane surfaces facing and surrounding the sinusoids. This basal or sinusoidal membrane accounts for approximately 70% of total hepatocyte membrane surface area. Each hepatocyte is in contact with other hepatocytes through the lateral membrane domain. The lateral membranes (15% of total surface area) of adjacent hepatocytes are interconnected by desmosomes and intercellular communication is provided by the presence of Gap-junctions. Enclosed in each plane between adjacent hepatocytes is a tubular space called a bile canaliculus, which is the start of the bile duct system. The hepatocyte apical membrane domains also called canicular membranes, line the canicular lumen and are the site of primary bile formation. The canicular membrane is separated from the lateral membrane domain by the tight junctions. They interconnect the lateral membranes of adjoining hepatocytes and form a selective paracellular barrier between blood plasma and primary bile. Thus, a canaliculus is a narrow sealed gap between the membranes of two hepatocytes. A few small microvilli protrude into the canaliculus from the hepatocytes.
The canaliculi form an anastomosing tree draining toward bile ductules. Bile secreted into the canaliculi flows parallel to the sinusoids, but in the opposite direction of the blood flow, and ends up in these bile ductules. Bile ductules thus begin in very close proximity to the terminal branches of the portal vein and hepatic artery. The grouping of bile duct, hepatic arteriole and portal venule is called a portal triad. These portal triads are surrounded by connective tissue, fine sheets of which interconnect different portal triads delineating polygonal columns. In 1833 Kiernan described these columns as the structural units of the liver and called them lobules (1). The lobule consists of a roughly hexagonal arrangement of plates of hepatocytes radiating outward from a central vein in the centre (figure 1). At the corners of the lobule are regularly distributed portal triads. These liver lobules were subdivided into a structural and functional unit by Rappaport et al. in 1954 (2). This concept of the "acinus" describes the efferent bile ductules as the centre of the basic structural and functional unit of microcirculation within the liver (3). The exact definition of liver unit was re-evaluated by Lamers et al. in 1989 (4) and their concept of a metabolic lobulus favours the concept of a liver lobulus to be the smallest structural and functional unit within the liver.

Figure 1: Schematic drawing representing the radial architecture of hepatocytes, sinusoids and bile canaliculi along portal-central axis. The large arrows indicate the direction of sinusoidal blood flow while small arrows indicate the direction primary bile flows towards the bile ducts. Reprinted from Bloom et al. (1975), "A textbook of Histology".
BILE FORMATION

Formation of bile by hepatocytes is a major, but not fully understood, function of the liver. It involves the vectorial transport of compounds such as bile salts, phospholipids, cholesterol as well as endo- and xenobiotics. Most biliary components are found in high concentrations and a steep concentration gradient is maintained between blood plasma and primary bile. Hepatocytes form the boundary between the plasma and bile compartment and because of their specialised structural and functional features they are able to maintain the steep concentration gradients that exist between sinusoidal blood and bile in the canicular lumen. Two main routes of transport from the blood plasma to the bile can be distinguished. First of all, tight junctions only separate both compartments from each other at the lateral membrane. These structures create a paracellular barrier that permits entry of water and small molecules like glucose. Tight junctions are also permeable for small ions although the permeability towards cations is much higher than towards anions. The potential difference between primary bile and blood plasma of approximately -5 mV generates a driving force for the passage of cationic counterions like Na\(^+\) into the canaliculus. Tight junctions thus function as a selective diffusion boundary (5) that cannot actively generate concentration gradients but is very important in maintaining them. The passage of molecules via the paracellular route becomes non-restricted upon opening of the tight-junctions, for instance when the liver is perfused with medium lacking Ca\(^{2+}\). Many organic and inorganic solutes must cross the hepatocyte boundary through an intracellular pathway in order to be excreted into bile. This transcellular route involves uptake at the basolateral domain, transport across the cytosol and excretion/secretion into the canaliculus at the canicular membrane. Uptake of substrates at the basolateral membrane domain occurs via endocytosis or in a carrier-mediated fashion. At the canicular membrane domain the substrates are secreted by the action of specific transporters or via exocytosis. The net result of the apical and sinusoidal localised transport processes determine the composition of primary bile. The endocytotic-exocytotic pathway mediates plasma to bile trafficking of receptor-bound ligands, fluid-phase proteins and other non-electrolytes. This transcytotic pathway involves transport of membrane vesicles from the basolateral to the canicular membrane domain of the hepatocyte. Like non-electrolytes, the transport of most plasma proteins destined for biliary secretion like albumin, α2-macroglobulin and α1-acid glycoprotein is mediated by the non-selective fluid-phase transcytosis (6). Invagination of
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the basolateral membrane entraps a part of the blood plasma in vesicles that enter the endosomal compartment. A more selective vesicle mediated uptake is used for IgA, transferrin, epidermal growth factor (EGF) and asialoglycoproteins (7). This vesicle transport is initiated by the formation of receptor-ligand complexes in subdomains of the basolateral membrane. Clathrin coated vesicles “pinch-off” from this membrane and enter the endosomal compartment where dissociation of receptors and ligands occurs. The endosomal compartment is the site of vesicle sorting and finally endosomes containing material destined for biliary excretion leave this compartment toward the canicular membrane. Most of the proteins captured in these vesicles, with the exception of IgA, are finally transported to the lysosomes where they are degraded. Transcytotic vesicles appear to enter into a subapical sorting compartment before the vesicles are inserted into the canicular membrane and release their vesicle content into the canicular lumen. The vesicular transcytotic pathway is dependent on the microtubular network, disruption of microtubules by colchicine results in inhibition of transcytosis.

Bile formation is an osmotic secretory process that primarily depends on the secretion of bile salts. The osmotic pressure that is generated attracts water through the tight junctions and through water channels (aquaporins) and this results in bile flow. The bile salt dependent flow is an important fraction of total bile flow and its relative contribution is species dependent. The non-bile salt dependent flow is generated by the active secretion of other osmotic active biliary constituents being mainly glutathione and bicarbonate (8). Bile flow as measured at the end of the biliary tract is not only determined by the hepatocyte secretion processes. En route through the biliary tract bile duct epithelial cells (cholangiocytes) lining this duct secrete into and extract from the primary bile. Cholangiocyte secretion of chloride and bicarbonate is thought to account for up to 40% of human bile flow (9). The transcytotic pathway only plays a minor role in the generation of basal bile flow (6-8%) (9). Blocking of the vesicular transcytosis with colchicine does not lead to significant changes in bile flow. However, biliary secretion of bile salts during high bile salt infusion can be partly inhibited by colchicine treatment and blocking of transcytosis increased with the hydrophobicity of the bile salt species. Physiological data do not provide conclusive evidence for microtubule dependent vesicular trafficking of bile salts. Moreover structural studies failed to provide evidence that bile salts sequester to a significant extent within the lumina of intracellular vesicles. The intracellular bile salt transport is reviewed by Ehrlinger in 1993 (10) and more recently updated by Crawford (6). Upon uptake at the basolateral membrane bile salts
bind to intracellular proteins and diffuse to the canalicular membrane where they are secreted (see further below). Under normal conditions bile salts are taken up very efficiently in the intestine resulting in the enterohepatic cycling (EHC) of a large bile salt pool. Therefore the contribution of de novo synthesised bile salts to total flux is less than 1% (11). Bile salts account for nearly 70% of the total dry weight mass of bile in humans. Besides bile salts, cholesterol and phospholipids are, in quantitative terms, the other major constituents of the organic fraction of bile 22% and 4% resp. (12).

Origin of biliary lipids

In all animal species studied, bile phospholipids almost exclusively consist of phosphatidylycholine (PC) molecules that have a specific, relatively hydrophilic, fatty acid composition, that is different from the PC molecules found in the canalicular membrane (13) (see later). The bulk of biliary PC is derived from preformed hepatic and extrahepatic pools (14). The localisation of the putative precursor pool for biliary lipid within the hepatocyte is unclear but probably includes components of the plasma membrane, endoplasmic reticulum (ER) and Golgi apparatus. High-density lipoproteins seem to function as the main extrahepatic pool of biliary PC. Portal et al. described that HDL-PC contributes to 38% of total biliary PC under normal conditions in the rat (15). Biliary phospholipids from intrahepatic pools are primarily derived from extensive rearrangement of pre-existing acylglycerides in the endoplasmic reticulum (13). In rat, PC synthesis via the CDP-choline pathway, the main route for de novo PC synthesis, only accounts for 3-14% of biliary PC (16). However this relative small portion of de novo synthesised PC seems to be a requirement for normal PC secretion into bile. Bile salt induced PC secretion was reduced dramatically when PC synthesis was impaired by feeding choline deficient diets, although total hepatic PC content was not altered by this diet (17). The transport of biliary lipids from the endoplasmic reticulum and Golgi apparatus to the canalicular membrane is suggested to be mediated by vesicular transport (reviewed in (6,14,18)). This vesicular transport seems to be microtubule-dependent. In the presence of microtubule-disrupters like vinblastine and colchicine phospholipid transport is reduced to 40-60% (13). However, total inhibition of lipid transport by microtubule poisons is not reported and there is no evidence supporting the large amount of vesicle transport needed to maintain normal hepatobiliary lipid fluxes (6). Therefore the presence of alternative transport pathways cannot be ignored. In vitro studies by Cohen et al. (19,20)
showed that transport of monomeric phosphatidylcholine is promoted by cytosolic phosphatidylcholine-transfer protein (PC-TP). They observed transfer of phosphatidylcholine between donor and acceptor vesicles, with lipid compositions mimicking endoplasmic reticulum and canalicular membranes respectively. The lipid transfer could be markedly stimulated in the presence of bile salts at submicellar concentrations. This study provided in vitro evidence for the role of PC-TP in the supply of PC to the canalicular membrane. The abundant presence of PC-TP in developing and adult mouse liver is compatible with its proposed role in bile formation (21). However, if existing, this role is not of major importance for normal PC secretion into bile. Biliary lipid secretion is not disturbed in PC-TP knockout mice and increasing the demand for biliary PC by infusion of increasing amounts of bile salt does not result in significant decreases in biliary PC secretion (22). Another cytosolic protein, sterol carrier protein 2 (SCP-2) was suggested to serve as a scavenger for phospholipid delivery. In analogy to their previous studies with PC-TP, purified bovine liver, Leonard and Cohen showed that SCP2 promoted the transfer of PC from donor to acceptor model membrane vesicles (23). It is possible that the combined action of these processes take care of biliary PC delivery to the canalicular membrane but considering the massive fluxes needed for biliary PC secretion other, yet unknown, routes might be present. Cholesterol is present in bile exclusively in its unesterified form. In both animals and humans the contribution of de novo cholesterol synthesis to biliary cholesterol secretion is of minor quantitative importance (10%-16%) (24,25) although the secretion of newly synthesised cholesterol is promoted by increased bile salt flux (25).

In 1985 Robins et al. reported that the cholesterol from preformed pools preferentially ended up in bile as opposed to newly synthesised cholesterol (26). This is in line with other data describing that almost all of the biliary cholesterol is derived from plasma lipoproteins (11,24,27-29). Lipoproteins enter the cell via receptor mediated transcytosis. Free cholesterol released from lysosomal degradation of the esterified cholesterol in the lipoprotein core distributes rapidly throughout the cell. There is a possible involvement of cytosolic cholesterol carriers like SCP-2 in the transport of this free cholesterol to the canalicular membrane (30,31). However the contribution of this route in biliary cholesterol secretion is very minor since it is known that biliary cholesterol is principally derived from high-density lipid proteins (HDL) and not from other lipoproteins (32). It recently became clear that the hepatic class B type I scavenger receptor (SR-BI receptor) present in the basolateral membrane plays an important role in the uptake of HDL-lipids.
SR-BI is a member of the CD-36 superfamily of proteins and is a high affinity cell surface high-density lipoprotein (HDL) receptor that mediates selective lipid uptake (33). The mechanism of selective uptake is fundamentally different from that of classic receptor mediated uptake via coated pits and vesicles (e.g. the low-density lipoprotein receptor pathway) in that it involves efficient transfer of lipids, but no apolipoproteins from HDL to cells. The efficient cellular uptake of HDL lipids via SR-BI requires not only receptor mediated surface binding, but also receptor-specific lipid transfer mediated by its extracellular domain (34). Several authors have described the physiological relevance of this process. The overexpression of the SR-BI receptor in mouse hepatocytes led to doubling of the biliary cholesterol (35) and Hillebrant et al. described that the HDL cholesterol is more related to the biliary secretion of cholesterol than to that of bile acids (36). It is not clear yet how cholesterol is handled by the hepatocyte after uptake via SR-BI, but previously it was already suggested that cholesterol is transported by lateral diffusion through the basolateral membrane to the canalicular membrane. Recent studies using rat livers perfused with reconstituted HDL, made with radiolabeled cholesterol (both unesterified and esterified), seem to delineate a novel hepatic route for the selective uptake of unesterified sterols from HDL to bile, that is consistent with a direct plasma membrane pathway (37).

The composition of HDL-PC is very similar to that of biliary PC and the role of HDL derived PC in biliary PC secretion is documented by Portal et al. (15). Therefore it is possible that biliary PC also originates from HDL-PC which is transported by lateral diffusion, similar to cholesterol. PC then has to be transferred via the inner-membrane leaflet because it cannot pass the tight junctional barrier in the outer membrane leaflet (38). This kind of transport has been reported to occur in MDCK cells (39,40) and could explain the relative enrichment of the canalicular membrane with labelled PC, after injection of HDL containing labelled PC, in rats (15).

The spontaneous flipping rate of PC is much slower than that of cholesterol. It is therefore more likely that biliary type HDL-PC ending up in the outer membrane leaflet enters the vesicular route upon capturing into endocytotic vesicles. However this remains highly speculative and more insight in the mechanism of SRBI mediated lipid uptake is needed.

The secretion of lipids into bile is the main focus of this thesis and mechanistic and functional aspects of this process will be addressed in more detail in the section on biliary lipid secretion.
Chapter 1

Basolateral uptake carrier-systems involved in bile formation

The basolateral membrane contains a wide variety of proteins that are involved in the uptake of organic compounds from blood plasma. Organic anions are taken up by two different membrane proteins named Organic Anion Transporting Polypeptide 1 and 2 (OATP1, OATP2) (reviewed in (41) and (42)). These proteins are responsible for the electrogenic sodium independent uptake of bulky organic mostly anionic compounds that are structurally unrelated. Among their substrates are amphiphilic compounds like bromosulfophthalein (BSP), dibromosulfophthalein (DBSP), taurocholate, cholate and sulphated tauroliothocholic acid (43-45), uncharged cardiac glycosides (46), steroid hormones (47), some amphiphilic organic cations (47) and leukotriene C4 (48). Bile salts are preferentially taken up by the Na\(^+\)/Taurocholate Cotransporting Protein (NTCP) (49-51). NTCP is involved in the sodium dependent uptake of conjugated bile salts like taurocholate and, to a lesser extent in the uptake of unconjugated bile salts like cholate (49,50,52,53). Finally, the organic cation transporter (OCT1) transports small organic cations in an electrogenic, sodium independent manner (54-56).

Canalicular transporters are members of the ATP-binding cassette transporter family

Hepatobiliary excretion at the canalicular membrane is driven at the expense of ATP-hydrolysis to generate the high concentration gradients of most substrates between cytosol and bile (57). The canalicular transporters that mediate this active transmembrane transport are members of a large and diverse group of proteins that mediate the selective movement of solutes across biological membranes (58). These proteins are found in many different species ranging from plants, bacteria, yeast and mammals and are specific for a single substrate or group of related substrates ranging from sugars to complex polysaccharides, amino acids to proteins or (in)organic ions. These transporters share a common organisation into four core domains. Two sets of transmembrane domains can be distinguished that span the membrane multiple times. Located at the cytosolic face of the membrane are two ATP-binding domains that contain the strongly conserved Walker A and Walker B motifs (59). This region is involved in ATP-binding and hydrolysis (60-62). On the basis of these commonly shared features these proteins were classified as the superfamily of ATP-binding cassette transporters or
ABC-transporters.
The ABC-transporters that are known to be involved in hepatobiliary secretion belong to two families of this large superfamily, the P-glycoproteins (P-gp) and the multidrug resistance proteins (MRP) (63,64) and to a single gene, that is very homologous to the P-gp family, called Sister of P-gp (65).

P-glycoproteins

It is known already for a long time that mammalian cells can develop resistance against a variety of functionally and structurally unrelated drugs upon selection with a single cytotoxic drug. This phenomenon of acquired resistance is known as multidrug resistance (MDR). In 1976 Juliano and Ling (66) discovered a plasma membrane protein that is overexpressed in colchicine-resistant tumour cells. They called it P-glycoprotein (P-gp) because they assumed it was involved in the permeation of these drugs across the plasma membrane. It is now clear that one of the major mechanisms of MDR is the overexpression of P-glycoproteins (170 kD) which actively pump these compounds out of the cell, maintaining a low intracellular concentration and thereby protecting the cell against toxicity (reviewed in (66-70)). Throughout the years several P-gp genes have been identified and because of historical reasons the nomenclature of these P-gps is not very consistent and alternative names are used throughout the literature. This thesis only deals with some of the mammalian P-glycoproteins which are listed below. In mammals the numbers of P-gp encoding genes ranges from two in human to five in pig (71,72). These highly homologous genes encode a small group of isozymes. In mice and rats three genes have been identified: Mdr1a (mouse (73); rat (74,75)), Mdr1b (mouse (76); rat (77)) and Mdr2 (mouse (78); rat (79), also known as Pgp3). Two genes were characterised in humans, MDR1 (80) and MDR3 (81,82). P-gps show a very high degree of inter- and intraspecies homology (83); nevertheless there is a striking functional difference between the individual MDR genes. Transfection of the human MDR1 or mouse Mdr1a (class I) or Mdr1b (class II) in cell lines reveals that these genes can confer multidrug resistance against amphipatic drugs (73,84,85). However, the pattern of drug resistance is not similar in class I and class II transfected cells (73). Three-dimensional structural analyses of compounds that interact with these P-gps revealed several shared characteristics. From this it was postulated that the prototype P-gp substrate is a cationic, hydrophobic, 400-1000 Da molecule containing at least two planar rings (86).
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In contrast, genes encoding class III type P-gps apparently cannot confer multidrug resistance upon transfection in drug sensitive cell lines (78,87-89). Because of this inability the question rises why class III type P-gps are upregulated in multidrug resistant cells. One explanation could be that these gene products could transport the drugs, but that the affinity/rate of transport for these substrates is too low to protect the cells sufficiently. A correlation between daunorubicin transport and overexpression of MDR3 was observed in some drug selected B-cell leukaemia’s (90,91). In 1996 Kino et al. described transport of the antifungal antibiotic aureobasidin A, in transfected yeast cells by both MDR1 as well as MDR3 P-gp (92). Smith et al. showed that LLC-PK1 cells stably expressing the MDR3 P-gp were able to transport some MDR1 P-gp substrates. The rate of this transport was low for most of the drugs that were tested and this explains the apparent inability of MDR3 P-glycoprotein to confer a multidrug resistant phenotype {submitted; JBC}. Another explanation for upregulation of class III P-gp genes in some MDR cells could reside in the fact that the genes encoding the P-gps that do confer multidrug resistance (class I and II) are genetically closely linked with the gene of non-MDR conferring P-gp (class III). Overexpression of class III MDR genes in multidrug resistant cells appears to occur only when there is amplification of class I or II MDR genes (93-96). Analysis of the amplification event has shown that not only Mdr genes are amplified but also nearby genes are part of the amplicon (73,97,98). Therefore it is possible that increased levels of MDR3/Mdr2 P-gp in MDR-cells are the result of co-amplification with MDR1/mdr1a/b in the same gene cluster.

The apparent functional difference between class I and II type P-glycoproteins on the one hand and class III type P-gps on the other hand seems to be reflected by the difference in expression patterns in normal tissue. The human MDR1 is expressed throughout the body. Immunohistochemical and RNAse protection assays show MDR1 P-gp expression in the proximal tubuli of the kidney, tissues in the gastrointestinal tract, bile canaliculi and bile ductules, the capillary endothelial cells of testis and brain, endometrium of the uterus, and adrenal gland (99-107). The murine counterparts of MDR1 P-gp, Mdr1a and Mdr1b P-gp, match the expression of the human gene although they seem to have a complementary and partially overlapping distribution. The non-MDR conferring mouse Mdr2 and human MDR3 are predominantly expressed in the hepatocyte and to a lower extent also in muscle, and spleen (82,108,109). Their rat homologue Mdr2 (Pgp3) was most highly, and almost exclusively expressed in liver and gastrointestinal tract. In the latter, a gradient of expression was observed with the highest level of expression in the
small intestine with decreasing levels in the more distal regions of the gut (79). Reports on the expression of rat *Mdr2* (*Pgp3*) in spleen and muscle are inconclusive. High levels of expression were found in Fischer rat muscle and spleen (110) while this was not the case in Sprague-Dawley rats (79). At present it is not clear whether these differences are caused by differences in the rat strains or resulting from analytical imperfections, although data available underline the first option. In both rat strains expression of *Mdr2* (*Pgp3*) was detected in brain tissue while this could not be detected in mouse brain (108). However, there is no doubt that in all species studied thus far, the high expression of *Mdr2*/MDR3 P-gp in the hepatocyte is exclusively restricted to the canalicular membrane (79,101,108,109) suggesting a role in hepatobiliary transport.

**Mechanisms of P-glycoprotein mediated transmembrane transport**

Data derived from many studies (reviewed in (70,83,111) were used to deduce a hypothetical model for P-gp structure. P-glycoproteins are glycosylated plasma membrane proteins (140-170 kDa) of about 1280 amino acids long. To date this structure model alone is not sufficient to elucidate the mechanism of drug transport. The initial idea that the transmembrane domains of P-gps simply formed a pore (76,80) is now considered to be incorrect. One of the main drawbacks of this pore theory is it could not explain why there was no intracellular accumulation of substrate in MDR cells. Substrates entering the cell would diffuse throughout the cytoplasm and build up to a certain level before the excess of substrate is removed through the channel. However, experimental data showed that the influx of substrate into the multidrug resistant cell was already lower than in sensitive cells (66,112).

It remains to be established whether the P-glycoprotein really functions as a conventional transporter or, alternatively, acts as a membrane extruder that merely expels amphiphilic compounds entering the membrane by non-ionic lipid diffusion. Higgins and Gottesman captured the latter concept of a P-gp moving around the membrane as a “hydrophobic vacuum cleaner” in a new model for P-gp transmembrane transport in 1992 (113). They proposed that P-gps might act as drug translocators or ‘flippases’. This concept implies that access of substrate to the transport protein occurs directly from the lipid phase and that the drug is flipped/translocated from the inner leaflet of the bilayer to the outer leaflet. The amphipatic or hydrophobic character of MDR substrates allows them to partition into hydrophobic membranes quite easily (86). From the outer membrane leaflet
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drugs could simply enter the extracellular space by diffusion (113). This model also predicts that the P-glycoproteins could decrease the initial uptake rate of the substrate in cells in vitro since it would not differentiate between drug entering the membrane from the inner or outer cellular space (114). Valverde et al. (115) and Gill et al. (116) reported an increase in cell swelling-activated Cl⁻ currents in several cell-types expressing P-glycoprotein. Gottesman and Pastan (111) have tried to combine the different reported transport functions of P-gp for protons, chloride and amphipatic compounds in an alternative model. In this hypothetical model ATP hydrolysis is linked to the transport of protons into the transporter, with chloride following passively. A water flow is generated by the osmotic gradient resulting from the ions inside the lumen of the transporter. Consequently membrane localised amphipatic drugs entering the water phase are excreted. Both the flippage model proposed by Higgins and Gottesman and the model proposed by Gottesman and Pastan could explain the broad specificity for amphipatic compounds but more recent data shed doubt on the latter model. These studies suggested that the chloride conductance is not a feature of P-glycoprotein itself but that P-gp expression regulates an endogenous channel (117-120).

In contrast a series of additional findings emerging in recent years, provide evidence that supports the idea that MDR1 mediated transport is adequately described by the flippage model of Higgins and Gottesman.

Homolya et al showed that MDR1 P-gp extrudes fluorescent cellular indicators from the cell before they can enter the cytoplasm (121). This indicates that the dye is rapidly taken up from the cytoplasmic leaflet of the membrane and transported to the exoplasmic membrane leaflet. Van Helvoort et al. studied the transport of several short-chain lipid analogues in polarised epithelial LLC-PK1 cells that were stably transfected with MDR1 (122). At 15°C, newly synthesised short chain analogues of various membrane lipids could be recovered from the apical membrane using medium containing albumin as a lipid acceptor. No lipid was recovered from control cells. Since no vesicular transport occurs at 15°C, the short-chain lipid metabolites that are formed within the cell must have been translocated across the plasma membrane before they could be extracted by the albumin-containing medium. This study generated direct evidence that MDR1 P-gp functions as a translocator.

However, the model of the mechanism by which MDR-1 functions is still evolving. Lactococcus lactis possesses an ATP-dependent drug extrusion system (LmrA) which shares functional properties with the mammalian MDR1 P-gp. Expression of LmrA in
mammalian cells resulted in a MDR-profile that is comparable with the profile of cells transfected with MDR1 (123). The biophysical studies of Bolhuis et al. (124) on the extrusion mechanism of LmrA demonstrated that this lactococcal MDR transporter functions as a "hydrophobic vacuum cleaner", expelling drug directly from the inner leaflet of the lipid bilayer. Their data support idea that P-gp does not function as translocase (flippase) but functions as a genuine export-protein. Studies of the ultrastructure of MDR1 P-gp by electronmicroscopy are not definite because of the relative low-resolution of these images but several key features could be identified (125). The membrane spanning domains of P-gp seem to form a pore like structure from which the cytoplasmic side is sealed off. The exoplasmic end of this barrel like structure is accessible from the exoplasmic side. This aqueous chamber possibly allows the entry of more soluble substrates directly into the extracellular space. Additionally, an opening in the longitudinal plane of the pore exist at the site where it crosses the cytoplasmic membrane leaflet. This cytoplasmic opening could be the entry-site of substrates residing in the inner membrane leaflet, but this remains highly speculative. In accordance with the structure deduced from hydrophobicity plots, the ATP-binding domains are sticking into the cytoplasm. Recent studies using predictive modelling techniques or circular dichroism methods substantiate these ultrastructural features of P-gp (126-128).

The function of P-glycoproteins in bile formation

The localisation of MDR1/mdr1a-mdr1b P-glycoprotein and their ability to transport naturally occurring cytotoxic compounds across membranes in vitro, suggests a physiological role in the protective mechanism against toxic insults or transport of endogenous substrates (129-133). The pursuit of the physiological function of mdr2 P-glycoprotein succeeded in 1993 when knockout mice for the mdr2 gene were produced (134). The phenotypic characteristics of mdr2 knockout mice almost directly reflected its putative physiological function. Bile formation is severely affected in these animals. The most prominent change in bile composition represents a virtual absence of phospholipid and a dramatic decrease in cholesterol, while bile salt secretion is normal. Glutathione secretion is severely decreased as well. Analysis of bile of mice heterozygous for mdr2 gene showed a normal composition except a 40% decrease in the phospholipid content.
Chapter 1

This strongly suggested that mdr2 P-gp is primarily involved in the biliary secretion of phospholipids. Based on its homology with the other P-glycoproteins it was hypothesised that mdr2 P-gp is a transporter as well and thus, that it might function as a flippase which translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane (134,135) (see later).

Because the mdr1a P-gp is also expressed in the canalicular membrane it was interesting to evaluate its physiological function in hepatobiliary transport. Once again generation of genetically modified animals provided some straightforward answers. The phenotype of mdr1a knockout mice was entirely different from the mdr2 knockout. Normal bile secretion parameters in mdr1a knockout mice were not affected and no liver pathology was found. These animals showed accumulation of amphipatic drugs like ivermectin and vinblastine in brain tissue, which suggests that mdr1a P-gp plays an important role in extrusion of these compounds across the blood brain barrier (136,137). The role of this P-gp in canalicular secretion of amphipatic drugs could not be elucidated because in the mdr1a knockout increased expression of the mdr1b gene was observed. In order to circumvent this problem, mice with the combined disruption of both mdr1a and mdr1b genes were produced (138). Phenotypically these mice were normal, they turned out to be healthy and fertile. These mice turned out to be hypersensitive to several exogenously administered toxic substances. In these double knockouts the absence of Mdr1a/b from the endothelial cells comprising the blood brain barrier resulted in increased permeability of this barrier to neurotoxic drugs consequently leading to fatal drug-induced neurotoxicity. To date no physiological roles of class I and II type P-glycoproteins have been determined. However, in general, they seem to be involved in the protection of tissues and organs against intoxication by potentially hazardous exo- and endogenous compounds, either by preventing uptake or by active excretion. This could very well also be the functional role of these P-gps in bile formation. Bile is one of the excretory routes by which compounds can be eliminated from the body. The transport of amphiphilic cations across rat hepatocyte canalicular membranes is mediated by Mdr1 P-gp (139). Additionally, heterologous expression of various P-glycoproteins in polarised epithelial cells induces transport of cationic drugs (140). Modulators of protein kinase C as well as MDR reversal agents have a major influence on the biliary excretion of several cationic drugs (141). The impaired hepatobiliary clearance of these amphiphilic cations, in Mdr1a (142) knockout mice as well as in Mdr1a/b double knockout mice (138,143), support the role of class I and II type P-gps in excretion of drugs into bile.
General Introduction

**Canicular Bile salt secretion is mediated by a protein closely related to P-glycoproteins**

The canicular secretion of bile salts was initially thought to be mediated by an electrogenic canicular organic anion carrier (144,145). In 1987, this putative canicular bile salt transporter was isolated from rat liver (146). Reconstitution of this 100-kDa canicular membrane protein into proteoliposomes confirmed the ability of this transporter to confer the transport of bile salts like taurocholate (147). This electrochemical transport alone could not be responsible for the high bile salt fluxes needed to generate the high bile salt concentrations that sometimes are reached in the canaliculus. Later, several groups demonstrated that uptake into isolated canicular membrane vesicles of both rat and human was ATP-dependent (148-153). Kaste et al (154) demonstrated that electrogenic bile salt transport was localised in the endoplasmic reticulum while ATP-dependent transport was canicicularly localised. The previously observed electrogenic transport in canicular membranes was probably due to contamination of these vesicles with ER derived membranes.

Several attempts were made to isolate the ATP-driven canicular bile acid transporter (cBAT) and these resulted in a putative candidate by the isolation and characterisation of a 110-kDa ecto-ATPase (148,155-157). The above mentioned subfractionation of canicular membrane vesicles revealed, however, that the ecto-ATPase activity and the taurocholate transport were not coupled within the same vesicle population (42,51,154).

A yeast ATP-binding cassette protein mediating ATP-dependent taurocholate transport was isolated by Ortiz et al. (158). In 1995 the isolation of an ABC-transporter closely related to the subfamily of P-glycoprotein from pig liver was described by Childs et al (65). The expression of this gene was solely restricted to liver tissue. Further analysis showed that the protein encoded by this gene was localised in the canicular membrane (159). Functional characterisation of spgp by Gerloff et al (160) revealed that ATP-dependent bile salt transport was mediated by spgp and they concluded that spgp represented the bile salt export pump (BSEP) of mammalian liver.

This conclusion seems to be substantiated by a series of findings concerning the genetic basis for a subtype of progressive familial intrahepatic cholestasis (PFIC), an autosomal recessive form of severe cholestatic liver disease. PFIC presents in infancy with intermittent jaundice and cholestasis and progresses to end-stage liver disease and death in childhood. It was first described in a large Amish kindred and was referred to as Byler disease, after the family name (161). Subsequently it was described in many populations.
Although the mechanistic basis is unknown, it was presumed to represent a defect of bile salt transport. Both Byler disease and benign recurrent intrahepatic cholestasis (BRIC) were found to map to chromosome 18q21-q22. Mapping to this area was excluded, however, in kindreds from the Middle Eastern, Sweden and Greenland (162) and (163). By homozygosity mapping in 6 Middle Eastern kindreds and conventional linkage analysis, Strautnieks et al. mapped a second locus for PFIC (PFIC2) to chromosome 2q24 with a maximum lod of 7.1 (164). One pedigree was unlinked, suggesting the existence of a third locus. A short time later, Strautnieks et al. reported that the form of progressive familial intrahepatic cholestasis that is linked to 2q24 is caused by mutations in BSEP, the human orthologue of sPGP (165). The functional studies in the rat and the phenotype seen in these patients provide evidence that BSEP/sPGP is the major canalicular bile salt export pump in man.

Multidrug Resistance-associated Protein and non-bile salt organic anion transport

The notion of distinct canalicular transport systems for bile salts and organic anions came with the recognition of a rare recessive human hyperbilirubinemic disorder known as Dubin-Johnson syndrome (166). The characteristics of the disorder are hyperbilirubinemia, deposition of melanin-like pigment, in otherwise normal liver cells and sometimes hepatomegaly and abdominal pain. Dubin-Johnson patients show prolonged hepatobiliary clearance of organic anions like bromosulfophthalein (BSP), bilirubin-glucuronide and indocyanine green (reviewed in (13)), while other liver function is normal, including the secretion of bile salts (167).

The biochemical characterisation of this defect in the ATP-dependent transport of organic anions from liver into bile started with the discovery of a jaundiced inbred rat subpopulation from the Wistar strain called TR- rat (168). The jaundice was caused by elevated serum levels of bilirubin, the breakdown product of haemoglobin that is normally secreted by the liver into bile. Further analysis showed that the hereditary chronic hyperbilirubinemia in these mutant TR- rats was caused by defective hepatic anion transport (168,169). The genetic defect only affected transport of organic anions by the liver; no changes in renal or intestinal organic anionic transport was observed (170). Furthermore, the impaired clearance was caused by impaired transport across the canalicular membrane as was shown in studies using isolated canalicular membrane
vesicles (reviewed in (13)). Subsequently, two other mutant rat strains were identified, the Groningen Yellow (GY-rat) (171) and the Eisai Hyperbilirubinemic Rat (EHBR-rat) (172) which both showed disturbed hepatic clearance of organic anionic solutes. Cross breeding of these different mutant rat strains resulted in a hyperbilirubinemic offspring which indicated that the defect had the same allelic localisation (173,174). The mutant rats showed an impaired hepatobiliary clearance for a broad range of organic anions (reviewed in (13)) ranging from conjugated bilirubin, glutathione, bile salt conjugated with glucuronides or sulphates to exogenous compounds like ceftriaxone and dibromosulphophthalein (DBSP) to metal ions like zinc, copper and manganese. For this reason the ATP-dependent transporter of these non-bile-salt organic anions has also been called canalicular Multispecific Organic Anion Transporter (cMOAT).

In 1994 it became evident that cMOAT activity could possibly be attributed to a recently cloned ABC-transporter (175). It was shown that a human ABC-transporter, Multidrug Resistance-associated Protein (MRP), could function as an ATP-dependent export pump for substrates like leukotriene C4 and glutathione S-conjugates like dinitrophenyl-glutathione (GS-DNP) (176-178). These substrates were also known as substrates for cMOAT. This made MRP a potential candidate for cMOAT. However, the low expression of MRP1 in liver tissue rendered it unlikely to be responsible for biliary secretion of organic anion transport (179). Furthermore, the transport defect in TR- rats was liver specific, while MRP1 is ubiquitously expressed in almost all tissue (180).

Assuming that the putative rat cMOAT gene was a homologous but distinct member of the MRP family, Paulusma et al. obtained a rat Mrp1 probe amplified from rat lung cDNA. Using this fragment as a probe, they screened rat liver cDNA libraries and finally isolated a full length cDNA with a single open reading frame of 1541 amino acids (181). The sequence data showed that this putative cMOAT protein had an overall identity of ± 48% with MRP1, making it unlikely that they are derived from a single gene by differential splicing and suggesting instead that MRP and cMOAT are encoded by 2 different genes. This new gene is a member of the MRP-subfamily of ABC-transporters and also known as Mrp2 (176,182,183). It was shown that Mrp2 mRNA levels were strongly reduced in liver tissue from TR- rats and that the Mrp2 protein was exclusively expressed in the canalicular membrane, while in TR- liver no canalicular staining is observed. To demonstrate that the Mrp2 cDNA indeed encoded an organic anion transporter it was transiently expressed in COS-7 cells and efflux of GS-DNP was studied. Cells expressing the cMOAT protein showed a significant increased transport of GS-DNP compared to
mock transfected cells. Similar findings were also reported by Buchler et al. (184) who also reported on the cloning of the hepatocyte canalicular isoform of the multidrug resistance protein and its characterisation as a conjugate export pump that was deficient in hyperbilirubinemic mutant rats (183-185). Finally mutation analysis of TR- cDNA by Paulusma et al. (181) revealed that a single basepair deletion at amino acid 393 resulted in a frameshift and subsequent introduction of a premature stop codon at position 401 that led to a truncated protein lacking both ATP-binding domains. Thus a truncated nonviable protein was responsible for the impaired organic anion transport in the TR- rats. Not much later it was reported that also the EHBR strain had defects in the Mrp2 gene (186). In these rats a single nucleotide substitution leads to a truncated protein lacking the second ATP-binding domain. Consistent with these findings in the rat models of congenital hyperbilirubinemia mutations in MRP2 genes of patients with Dubin-Johnson syndrome were reported (187,188).
HEPATOBILIARY LIPID SECRETION

Bile salts and lipid secretion

Both in physiological and experimental conditions the secretion of biliary lipids appears to be determined by the secretion of bile salts. When bile salt secretion is low, e.g., during fasting or when the enterohepatic circulation of bile salts is interrupted, biliary lipid secretion is low and when bile salt secretion increases, e.g. after a meal or administration of bile salts, biliary lipid secretion increases (reviewed in (14)). The mode of action by which bile salts regulate lipid secretion is still a matter of debate. In most species the relationship between bile salt secretion and that of cholesterol and phospholipids appears to be curvilinear. In addition, many studies have demonstrated a direct positive relation between bile salt hydrophobicity and the amount of lipid that can be secreted (14,189-192). The relative hydrophobicity of a bile salt is reflected in its critical micellar concentration (CMC). The observed quantitative differences between the amount of lipid secreted per amount of bile salt in different species could be explained by differences in bile salt species present in the bile salt pool (193). Data from many studies suggest that the secretion of phospholipid and cholesterol occur in a coupled fashion (14). Experimentally induced alterations in biliary phospholipid secretion result in a similar alteration in cholesterol secretion (17). It has been recognised however that under certain metabolic conditions deviations can occur (194-197).

In all animal species studied, bile phospholipids almost exclusively consist of phosphatidylcholine (PC) molecules that have a specific, relatively hydrophilic, fatty acid composition. The sn1 position of biliary PC usually is occupied by the saturated fatty acid species palmitate (16:0) whereas the sn2 position invariably contains an unsaturated species, predominantly oleate (18:1) or linoleate (18:2) (12). The fatty acid composition of biliary PC contrasts with that of PC in the canalicular membrane which has a high content of arachidonate (20:4) in the sn2 position (14,198,199). Biliary phospholipids consist almost exclusively of phosphatidylcholine (PC; >95%) whereas the canalicular membrane, besides PC (±33%), also contains sphingomyelin (SM; ±20%), phosphatidylethanolamine (PE; ±20%) and phosphatidylyserine (PS; ±20%) (198-200). These lipids are not homogeneously distributed through the canalicular membrane, PE and PS are almost exclusively found in the cytosolic leaflet of the canalicular membrane whereas the (glyco)sphingolipids are predominantly residing in the exoplasmic membrane leaflet of the
canalicular membrane. The distribution of PC over both membrane leaflets seems to be non-discriminating. Two major constituents of hepatic membranes, sphingomyelin and phosphatidylethanolamine, are present in bile only in small amounts under normal conditions. Apparently hepatocytes selectively recruit specific PC species for secretion into bile and/or effectively exclude other phospholipids from entering this pathway. Cholesterol is present in bile exclusively in its unesterified form.

The lipids in bile are present in different aggregated states. Shape, size and composition of these aggregates is determined by the concentration of these lipids relative to that of the biliary bile salts as well as by their physicochemical characteristics. A lot of evidence is available to indicate that simple micelles, consisting of bile salts and cholesterol, mixed micelles, containing bile salts, cholesterol and phospholipids, and unilamellar vesicles, consisting of cholesterol and phospholipids and trace amounts of bile salts, can co-exist as biliary lipid carriers in a dynamic, i.e. interchangeable, form (201-203). An important observation was made by Coleman et al. (204) who described that retrograde injection of bile salts into the biliary tree was sufficient to cause biliary phospholipid secretion. Among other data (reviewed in (200)), this was an indication that the secretion of cholesterol and phospholipids is, at least in part, governed by bile salts present in the canalicular lumen. From these observations a commonly accepted model for biliary phospholipid secretion was drawn that was based on the passive extraction of canalicular membrane PC by bile salt micelles present in the canalicular lumen (reviewed by Coleman (14)). Biliary type PC molecules were thought to be transported to the canalicular membrane by means of vesicular traffic (see also section on the origin of biliary lipids). After fusion of these vesicles with the canalicular membrane the PC was thought to merge into microdomains. The presence in the membrane of sphingomyelin, cholesterol and PC makes this part of the membrane more rigid than the proposed microdomains, which are particularly rich in PC with shorter acyl chains. As a consequence, extraction of PC from these fluid microdomains by luminal bile salt micelles was thought to proceed much easier than from other parts of the membrane. The action of bile salts was thought to consist of an interaction of bile salt micelles with the microdomains leading to budding of the microdomain bilayer and subsequent pinching off of biliary type PC-rich vesicles. In this way the canalicular membrane was able to withstand the bile salt rich external environment. The presence of phospholipid vesicles in bile was observed, although their emanation from the canalicular membrane is hard to prove (205,206). Although never demonstrated in canalicular membranes up to now, microdomains have been observed in
other, in vitro systems (207). Additionally bile salts have been shown to induce the pinching-off of "bile-type" membrane lipids from red blood cell membranes in the form of vesicles (208,209).

**Class III type P-glycoprotein and biliary PL secretion**

With the finding, in 1993, that biliary PL secretion was abrogated in \( Mdr2 \) knockout mice and that mice heterozygous for the Mdr2 disruption excrete about half the amount of PC of wild type mice, it became clear that bile salt secretion was not the only factor regulating biliary lipid secretion. It was proposed that Mdr2 P-gp functioned as a membrane translocator for PC, transporting PC from the inner to the outer membrane leaflet and thus providing sufficient PC in the outer leaflet to be extracted by bile salts. This hypothesis is supported by several pieces of experimental evidence. The first came from experiments of Ruetz and Gros (210) who transfected the \( Mdr2 \) gene in a yeast secretion mutant. Using this experimental model the translocation of NBD-labelled phosphatidylcholine (NBD-PC) from the outer to the inner leaflet of the secretory vesicles was determined. ATP-dependent translocation of NBD-PC was observed which was specific for Mdr2 P-gp. Vesicles from yeast cells that were transfected with \( Mdr1 \) or with the transfection vector alone were not able to transport the substrate in an ATP-dependent fashion. A small fraction of PC-molecules was translocated in this assay. This may, however, be expected since the translocation process induces a phospholipid imbalance between the inner and outer leaflet, which is thermodynamically highly unfavourable. Thus, in the absence of net extraction of phospholipid from the trans-side of the bilayer, the translocation will halt. Unfortunately, little information was obtained on the substrate specificity of transport. It was not described whether the NBD-moiety was present in the head group or in the fatty acid tail of phosphatidylcholine. Unexpected was the inhibition of translocation by low concentrations of verapamil, which is an inhibitor of Mdr1 P-gp. This suggests that verapamil is able to inhibit Mdr1 as well as Mdr2 P-gp as was confirmed by van Helvoort et al. (122). Using the same yeast-vesicle system it was also shown that mouse Mdr2 mediated PC translocation was enhanced by the bile salt taurocholate (211). This stimulatory effect of bile salts on ATP-dependent PC translocation was also shown in rat canalicular vesicles (212). A second piece of evidence supporting the flippase function of Mdr2 P-gp was provided by Smith et al. (213)
who used fibroblasts from transgenic mice that express MDR3, the human homologue of Mdr2 P-gp. After metabolic labelling of intracellular phosphatidylycholine with radioactive choline, translocation from the inner to the outer leaflet was assayed and this was compared with normal mouse fibroblasts that do not express Mdr2. Translocation of radioactive phosphatidylycholine to the outer leaflet was measured by the possibility to exchange with phosphatidylcholine-transfer protein and liposomes in the medium. In MDR3 expressing fibroblasts a more rapid translocation of PC was observed than in control fibroblasts. Van Helvoort et al. studied the translocation of NBD-labelled lipids in polarised pig kidney cells that were stably transfected with MDR1, MDR3 or Mdr1a P-gp. These cells were loaded with the fluorescently labelled short chain lipid precursors, C6-NBD-diacylglycerol (DAG) or C6-NBD-ceramide (Cer). At 15°C, newly synthesised analogues of these precursors were recovered in the apical medium that contained albumin as a lipid acceptor. Because vesicular transport is absent at this temperature lipid molecules delivered to the apical membrane had to undergo membrane translocation before extraction into the medium could take place. With this assay it was demonstrated that MDR1, Mdr1a and MDR3 P-gp were all able to translocate short-chain labelled PC. Other lipid analogues like C6-NBD-phosphatidylethanolamine, C6-NBD-sphingomyelin (SM) and NBD-glucosylceramide (GlucCer) were only translocated by MDR1 and Mdr1a P-gps while MDR3 could not translocate these lipids. The key function of class III P-gps in hepatobiliary phospholipid transport was definitly confirmed by the finding that introduction of a human MDR3 transgene in Mdr2 knockout mice could restore hepatobiliary phospholipid to a level that correlates with expression levels of transgene expression (214). The role of both Mdr2 P-glycoprotein expression and bile salt secretion in hepatobiliary lipid secretion was studied in more detail using wild type mice, heterozygotes and homozygotes for mdr2 gene disruption (215). The PC and Cholesterol secretion in these mice was studied at various bile salt secretion rates generated by venous infusion of bile salts at stepwise increasing rates. Even at high bile salt secretion rates, PC secretion rates remained extremely low in mdr2 -/- mice. The curvilinear relation between BS and phospholipid secretion was maintained in wild type and +/- mice. Not only maximal phospholipid secretion was reduced in +/- mice but at all bile salt secretion rates the phospholipid output was reduced compared to wild-type mice. This indicated the strong control Mdr2 exerts over phospholipid secretion.
A new model for biliary PC secretion

The classical concept of bile salt induced secretion of PL and cholesterol through budding of the membrane involves both leaflets of the membrane. Inclusion of flippase action of mdr2 P-gp in this model has no functional relevance because it could not explain the absence of PL secretion in the absence of mdr2 P-gp. Therefore a new model was generated that recognised the involvement of asymmetry of the canalicular membrane bilayer (215,216), and is outlined in figure 1.

The first step in this model is the supply of PC to the canalicular membrane. The inconclusiveness of experimental data on the mechanisms of intracellular trafficking has been addressed before in, but generally three possible routes were proposed (22,200,215-217): -1- monomeric transport mediated by specialised PC transfer proteins; -2- vesicular transport directly from the site of synthesis to the canalicular membrane and -3- lateral diffusion from the basolateral membrane to the canalicular membrane via the inner membrane leaflet.

In case PC is supplied to the canalicular membrane via transfer proteins it will be delivered to the inner leaflet and Mdr2 P-gp than translocates PC from the inner to the outer leaflet of the membrane. Recent data from PC-TP knock out-mice show that the main candidate for this protein mediated transport PC-TP is not important for PC supply to the canalicular membrane (22). The role of other transport proteins remains to be proven.

If PC is delivered to the canalicular membrane by means of vesicular transport, one would expect that 50% of the PC is delivered to the outer leaflet of the canalicular membrane because PC will be present in both leaflets of such a vesicle. These lipids would be directly available for secretion into bile disregarding the presence or absence of the PC-translocating activity of Mdr2-Pgp. However, no biliary PC secretion was found in Mdr2-Pgp (-/-) mice. A possible explanation for this was proposed by van Helvoort et al. (22). It was suggested that Mdr2 Pgp is not only actively flipping PC from the inner to the outer membrane leaflet but additionally destabilises the outer membrane in this process. Destabilisation of the outer membrane could facilitate the extraction of PC from the outer membrane leaflet by bile salts. Because of the sheer impossibility to identify and isolate intracellular vesicles that might deliver lipids, destined to be secreted into bile, to the canalicular membrane it cannot be ruled out that these type of vesicles are asymmetrical.

The third option by which PC could be delivered to the canalicular membrane is by lateral diffusion in the inner membrane leaflet from the basolateral to the canalicular membrane.
domain. Lateral diffusion between these domains in the outer membrane leaflet is prevented by the tight-junctions which do not interfere with the lateral diffusion processes of PC in the inner membrane leaflet (38). In this way the PC molecules would reach the Mdr2 P-gp in the canalicular membrane to be flipped to the outer membrane leaflet.

![Diagram of lipid binding proteins and phospholipid secretion](image)

Figure 2. Model for the proposed mechanism of class III P-glycoprotein mediated phospholipid secretion. Adapted and modified from Oude Elferink et al. (216).

The process of Mdr2/MDR3 P-gp mediated translocation of PC from the cytoplasmic membrane leaflet to the exoplasmic membrane leaflet is the second step in the proposed mechanism of class III P-glycoprotein mediated phospholipid secretion. The current concept on biliary lipid secretion regards the role of Mdr2/MDR3 P-gp in this process to be the inductio of instable microdomains in the outer leaflet of the canalicular membrane.
through the production of local excess of PL. Then in the final step, these instable structures ultimately shed from the membrane in a vesicular form under the influence of bile salts, either monomeric or micellar, present in the canalicular lumen.

As yet, the mechanism by which bile salts actually induce lipid secretion has not been elucidated. The cooperative relationship between \textit{mdr2} P-gp expression and bile salt in the process of biliary lipid secretion was studied by Ruetz and Gros (218). Using the same heterologous Mdr2 P-gp expression system in yeast sec6-4 mutants as mentioned before they showed that secretion of PC in secretory vesicles transfected with \textit{mdr2} P-gp is significantly stimulated by the presence of taurocholate in these vesicles. The non-micelle forming bile salt taurodehydrocholate did not cause such stimulation.

With the use of electronmicroscopy of cryofixed liver, Crawford et al. provided ultrastructural evidence for bile salt induced vesiculation in the rat (219). They demonstrated the presence of electron-lucent vesicular structures, in the lumen of the canaliculus, that were connected, by an electron-dense base, to the exoplasmic leaflet of the canalicular membrane. The number of vesicular structures increased when rats were infused with taurocholate and the vesicles were infrequently found in bile salt-depleted rats. In a more recent study, it was demonstrated that the hepatobiliary secretion of these vesicles in the mouse was critically dependent on \textit{Mdr2} or \textit{MDR3} expression (220). Vesicle numbers were decreased in \textit{Mdr} \((+/-)\) and \((-/-)\) mice; 55% and 12% resp. of the numbers in wild type mice. Moreover in a \textit{MDR3} transgenic mouse strain, that had PC secretion levels of about twice that in wild-type mice an increased number of vesicular structures was observed. These observations are supported by the fact that also in primary bile of humans, biliary lipids exist as vesicles. Although the data look very promising, it still remains to be solved whether vesicle formation is the primary step in biliary lipid secretion.

Recent data support the concept that the very last step of canalicular phospholipid secretion is mediated in vivo by bile salt-induced vesiculation of PC-enriched microdomains from the outer leaflet of the canalicular membrane (221). Incubation of isolated rat hepatocyte canalicular and basolateral membrane vesicles with taurocholate showed that bile salt can preferentially solubilise PC from rat canalicular membranes; a four-fold higher PC release occurred from canalicular as compared to basolateral membranes. Compared with the contribution of PC to overall membrane lipid composition, taurocholate preferentially induced a two fold higher PC release from canalicular membrane vesicles. In contrast, taurocholate induced PC release from rat liver.
microsomal and kidney brush border membranes was proportional to their membrane contents. The use of 3-[(3-cholamidopropyl)dimethylammonium]-propane-1-sulphonate (CHAPS), a non-bile salt detergent, led to non-selective lipid solubilisation in both canalicular and basolateral membrane vesicles. Comparison of membranes from different organs showed an inverse exponential relationship between sphingomyelin content and taurocholate induced PC release while PC release from canalicular membranes did not fit this relationship. This indicates that the canalicular membrane is especially sensitive to taurocholate induced PC release.

From the model describing the biliary PC secretion it can be inferred that in the absence of PC-translocation activity, an increase in outer membrane leaflet cholesterol and sphingomyelin is induced. It is known that these types of membranes are highly resistant to the detergent action of bile salts (14). This is in agreement with the observation that infusion of the liver of mdr2 (-/-) knockout mice with bile salts that are more hydrophobic than the ones present in their endogenous pool still resulted in an almost negligible secretion of phospholipids. The data presented in chapter 5 indirectly support the view that in Mdr2 (-/-) mice the outer leaflet of the canalicular membrane is devoid of PC molecules. We show that the biliary secretion of short-chain labelled phospholipid analogues was disturbed in the perfused liver of Mdr2 -/- mice while it is known that these lipids are no substrates for Mdr2 P-gp. We hypothesised that a reduced PC content of the exoplasmic canalicular membrane leaflet in these mice prohibited bile salt mediated extraction of the NBD-lipid analogues from the membrane. Because it is extremely difficult to study lipid asymmetry in canalicular membranes in situ we studied tauroursodeoxycholate (TUDC) mediated extraction of one of these short chain lipid analogues, i.e. NBD-C6-SM, from small unilamellar vesicles of different lipid composition. This showed that NBD-C6-SM could be extracted from vesicles with a lipid composition roughly resembling that of the canalicular outer membrane leaflet (PC:Chol:SM (1:2:2)), while its extraction from pure Chol/SM vesicles (2:3) was reduced by 65%.

**Hepatobiliary cholesterol secretion**

Because the passive transmembrane diffusion of PC-molecules is supposed to be slow (i.e. hours to days; reviewed in (222)), the PC-translocation activity of Mdr2/MDR P-gp is probably needed to generate enough lipid flux to keep up with the high PC demand in normal biliary secretion. In contrast, neutral lipid species (i.e. with no ionised residues), like
cholesterol, can diffuse very rapidly through the membrane, and therefore no translocator is required to maintain normal levels of cholesterol in the outer membrane leaflet. The model in figure 1 assumes that cholesterol molecules ending up in the outer membrane leaflet of the canalicular membrane will enter the PL-microdomains by means of lateral diffusion, and subsequently are secreted into the bile together with the phospholipids. In this model a close relation between cholesterol and phospholipid secretion rates is expected which will be determined by the cholesterol content of the canalicular membrane. This model is supported by the observation in many studies that cholesterol and phospholipid secretion are tightly coupled. However, the model can not explain the substantial cholesterol secretion that was observed in mdr2 knockout mice, during bile salt infusion, in the virtual absence of phospholipid secretion (215). Furthermore, already at low rates of phospholipid secretion cholesterol secretion rates normalise to wild type levels. Mdr2 (+/-) mice have a 50% reduction in biliary PC but have normal cholesterol output value (134). With the generation of different strains of MDR3 rescue mice (transgenic mice expressing MDR3 at various levels) an animal model became available that allowed the study lipid secretion at different levels of PC/cholesterol secretion while BS output or composition could remain fairly equal (214). With these mice it was also shown that, especially at low PL secretion rates, a small increase in PL secretion results in a significantly larger increase of cholesterol secretion.

An explanation for these observations could be that, besides the proposed vesicular secretion via PC/Chol microdomains, an alternative mechanism exists. This mechanism could be that bile salt micelles directly extract cholesterol from the outer leaflet of the membrane. The efficiency of this extraction is determined by the cholesterol solubilising capacity of bile salts and the presence of phospholipids further increases this efficiency because mixed micelles of bile salts and phospholipids have a higher cholesterol solubilising capacity (81). In the latter model cholesterol secretion would thus only be secondarily dependent on phospholipid secretion. The data obtained thus far, suggest that under normal conditions both mechanisms could be active simultaneously although it is not clear what their individual contribution to total biliary cholesterol secretion is.
Mdr2/MDR3 P-glycoprotein and liver disease

The absence of biliary lipids in the bile of Mdr (-/-) mice leads to liver disease, which becomes manifest shortly after birth and progresses to an end stage within 3 months after birth (214,223). The liver pathology is that of nonsuppurative inflammatory cholangitis with portal inflammation and ductular proliferation. At an age of 4-6 months these mice start to develop nodular outgrowths in the liver parenchyma, which progresses to metastatic liver cancer (223). It was hypothesised that the cells lining the biliary tree react to the lipid-free bile by an inflammatory response and induction of bile duct proliferation. Several studies have demonstrated that the cytolytic effect of bile salt micelles can be counteracted by addition of phospholipid and cholesterol (224-226). As mentioned before, the ability of bile salt to extract phospholipids from membranes depends on its relative hydrophobicity. Manipulation of the overall hydrophobicity of biliary bile salts influences the severity of liver pathology in Mdr (-/-) mice. Feeding cholate, a relative hydrophobic bile salt increases the pathology, while decreasing overall hydrophobicity by feeding ursodeoxycholate (hydrophilic) improves the histological picture (227). The bile salt pool of mice is relatively hydrophilic; it is composed of the very hydrophilic muricholic acid (60-70%) and taurocholate (30-40%). Interestingly, female Mdr (-/-) mice seem to develop a more severe liver pathology than male Mdr (-/-) mice (228). These female mice also show an increased bile salt hydrophobicity that is caused by the presence of more taurocholate as opposed to the male mice (227).

The importance of biliary lipid secretion and the development of liver pathology was further emphasised by de Vree et al, who showed that a minimal level of phospholipid secretion (15% of normal secretion) is absolutely required to prevent liver pathology in MDR3 transgenic mice. ((214) and thesis J.M.L de Vree).

The phenotypical features of the progressive liver disease found in Mdr2 (-/-) mice is very similar to that found in human patients suffering from a subtype of progressive familial intrahepatic cholestasis (PFIC). These patients can be distinguished from the other 2 types of PFIC patients by their high serum γ glutamyl-transferase activity (γ-GT) and liver histology that shows portal inflammation and ductular proliferation in an early stage (229,230), and therefore this disorder was called “progressive familial cholestasis with high γ-GT” or PFIC type 3. Deleuze et al. (231) described a lack of MDR3 mRNA in the liver of patients with a this type of PFIC. Subsequently, de Vree et al. (232) demonstrated that negative canaliculal staining for MDR3 P-glycoprotein in PFIC 3
patients was the result of defects in the MDR3 gene caused by mutations or deletions. In these patients reduced phospholipid concentrations (1-7% of normal) were observed.

**Functional aspects of lipid secretion**

Conversion of cholesterol into bile salts is the major degradation pathway for this lipid. Bile salts are almost exclusively secreted into bile. Thus, the biliary pathway represents the major route for the removal of cholesterol, either as such or in the form of bile salt, from the body and functions as a crucial factor in the maintenance of cholesterol homeostasis (233). The highly efficient reabsorption of bile salts from the intestine appears to be somewhat contradictory to the homeostatic function of biliary cholesterol secretion. However, the controlling step in cholesterol metabolism could be mainly located in the intestine and to a lesser extent in the liver. Biliary phospholipids play an important role in the absorption of dietary lipids from the intestine, in addition to the well-established function of bile salts in this process (234-236).

Studies by Davidson et al. (237,238) have provided evidence for a physiologic role of biliary phospholipids in the regulation of intestinal apolipoprotein B48 expression, an apolipoprotein essential for proper assembly and secretion of chylomicrons containing the absorbed dietary fats. Furthermore, Voshol et al. (gastroenterology, in press) showed that the production of chylomicrons by enterocytes is delayed in Mdr2 (-/-) mice. They concluded that this was most likely explained by the absence of biliary phospholipids from the gut in these mice. This is very interesting because chylomicron surface lipid is an important source of HDL-lipids and these authors previously reported strongly reduced plasma HDL levels in the same mouse strain (239).

Finally the biliary lipids could serve as carriers for lipophilic compounds in their routing from hepatocyte to the intestine. Based on both in vitro and in vivo studies it has been proposed that lipid vesicles may act as transport carriers for hydrophobic anions such as indocyanine green (ICG) and protoporphyrin (PP). As shown by Tazuma et al. (240,241), Berenson et al. (242) and Beukeveld et al. (243), these hydrophobic organic anions show a high affinity for the (vesicular) lipid fraction in bile and it has been suggested that vesicular structures act as carriers of these compounds during transit from the liver to the intestine. Although this is a new concept, structural and/or secretion characteristics of several compounds make them candidate compounds for such an excretion pathway. Mechanistic aspects of biliary protoporphyrin excretion can be used as a model for lipid coupled hepatobiliary
transport.
Protoporphyrins are large planar hydrophobic molecules that are virtually insoluble in water at physiological pH. Protoporphyrins are intermediates in haem synthesis and excreted from the body solely by secretion into bile and subsequent elimination via faeces. It is known that biliary protoporphyrin excretion in rat liver is greatly increased by infusion of bile salts and that protoporphyrin secretion shows a curvilinear relationship with bile salt secretion. Protoporphyrin secretion reaches a plateau phase at high bile salt secretion rates and taurocholate (TC) is a more potent inducer of protoporphyrin secretion than the more hydrophilic ursodeoxycholate (244-246). Infusion of taurocholate induces a 150-fold increase in protoporphyrin excretion. Berenson et al. (247) studied the relation between canalicular bile salts and protoporphyrin excretion in the perfused rat liver. These elegant studies revealed that bile acids facilitate the biliary translocation of protoporphyrins in the same manner as it affects biliary phospholipid secretion. Injection of TC together with protoporphyrin to the portal venous catheter resulted in a peak in biliary porphyrin secretion and this peak coincided with the peak of PL secretion. Injection of TC 12 minutes after injection of protoporphyrin caused an equal delay in PL and protoporphyrin secretion.

Because of their strong relation it was speculated that Mdr2 P-gp may both influence PL and protoporphyrin secretion by a similar mechanism. This possibility was further investigated by Beukeveld et al. (243). They determined the secretion of protoporphyrin in mdr2 knockout mice and controls. Biliary secretion of endogenous protoporphyrin was strongly reduced (90%) in knockout mice compared to the controls. The precursor molecules of protoporphyrin, coproporphyrin isomers I and III, showed no significant reduced secretion in the mdr2 knockout mice compared to controls. The mechanism of protoporphyrin excretion seems to be different from the coproporphyrin isomers. In contrast to protoporphyrin, the coproporphyrin isomers can also be excreted via the urine and they seem to be excreted into bile by Mrp2 while protoporphyrin can not be excreted via this mechanism (243).

Whether protoporphyrin is transported directly by Mdr2 P-gp or is translocated through the membrane by another mechanism is not known. Secretion of protoporphyrin could be explained by two mechanisms: 1) vesicles present in the canaliculus could accept protoporphyrin, residing in the canalicular membrane or 2) protoporphyrin and (vesicular) lipids could be simultaneously discharged from the outer leaflet. In both mechanisms lipid vesicles would act as carriers and/or acceptors of amphipathic compounds that end up in the outer membrane leaflet of the canalicular membrane. Either spontaneous or facilitated
translocation of such a compound to the outer membrane leaflet would be sufficient to eliminate them from the membrane. It could very well be that the amphipathic substrates of class I and II P-gps (129-133,248) are expelled from the membrane upon acceptance or incorporation into nascent lipid vesicles. Capture of the compound in a lipid environment also impedes the substrate from re-entering the plasma membrane by diffusion. The transport of hydrophobic compounds through the biliary tract in lipid vesicles could be a general mechanism. Such a mechanism would imply that the combined action of Mdr2 P-gp and bile salts facilitates the biliary disposition of such compounds. While the reduced biliary secretion of protoporphyrin transport or vit D in Mdr2 knockout could possibly be attributed to absence of Mdr2 P-gp mediated translocation of these substrates to the exoplasmic leaflet, data in chapter 5 suggest that the secretion of lipoidic non-Mdr2 substrates is also decreased in these mice. This might indicate that the mere prescence of a normal biliary lipid environment facilitates the extrusion of these amphipathic compounds.
REGULATION OF BILIARY LIPID SECRETION

An important role for bile salts

It is clear that biliary secretion of phospholipids is controlled simultaneously by type and amount of bile salt present in the canalicular lumen and Mdr2/MDR3 P-gp activity. Among the various species bile salt composition is not identical and in some cases bile salt composition seems to be gender-related (228) and (249). However, in different species marked quantitative differences exist in the amount of lipid secreted per amount of bile salt (193). Therefore we investigated the possibility that bile salt composition had regulatory effects on Mdr2 expression ((250,251) and chapter 2). To this end male mice (FVB) were fed a purified diet to which either 0.1% (\%/w) cholate or 0.5% (\%/w) ursodeoxycholate was added. This led to a near complete replacement of the endogenous bile salt pool (mainly tauromuricholate) by taurocholate or taourursodeoxycholate resp. After chronic feeding the maximal phospholipid secretion capacity in these mice was determined by infusion of increasing amounts of taourursodeoxycholate. Cholate-feeding resulted in a 55% increase in the maximal phospholipid secretion compared to control diet. Northern blotting revealed that with cholate-feeding Mdr2 P-gp mRNA was increased by 42%. Feeding ursodeoxycholate did not influence the maximum rate of phospholipid output nor Mdr2 mRNA content. Female mice had a higher basal Mdr2 P-gp mRNA level than male mice and this also correlated with a higher phospholipid secretion capacity. This could be explained by the higher basal cholate content in the bile of female mice compared to male mice. From these results we deduced that the type of bile salts in the circulation influences the expression of the Mdr2 gene. The mouse model did not allow us to study the regulatory effects of bile salts at low bile salt fluxes. To investigate this we chose to study the relation between bile salt and Mdr2 (PGP3) expression in a bile diverted rat model as described in chapter 3. The content of the bile salt pool was decreased by long term interruption of the enterohepatic circulation. The ability of bile salts to elicit phospholipid secretion was studied again by application of a standard infusion protocol by which TUDC was infused at stepwise increasing rates. Long-term bile diversion resulted in strongly decreased maximal PL secretion capacity that coincided with low levels of rat Mdr2 RNA expression. When the enterohepatic circulation was restored again both Prsecmax and Mdr2 mRNA levels were increased significantly. To show that this regulation was only depending on endogenous bile salt concentration, taurocholate was infused into the duodenum of bile depleted rats during the last 48 hours of the bile
diversion period. As expected, duodenal TC infusion was sufficient to increase both Plicemax and expression of Mdr2 mRNA. In this study the levels of Mdr2 expression reflected nicely the sub- and supramaximal PL secretion capacities reached in this model. Both studies provide evidence that bile salts are able to regulate Mdr2 P-gp expression. In intact rat liver high levels of Mdr2/PGP3 mRNA are found while in cultured rat hepatocytes Mdr2/PGP3 mRNA levels showed a gradual decline between 4 hours and 24 hours in culture (252). In another study no PGP3 mRNA could be detected in rat hepatocytes after 40 hours of culture (253). The time dependent decreased expression levels are in line with the regulatory effect of bile salts on class III P-glycoproteins, because no bile salts were present in the culture medium. Similar results were found for Mdr2 mRNA expression in cultured primary mouse hepatocytes. However, when bile salt was added to the culture medium a slower decline in Mdr2 mRNA levels was observed (C. Frijters and O. Mook; unpublished observations). Chung et al demonstrated that hydrophobic bile salts like cholic acid and chenodeoxycholic acid, but not the hydrophilic bile salt ursodeoxycholic acid, increase human MDR3 mRNA expression in HEPG2 cells (254). However, using a luciferase reporter gene controled by a 3 kB promotor fragment of MDR3 no effects of bile salts on luciferase activity could be detected (254). The expression of Mdr2 in rat liver is reported to be increased during bile duct ligation (255) and this might this be caused by increased intracellular bile salt concentrations.

The molecular mechanism by which bile salts regulate the expression of class III P-gps is not clear. It is known that bile salts are potent regulators of key enzymes in their own biosynthetic pathway, 7α-hydroxylase and sterol 27-hydroxylase (256-262). The regulatory capacity seems to be dependent on the hydrophobicity and structure of bile salts. A bile acid responsive element (BARE) residing in the proximal 5'-flanking regions of rat cholesterol 7α-hydroxylase (CYP7a) was identified that seemed to be involved in regulation of this gene by bile salts. It was proposed that binding of bile salt to an unidentified bile salt receptor eventually would lead to the release of a positive trans-acting factor from the cholesterol 7α-hydroxylase gene. Recently, it has been demonstrated that bile acids also function as "hormones" that bind to nuclear receptors and, through that mechanism, modulate expression of proteins involved in cholesterol homeostasis. Several orphan nuclear receptors have been shown to bind bile acids (cholic and chenodeoxycholic acids), including the farsenoid X receptor (FXR) (263-265) and the LXRalpha receptor (266-268). Furthermore, the resulting bile acid-receptor complexes have been shown to be capable of binding to promoter regions of specific genes and
either to stimulate or suppress their transcription (268). In essence, bile salts can function as steroid hormones. A repressive action of bile salts on a luciferase reporter controlled by the CYP7a gene promoter was only observed when FXR, but not other nuclear receptors was expressed (266). On the other hand, it has been shown that bile salts can have a stimulating effect on genes involved in cholesterol homeostase. A majority of the bile salts delivered into the intestine are recycled by absorption in the ileum (enterohepatic circulation). The transport of bile salts through the cytosol of intestinal enterocytes is thought to be facilitated by specific binding proteins like the intestinal bile acid-binding protein (I-BABP). Expression of the gene encoding this I-BABP increases in response to bile salts. The promoter of the I-BABP gene also binds the FRX-bile acid complex, which activates transcription. In this case, bile salts induce transcription in order to enhance their reabsorption from the gut. Database comparison could not identify a sequence similar to the BAREs reported to function in CYP7a (262) (TCAAGTTCAAGT) or I-BABP (269). (AGGTGAATAACCT) (-142-130) in the human MDR3 (270) or rat Mdr2 promoter regions (271) (personal observation).

It is well known that hypolipidaemic drugs such as fibrates induce several genes required for peroxisomal β-oxidation of long chain fatty acids, genes of the P-450 family and genes encoding proteins involved in cell growth and proliferation (272-275). Chianale et al. described that feeding mice during one week with clofibrate or some of its structurally related compounds increased biliary phospholipid output independently of bile salt secretion rates and this was not related to changes in biliary bile salt pool composition (276). These changes were accompanied by increased steady-state levels of Mdr2 mRNA in the liver (276). However, there seems not to be a strict correlation between Mdr2 mRNA expression and biliary lipid secretion. While the former is elevated to 5 or 6 times the normal situation, biliary phospholipid secretion is only increased twofold. Furthermore, cholesterol output in these mice was not affected by the fibrate diets. In these studies the maximal PC secretion capacity was not determined. In a later study it was shown that, besides fibrates, also other peroxisome proliferators were able to induce the mouse Mdr2 gene (277). It was suggested that modulation of Mdr2 gene expression might be part of the pleiotrophic response of peroxisome proliferation in mice liver.

Carrella et al. (278) described that sustained treatment of rats with pravastatin, a statin type 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA-reductase) inhibitor, led to increased biosynthesis of PC and cholesterol. In addition, biliary secretion of both cholesterol and PC were increased to approx. 250% of controls while bile salt synthesis
was not affected. These increases were accompanied by a sevenfold increase in Mdr2 mRNA. The question was raised whether there exists co-ordination between synthesis, canalicular translocation and biliary secretion of phospholipids. This suggestion would fit with the effects found in the mice fed fibrates because an enhancement of cytidyltransferase has been shown by fibrates (279).

Recently, it was shown by Hooiveld et al. (abstract Dutch Society for Hepatology; 1999) that induction of hepatic Mdr2 by statins is, at least partially, under control of sterol regulatory element binding proteins (SREBPs). A novel family of bound transcription factors that are known to regulate multiple genes involved in cholesterol biosynthesis and uptake (reviewed in in (280)). Transient transfection studies with HepG2 cells revealed that statins stimulated Mdr2 promotor activity and that this stimulation was increased by co-tratrasfection with SREBP1. Whether the statin related effect and the bile salt mediated regulation of Mdr2 are connected remains to be established.

The expression of \textit{Mdr2} mRNA is increased following partial hepatectomy in rats (74,281) and it is not known which factors regulate this induction. Because in this situation also Mdr1a and Mdr1b mRNA levels were elevated it is unclear whether the induction of Mdr2 mRNA is specific. Gant et al. (282) studied the gene expression of P-glycoproteins in rhesus monkeys after treatment with xenobiotics that are excreted via the bile. After treatment with erythromycin, rifampicin, tamoxifen and probenecid an increase in Mdr2 mRNA was observed. Interestingly, this increase in Mdr2 mRNA was accompanied by decreased levels of Mdr1 expression. The results suggested that expression of Mdr2 is responsive to xenobiotics or their metabolites that require biliary excretion.

\textit{Uncoupling of PC and cholesterol excretion}

It is well known that several hydrophilic organic anions can inhibit biliary lipid secretion without affecting bile salt secretion (197,283-286), a process referred to as "uncoupling". This phenomenon has been used experimentally to determine the site of action of bile salt induced lipid secretion. Evidence has been provided that, under physiological conditions, bile salt induced lipid secretion is regulated at the level of the canaliculus and within the hepatocyte. In these studies, reviewed by Verkade \textit{et al.} (197) it was first shown that hydrophilic organic anions, that predominantly interact with biliary bile salts, are able to
uncouple lipid from bile salt secretion in normal but not in mutant (GY/TR) rats (168,287). These rats lack canalicular ATP-dependent organic anion transport and therefore show impaired bile secretion and increased intracellular concentrations of the uncoupling agents (213,288). This observation demonstrates that uncoupling takes place within the canaliculus and strongly suggests that the interaction of organic anions with lipid secretion is exerted after secretion of the bile salts across the bile canalicular membrane.

The uncoupling of PL and cholesterol from bile salt secretion has been reported as a side effect for therapeutical use of organic anions. Patients receiving ioglycamide, a compound used as contrast medium for radiographic inspection of the biliary tree showed a depression of phospholipid and cholesterol secretion while bile salt secretion remains normal (289).

This anion is similar to iodipamide, which in addition to uncoupling activity in rats (290) can cause hepatotoxicity (291). Theoretically, liver damage could occur by reducing the lipid to bile salt ratio in the canalicular lumen. The biliary lipids are less able to counteract the detergent action of the canalicular bile salts, which can become toxic to membranes lining the lumen of the biliary tract. Although this pathophysiological mechanism is speculative, iodipamide has been shown to induce liver damage in rats injected with iodipamide. Electronmicroscopic analysis of the liver revealed accumulation of lipid droplets 17 days after the injection of iodipamide (292). Thus, the uncoupling effect of certain cholephilic anions calls for care in the chronic use of these compounds.

In contrast to hydrophilic organic anions, hydrophobic organic anions do not interfere with biliary lipid secretion in rats. Upon gel filtration of bile, hydrophilic organic anions show high affinity for the bile salt micellar fraction whereas hydrophobic species preferentially associate with the vesicular, phospholipid and cholesterol containing fractions (197,286,293). The mechanism by which hydrophilic organic anions evoke uncoupling of biliary lipid secretion from bile salt secretion is not clear. As described previously, biliary lipid secretion probably involves both the formation of vesicles and micellar extraction of lipids from the canalicular membrane. To test whether organic anions interfere with the latter Verkade et al. (294) studied the interaction between organic anions, micelles and vesicles in model bile systems. From these studies it was concluded that the effects of organic anions in vivo are unlikely to be based on the inhibition of micelization of bile canalicular membrane lipids.

From several studies in different animal species it is known that there exists an inverse relationship between bile salt independent flow and the capacity of bile salts to induce lipid
secretion. Although these data are generated in different experimental systems and bile salt composition can differ significantly, some correlation indeed seems to exist (295). This is substantiated by the observation that the hydrophilic anion ampicillin uncouples biliary lipid from bile salt secretion and at the same moment increases the bile salt independent flow (293). Control analysis of the process of biliary lipid secretion suggested that, besides bile salts and Mdr2 expression, canicular water flux is an important factor controlling this process (296). These combined findings led us to hypothesize that hydrophilic organic anions might interfere with water flux and thus secondarily affect the bile salt mediated uptake of biliary lipids. The data presented in chapter six provide experimental proof that certain organic anions influence the canicular bile salt concentration and therefore induce an “apparent” uncoupling of bile salt and lipid secretion. However it should be stressed that this mode of action is not the universal way by which organic anions interfere with biliary lipid secretion. Sulphated tauroliothocholic acid (STLC), a hydrophilic organic anion, has uncoupling ability but does not induce bile flow ((288) and chapter 6). If this organic anion would affect the direct extraction of canicular lipids by micelles, the uncoupling effect should become less with increasing concentrations of bile salt molecules in the canicular lumen. At high bile salt output rates the uncoupling effect of STLC was indeed smaller than at low secretion rates. However, as discussed before, the formation of canicular vesicles seems to be the major route by which biliary lipids are released into bile and our results do not exclude the possibility that STLC influences the vesiculation process.
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