Histol Histopathol (2000) 15: 761-770

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Invited Review

Microstructural analysis of bile: relevance to cholesterol gallstone pathogenesis

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Summary. The study of physical-chemical factors and pathways leading to cholesterol crystallization in bile has important clinical relevance. The major processes in cholesterol gallstone formation can be subdivided into nucleation, formation and precipitation of solid crystals (crystallization), crystal growth, crystal agglomeration and stone growth. A clear understanding of the microstructural events occurring during the earliest stages of these processes in bile is crucial for the identification of factors possibly delaying or preventing precipitation of cholesterol crystals and, therefore, gallstone formation in bile.

Detection and characterization of microstructures in native and model biles can be achieved by both direct and indirect techniques. Direct imaging techniques provide more readily interpretable information, but sample preparation problems, particularly for electron microscopy, are a source of artifacts. Moreover, microscopic techniques provide only qualitative data without the possibility to quantitate or to analyse the composition of microstructures. Several indirect techniques have been used to obtain additional microstructural information about nucleating bile. These techniques have the disadvantage of often being model dependent in addition to constraints specific for each method.

The systematic, judicious use of a combination of complementary direct and indirect techniques have led to a comprehensive understanding of the various microstructural processes and interactions occurring during bile secretion, flow in the biliary tract and storage in the gallbladder. This forms the basis for our current understanding of cholesterol nucleation, crystallization and gallstone formation.

Key words: Bile, Crystallization, Gallstone, Cholesterol, Imaging

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Introduction - The pathway to cholesterol gallstone formation

Bile is secreted from the liver into the biliary tree, through which it flows to the duodenum. In the interdigestive period bile is stored in the gallbladder. Following cholecystokinin stimulation during digestion, it is delivered in a concentrated form into the gut (Carey and Cahalane, 1988). Bile is composed of over 90% water and about 10% solutes, mainly biliary lipids-cholesterol, bile acids and phospholipids. Other components of bile include proteins (glycoproteins, mucins), pigment (bilirubin), electrolytes and xenobiotics (Harvey et al., 1985; Carey and Cahalane, 1988). Cholesterol in bile is present as free cholesterol. Bile acids are present as salts of primary bile acids (cholic and chenodeoxycholic acid) and their intestinal dehydroxylation products (deoxycholic and lithocholic acid, respectively). All bile acids in bile are conjugated with glycine or taurine (Carey and Cahalane, 1988). Biliary phospholipids are composed mainly of phosphatidylcholine (about 95%) and small amounts of phosphatidylethanolamine and phosphatidylserine (Angelico et al., 1992). These phospholipids are a mixture of molecular species having primarily palmitic or stearic acid in the sn-1 position and less saturated fatty acyl chains in the sn-2 position (Hay and Carey, 1990).

Bile is the only significant pathway for the excretion of cholesterol, and about half of the biliary cholesterol is lost in feces. The hydrophobic cholesterol is solubilized in bile at a concentration of 5-50 mM, which exceeds over 100,000 fold its water solubility. This solubilization of cholesterol molecules is made possible by the two ampiphilic lipid molecules-bile salts and phospholipids (Cabral and Small, 1989). Together, the three biliary lipids form micellar and lamellar-vesicular structures that serve as cholesterol "carriers" in bile (Admirand and Small, 1968; Carey and Small, 1978; Somjen and Gilat, 1983, 1985). These structures incorporate the insoluble cholesterol molecules during bile secretion, flow, and bile storage in the gallbladder.

The sequence of physical-chemical events during lipid secretion into bile is still being investigated. Crawford et al. (1995) showed that biliary phospholipid molecules are secreted by hepatocytes into the bile canalicular lumina as 63-67 nm (diameter) unilamellar vesicles. This process is rapid and is facilitated by the detergent action of bile salts at the exoplasmatic part of the canalicular membrane. Cholesterol is taken up by the vesicles and secreted into hepatic bile, which normally has a cholesterol:phospholipid ratio of about 1:3. In lithogenic bile, which is supersaturated with cholesterol this ratio is higher especially in the vesicles, where it can reach about 2:1 (Donovan and Carey, 1990). During flow in the biliary tree vesicular cholesterol is gradually taken up into bile salt rich micelles. Since mixed micelle formation requires the solubilization of more phospholipids than cholesterol, the excess cholesterol remains to be solubilized in vesicles, which become supersaturated and thermodynamically metastable. Recently, some intermediate lamellar structures have also been described in model and native biles, but their nature and possible role in biliary cholesterol solubilization and transport is controversial (Somjen et al., 1990a,b; Cohen et al., 1993). When bile salt concentration is sufficient, all cholesterol will be solubilized in micelles. However, more commonly bile is supersaturated and only part of the cholesterol is solubilized in micelles, with the rest remaining in metastable vesicles. When the cholesterol carrying capacity of the lipid aggregates is exceeded, cholesterol may precipitate and form cholesterol monohydrate crystals (Small, 1980). Most available data suggest that biliary cholesterol crystallizes from cholesterol rich vesicles, although recently it has been shown that supersaturated mixed micelles may also be the source of cholesterol crystal precipitation (Ahrendt et al., 1994). Ultimately, within the gallbladder cholesterol crystals are agglomerated with an organic matrix of mucin glycoproteins to form cholesterol gallstones (Lee et al., 1979). Crystallization processes are additionally influenced by pro- and anti-nucleating factors, which are mostly believed to be biliary proteins (Portincasa et al., 1997).

The study of physical-chemical factors and pathways leading to cholesterol crystallization in bile has important clinical relevance. The major processes in cholesterol gallstone formation can be subdivided into nucleation, formation and precipitation of solid crystals (crystallization), crystal growth, crystal agglomeration and stone growth (Wang and Carey, 1996a). A clear understanding of the microstructural events occurring during the earliest phases of these processes in bile is crucial for the identification of factors possibly delaying or preventing precipitation of cholesterol crystals and, therefore, gallstone formation in bile.

Studying bile

To gain detailed information on cholesterol crystal formation different "bile models" have been investigated. These have been mainly of three principal types: human bile, animal bile, and artificial bile solutions.

Human bile can be obtained for examination by a variety of procedures: I. Gallbladder bile obtained at surgery or by percutaneous puncture. II. Duodenal bile obtained by nasoduodenal drainage. III. Hepatic bile obtained via a T-tube placed intraoperatively. IV. Bile obtained during endoscopic retrograde cholangiopancreatography (ERCP) and nasobiliary drainage. Examination of bile obtained directly from the gallbladder can provide the degree of cholesterol supersaturation, chemical estimation of biliary lipids, nucleation time and depict the cholesterol crystallization process as it occurs in the native enviroment (Holan et al., 1979; Van Erpecum et al., 1988). There are, however, several problems in studying native human bile. First, obtaining bile directly from the gallbladder requires the use of invasive methods such as surgery or gallbladder puncture. The use of percutaneous fine needle puncture of the gallbladder is not always successful and has been associated with bile leakage (Petroni et al., 1993). Moreover, the complex nature of native bile, in which many factors may change from sample to sample, makes it difficult to study nucleation and crystal growth systematically (Van Erpecum et al., 1988; Janowitz et al., 1990). Finally, human bile is usually obtained from patients who already have gallstones, and may thus not provide relevant information regarding early events in gallstone pathogenesis.

Duodenal bile can be obtained by nasoduodenal intubation and can be examined for cholesterol saturation, nucleation and crystal growth (Marks and Bonorris, 1984; Marks et al., 1991; Choudhuri et al., 1993) and also for gallstone type prediction (Agarwal et al., 1994). It is, however, dilute compared with gallbladder bile and contaminated with gastric, pancreatic and intestinal secretions. Since crystallization is affected by biliary lipids and proteins, and duodenal bile contains proteases and lipases, the investigation of duodenal bile is problematic and should be interpreted with great caution (Marks et al., 1991; Petroni et al., 1993).

An alternative approach is to obtain bile during endoscopic retrograde cannulation of the common bile duct or following nasobiliary drainage, thus avoiding contamination of bile with intestinal contents. A limitation of the method may arise from the necessity to selectively cannulate the biliary tract during ERCP, and bile collection can extend the time of the endoscopic procedure and involve side effects especially pancreatitis (Petroni et al., 1993). Moreover, aspiration of bile should be performed before injection of contrast medium in order to avoid bile dilution (Konikoff and Gilat, 1999). The composition of bile is similar to the bile obtained by drainage from T-tube, unless it is aspirated after cholecystokinin injection (Busccail et al., 1992).

Patients following cholecystectomy and choledocho-

tomy are left with an indwelling T-tube for variable time periods, providing an opportunity to study hepatic bile in humans. This form of biliary drainage, however, interrupts the enterohepatic circulation and may result in bile acid deficiency, cholesterol malabsorption and thus influence bile composition. To avoid this, it is important to collect the bile after clamping the T-tube for at least 3 days (Shaffer et al., 1972; Pakula et al., 1999). The use of a balloon occludable T-tube may permit complete collection of hepatic bile, controlled sampling, and return of the remaining bile to the common duct and duodenum (Soloway et al., 1972).

In order to study the formation of gallstones, various animal models have been used. The hamster has been, perhaps, the most widely used model since this species forms cholesterol gallstones, and the lithogenic diet fed to hamsters is easily altered (Cohen et al., 1989; Trautwein et al., 1993; Ayyad et al., 1996). Other traditional laboratory animal models include the prairie dog (LaMorte et al., 1993; Magnuson et al., 1995), mouse (Alexander and Portman, 1987; Kim et al., 1993; Wang et al., 1997), gerbil (Bergman and Van der Linden, 1971), guinea pig (Dorvil et al., 1983), various species of monkeys (Osuga and Portman, 1972; Pekow et al., 1995), dog (Englert et al., 1969), rabbit (Ozben, 1989) and squirrel (MacPherson et al., 1987). A few characteristics are essential in an animal model in order for it to be used for the study of gallstone production (Lipea et al., 1988). One has to be aware of the chemical composition of bile, since most animals do not have bile acids similar to those found in humans. Guinea pig and rabbit biles contain bile acids which are not present in human bile, although, guinea pig bile also contain the main four human bile acids (Lipea et al., 1988). The relative concentration of phospholipids, bile acids and cholesterol are similar between man and prairie dog (Lipea et al., 1988). Gallstone composition was found to be similar to that of human gallstones in the prairie dog, mouse, hamster, squirrel monkey and guinea pig. The induction of gallstones may be variable in terms of reproducibility and time. For example, prairie dogs developed gallstones within 2 weeks (Ayyad et al., 1996), hamsters within 1-2 months (Cohen et al., 1989; Ayyad et al., 1996), and mice after 2-8 weeks on a lithogenic diet (Alexander and Portman, 1987; Wang et al., 1997). Most animal models develop gallstones without any other ill effects. However, when stones appear in the prairie dog, a high incidence of fatty liver, hypercholesterolemia, and atherosclerosis occur (LaMorte et al., 1993). Obtaining sufficient quantities of bile, stones and tissue for analysis can be problematic in some small animals. This can be circumvented by pooling fluids and gallstones prior to analysis (Lipea et al., 1988).

In vitro studies with artificial bile models have been the mainstay of investigating biliary lipid interactions and have served to establish the importance of both bile salts and lecithin in the solubilization of biliary cholesterol (Admirand and Small, 1968; Carey and Small, 1978; Somjen and Gilat, 1983, 1985). Through the use of model bile systems the mechanisms of cholesterol solubilization and crystallization have been systematically investigated. The models have well defined components and concentrations, and the sequence of events and evolution of microstructures is assayed from a controlled "time zero". For example, dilution of a concentrated micellar solution causes bile salt depletion of micelles and subsequent cholesterol supersaturation that induces cholesterol crystallization (Konikoff et al., 1992). This well defined zero time provides a basis for comparison between several solutions of the same model as well as between different models (Gilat and Somjen, 1996; Wang and Carey, 1996a). The crystallization process in native human bile during *ex-vivo* incubation was found to resemble that in model systems, supporting the applicability of the models in the exploration of microstructural aspects of nucleating human bile (Gilat and Somjen, 1996; Wang and Carey, 1996a,b).

Analytical methods

Characterization of the lipid particles and estimation of their contribution to cholesterol crystallization has been done using various experimental techniques. In order to determine the proportions of lipids in the different aggregational forms, separations usually have to be done. The problems with separation are that these may influence proportions of compounds, change the volume of the original sample, introduce interactions with exogenous materials and create artifacts. These changes shift towards new equilibria and interconversions of particles are probable. However, if re-equilibration of particles after separation is slow, separation may not significantly disturb the initial distribution (Gilat and Somjen, 1996). Thus, all techniques used to study cholesterol crystallization have to be viewed in relation to their potential of introducing specific artifacts. In general, the methods can be divided into direct and indirect techniques. Direct techniques, such as morphological imaging, are more familiar and tend to receive precedence over indirect, usually model-dependent assessment methods, which often have specific constraints and require caution in data interpretation.

Direct techniques

Microscopic techniques

The microscopic examination of bile in the detection of biliary tract diseases dates to 1919 when Lyon devised a method for stimulation of gallbladder contraction and collection of bile specimens from the duodenum (Lyon, 1919).

Light microscopy examination of bile was found to be accurate in diagnosing gallstones, and stone type and predicting the outcome of gallstone dissolution therapy

(Ros et al., 1986; Ramond et al., 1988). Using polarized light microscopy cholesterol crystals and calcium bilirubinate granules can be identified in a size range from 2000 Å to several microns (Gogna et al., 1989). The major advantage of microscopy is that it is non-perturbing, and does not require sample processing.

A more recently introduced form of microscopy is *video-enhanced light microscopy* (VELM), which employs differential interference contrast (Nomarski optics) combined with digital subtraction of background noise by a computer. The resolution is at least twice that of conventional light microscopy with a lower limit of about 1,000Å. By this method dynamic particle movement in the sample can be observed and recorded by a video recorder for further analysis. The main disadvantage is that because of manipulations necessary in a multistep reproduction process, quality and contrast are somewhat compromised compared with primary images detected by light microscopy (Holzbach, 1990).

Light microscopic detection of cholesterol monohydrate crystals in bile was proposed to identify the rate limiting step in gallstone formation (Holan et al., 1979). The whole process can be divided into three successive steps: nucleation, crystallization, and crystal growth, which are difficult to observe or investigate separately (Konikoff, 1994). Most research on cholesterol crystallization to date has been based on the simple, but relatively practical assay of *nucleation time* (Holan et al., 1979). This assay measures the crystal detection time in crystal free bile samples incubated *ex-vivo*, and represents in fact a combination of the three steps mentioned, until the first appearance of crystals detectable by light microscopy. Patients with cholesterol gallstones have shorter nucleation times compared with equally saturated bile from persons without gallstones. In an attempt to gain more quantitative information the number of crystals may be counted. However, that is very time-consuming and crystal counts cannot be accurately done once the number of crystals exceeds 100. Busch et al. (1990) introduced a spectrophotometric method to ease the measurement of cholesterol *crystal growth*. It is somewhat less sensitive than the microscopic method because many hundreds of crystals are required before detection, but easier to perform on a large number of samples. The development of turbidity with time yields a sigmoidal crystal growth curve that reveals the onset time, the crystal growth rate, and the maximal final crystal concentration at equilibrium. Due to the presence of biliary pigments, this method cannot be utilized for nucleation assays in native biles. Recently, the assay was modified using microtiter plates and a reader providing a rapid high capacity method for detecting cholesterol crystal growth in model biles (Harvey and Upadhya, 1995; Somjen et al., 1997). These studies were extended by developing methods allowing early detection and quantitative measurement of cholesterol monohydrate crystal growth in human native gallbladder bile, essentially based on the combination of spectrometry with preparative ultracentrifugation (Corradini et al., 1994) or dilution of the samples (Ohya et al., 1994).

None of these assays, however, gives information about the actual mechanism by which cholesterol nucleates and crystals grow. Lichtenberg et al. (1990), have shown that complete solubilization of phospholipids and cholesterol by bile salts in the form of stable mixed micelles requires that the effective ratio of bile salt/lipids in the mixed micelles will exceed a critical value. To study the sequence of events leading to cholesterol crystallization, Van de Heijning et al. (1994) investigated the dynamics of different bile salts and vesicle interactions. It was shown that an instantaneous micellization of vesicular cholesterol and phospholipids occurred. The start of micellization was accompanied by an immediate rise of the vesicular cholesterol: phospholipid ratio due to the preferential solubilization of phospholipid into *de-novo* formed mixed micelles. This method was also used to study the effect of concavalin A binding glycoproteins on cholesterol crystallization (Zijlstra et al., 1996).

In a dilute bile salt rich model bile, cholesterol was found to crystallize initially as filamentous crystals, which gradually transform to classical plate-like crystals (Konikoff et al., 1992). The same crystallization pattern involving a variety of pathways and crystal habits was subsequently found in more concentrated model biles, as well as in native human bile (Konikoff et al., 1992; Portincasa et al, 1996; Wang and Carey, 1996a,b). Furthermore, the crystallization pathways were shown to be determined by the relative composition of cholesterol, phospholipids and bile salts in the bile (Wang and Carey, 1996a,b).

Electron microscopy (EM) is the single most widely used structural technique for morphological characterization of biological specimens. Yet, the material observed is generally different from the original sample. For particles in solution, damage is caused in particular by dehydration, adsorption onto the supporting film and attempts to increase the contrast. Chemical fixation, metal shadowing and negative staining are excellent methods, but change the specimen in order to make it suitable for observation (Adrian et al., 1984; Holzbach, 1990). Hence, conventional transmission EM is particularly prone to artifacts in biliary systems and its use is limited and controversial.

More elaborate approaches to sample preparation have enhanced the power of conventional EM and mitigated some of the problems. One of these newer techniques is *freeze-fracture EM*. Freeze fracture allows the examination of non-fixed lipid materials, thus circumventing the artifacts of fixation, dehydration and embedding of conventional transmission EM. In addition, the rate of freezing (100 K/s) allows a snapshot view of dynamic particles in the solution (Rigler and Patton, 1983). The drawback of this method is the possibility of generating some freezing related artifacts during sample preparation.

A more recent variant of the freeze-fixing approach

to the study of particles in solution, is *cryo-transmission electron microscopy* (cryo-TEM). In this method, extremely rapid freezing causes vitrification of the water, thus eliminating freezing artifacts caused by crystallizing ice. Cryo-TEM provides direct images of microstructures larger than 40 Å and has been used in the study of many surfactant systems, also with biological relevance, without introducing artifacts typical of drying of specimens, as with standard EM techniques (Talmon, 1983). During the specimen preparation process a drop of solution is blotted to form a thin aqueous film of 250-300 nm. The thickness of the film determines the size range of structures that are maintained on the grid prior to plunging and vitrification. Structures larger than 3,000 Å are likely to be excluded from the grid and are thus not observed by the technique (Kaplun et al., 1997). Therefore, Cryo-TEM is limited to structures smaller than about 3,000 Å. In comparison, using the freeze-fracture method particles are less likely to be excluded. The probability of observing microstructures in both cryo-TEM and freeze-fracture EM decreases at lower particle concentrations. Cryo-TEM seems to be promising but the cold stage equipment needed for the use of this approach is still not widely available (Talmon, 1983; Kaplun et al., 1997). It has been used successfully in bile samples to determine the presence and shapes of micelles, vesicles, as well as early crystals (Kaplun et al., 1994, 1997; Konikoff et al., 2000).

Scanning electron microscopy (SEM) has proved to be useful in gallstone research, in particular when investigating spatial relationships of microstructures or analyzing microareas by means of energy dispersive x-ray microanalysis. Surface areas (crystal orientation, pigmented and calcified layers), inner structures (crystal textures, arrangement and composition of layered structures), central regions (centrally located microstructures and their individual composition) have been the regions of interest using SEM. Bile sediments, crystal aggregates and microcalculi representing pre-stages in gallstone formation, as well as macroscopic gallstones (macroliths) have all been investigated by SEM (Osuga et al., 1974a,b; Wosiewitz, 1983). The main disadvantage of this method is the sample preparation, as in TEM, and hence it has mainly provided data about solid biliary structures, such as crystals and stones.

Using a combination of the above direct visualization techniques microstructures present at early stages of cholesterol crystallization in bile have been revealed (Konikoff et al., 2000). Artificial and native bile models were studied from the point of cholesterol supersaturation through the evolution of microstructures leading to cholesterol crystallization. Initially, small spheroidal micelles were observed by cryo-TEM in model (Kaplun et al., 1994, 1997) and in human bile (Kaplun et al., 1997). The presence of vesicles in model and native biles was observed after negative staining (Lee et al., 1987), freeze fracture fixation (Somjen et al., 1986) and by cryo-TEM (Talmon, 1983; Konikoff et al., 2000). By the latter, the morphology of vesicles could be seen in greater detail and non-spherical as well as open vesicles were found in native bile. During a dynamic phase of cholesterol crystallization, filaments, tubular and helical microstructures, as well as classical plate-like cholesterol monohydrate crystals were noted primarily by light microscopy (Kaplun et al., 1997).

The major limitation of all direct microscopic techniques is that they provide only qualitative data without the possibility to reliably quantitate microstructures. Microscopic techniques are also limited in terms of providing chemical analysis or composition. Therefore, additional indirect techniques are needed to complement the data obtained by microscopy.

Indirect techniques

Chromatography

The most commonly used chromatography techniques are gel filtration on columns, in which separation is based on size exclusion. Columns can be constructed of agarose, superose, sephacryl, sepharose and HPLC columns (Somjen et al., 1990a,b). Chromatographic separation resolves vesicles and micelles, and allows to determine their chemical composition. The resolution between simple and mixed micelles, as well as unilamellar and multilamellar vesicles is poor. Before application, native bile samples have to be purified from cell debris, mucus and crystals (Somjen et al., 1990a,b).

Elution buffers contain bile salts to protect against micellar dissociation. Most investigators use sodium taurocholate in a concentration close to its critical micellar concentration. Recently it was suggested that the elution buffer should be composed of intermicellar concentrations (IMC) of bile salts (Donovan and Carey, 1990). The IMC has to be determined individually for each biliary sample. It has been shown that using the IMC results in lower values of vesicular cholesterol (Donovan et al., 1991).

Chromatographic analysis estimated the vesicular cholesterol to be 6-53% in human gallbladder bile, and 27-71% in hepatic bile (Harvey et al., 1998). The cholesterol in vesicles was found to be higher in gallstone patients compared to controls (Gilat and Somjen, 1996). Vesicles having a high cholesterol/phospholipid ratio were found to be the metastable carrier from which cholesterol nucleates (Halpern et al., 1986; Peled et al., 1988, 1989).

Ultracentrifugation

Ultracentrifugation on a density gradient is a standard method for separation between lipid particles in plasma. It has also been used in bile. This method does not require the addition of bile salts. However, artifacts may develop due to the effects of centrifugal forces in the tube, the high hydrostatic pressure within the

medium, and dilution during long run times (Ayyad et al., 1996). Bile samples are layered on top of sucrose, metrizamide, CsCl and KBr gradients (Amigo et al., 1990) or on the bottom of sucrose gradients (Sahlin et al., 1991; Somjen et al., 1992). If samples are applied to the top of the gradient, phospholipid bilayers are kept intact but bile salts precipitate and micelles may dissolve. Analysis of rat and human hepatic biles on metrizamide gradients demonstrated 50% and 61-90% of biliary cholesterol in vesicles, respectively. Micellar cholesterol was scattered over a number of fractions (Amigo et al., 1990). When samples are applied to the bottom of a sucrose gradient, bile salt micelles are kept intact and phospholipid bilayers float toward the top (Somjen et al., 1992). Vertical and near vertical rotors can be used to obtain separations similar to those of the traditional swinging bucket rotor but with significantly reduced run times (usually 1-2 hours), and less artifacts (Ayyad et al., 1996).

Sequential density gradient ultracentrifugation of precipitable cholesterol-containing lipid aggregates has also been applied to investigate cholesterol crystallization pathways in model biles (Konikoff and Carey, 1994; Konikoff et al., 1997). This method can separate filamentous crystals from plate-like and transitional cholesterol crystals and provides a means of quantitating the process of cholesterol crystallization.

The characterization of lipid particles and estimation of their contribution to cholesterol crystallization by the above indirect techniques are performed after a separation process. There are also physical-chemical methods, which are able to measure lipid aggregates in whole, unprocessed bile. Measurements performed in the bulk sample usually allow identification of the more abundant lipid particle aggregates without separation.

Quasi-elastic light scattering (QLS)

QLS is a non-perturbing method for determining the sizes, diffusivity, polydispersities, shapes, molecular weights and interactions of particles in solution. It is based on inter-particle constructive and destructive interference of scattered light secondary to the motion of particles (Cohen et al., 1990). By utilizing laser light, the technique is suitable for particles with mean hydrodynamic radii (sizes) in the 10 to 2,000 Å range and is especially well applicable in micellar-vesicular systems (Cohen et al., 1990; Mazer, 1990). Heavily pigmented biles, or biles containing cell debris, mucus, or other particles are, however, unsuitable for QLS analysis. Hepatic bile, which is relatively dilute and lightly pigmented may lend itself for QLS measurements. QLS has contributed substantially to our understanding of biliary lipid aggregation in bile. In model systems, QLS facilitated precise definitions of phase boundaries in bile salt-lecithin-cholesterol-water phase diagrams (Mazer et al., 1984). By systematically investigating model bile systems, native bile from man, dog and other species, QLS has been able to reveal a rich spectrum of aggregative states including simple, and mixed micelles, as well as vesicles and early crystal formation (Cohen et al., 1990; Mazer et al., 1984, 1990; Konikoff et al., 1992). A remarkable similarity has been demonstrated in the sizes, structures and equilibria of biliary aggregates formed in artificial and native biles (Somjen et al., 1988; Cohen et al., 1990; Mazer et al., 1984, 1990).

Nuclear magnetic resonance (NMR)

NMR spectroscopy is especially sensitive to the small micelles, in contrast to QLS in which the larger particles in the dispersion provide major contribution (Holzbach, 1990). NMR and QLS techniques are therefore complementary. NMR can be used successfully in well defined artificial bile systems in which large vesicles and micelles coexist. Its use is, however, limited with native bile, because of natural abundance of paramagnetic ions in the bile that interact preferentially with lecithin (present in vesicles and micelles) (Groen et al., 1990; Holzbach, 1990). Groen et al. (1990) compared the amount of phospholipids in vesicles obtained by NMR to that obtained by gel filtration and ultracentrifugation. Results of these three methods were comparable with a divergence in dilute models. In concentrated models (mimicking gallbladder bile) 10-20% of the phospholipids were found in vesicles. In diluted models (mimicking hepatic bile) 10-70% of the phospholipids were non-micellar. Ellul et al. (1992) demonstrated the feasibility of measurements of micellar cholesterol by ^{1}H-NMR, but quantitative measurements were not performed.

Small-angle X-ray, neutron scattering and other physical techniques

X-ray and neutron scattering provide information about the shape and structure of the scattering particles in solution based on their electron density distribution. Both techniques rely on the basic principles of beam scattering and differ mainly in sample preparation and in the wavelength of the beam. These, like QLS, are model-dependent techniques. The problems in their application are that the equipment and expertise required for their use are not readily available, and the methods are both labor- and time-intensive (Holzbach, 1990). Experimental determination of biliary lipid aggregate structures has been attempted using small-angle neutron scattering (Hjelm et al., 1988; Long et al., 1994), occurring upon dilution of a concentrated lecithin-bile salt solution. Results have shown a continuous transition from cylindrical micelles to unilamellar vesicles. A recent study showed that crystallization of cholesterol from bile salt micelles could also be detected by electron spin resonance and X-ray scattering (Somjen et al., 1995). Synchrotron X-ray diffraction has been used to study the earliest stages of cholesterol crystallization in model bile (Konikoff et al., 1992). The diffraction

Table 1. Steps in cholesterol gallstone pathogenesis.

STEP	MICROSTRUCTURES	METHOD (REFERENCES)*
A. Bile secretion	vesicles	QLS (Cohen et al., 1989), TEM (Marzolo et al., 1990; Crawford et al., 1995)
B. Supersaturated bile and cholesterol nucleation	simple micelles, mixed micelles, unilamellar vesicles, multilamellar vesicles, cholesterol crystals	light microscopy (Gogna et al., 1989; Konikoff et al., 1992), TEM (Halpern et al., 1986; Holzbach, 1990), cryo-TEM (Kaplun et al., 1994, 1997; Konikoff et al., 2000), freeze-fracture EM (Rigler and Patton, 1983), chromatography (Harvey et al., 1985; Halpern et al., 1986; Peled et al., 1988, 1989), ultracentrifugation (Amigo et al., 1990; Sahlin et al., 1991; Somjen et al., 1992; Ayyad et al., 1996), QLS (Mazer et al., 1984; Somjen et al., 1988; Cohen et al., 1990; Mazer, 1990), NMR (Groen et al., 1990; Ellul et al., 1992), X-ray scattering (Somjen et al., 1995), neutron scattering (Hjelm et al., 1988; Long et al., 1994), synchorotron X-ray diffraction (Konikoff and Carey, 1994) nucleation time assay (Holan et al., 1979)
C. Crystallization and crystal growth	filaments, helical ribbons, tubules cholesterol-monohydrate	crystal growth assay (Somjen et al., 1988; Busch et al., 1990; Corradini et al., 1994; Ohya et al., 1994; Harvey and Upadhya, 1995), light microscopy (Konikoff et al., 1992), ultracentrifugation (Konikoff and Carey, 1994: Konikoff et al., 1997)
D. Crystal agglomeration and stone formation	crystal aggregates bile sediments, stones	SEM (Osuga et al., 1974a,b; Wosiewitz, 1983)

*: for abbreviations see text.

pattern of early filaments suggests the presence of anhydrous cholesterol or that of a cholesterol monohydrate polymorph. These findings indicate that crystalline cholesterol in bile may not be completely mature or hydrated initially, and that it undergoes a series of transitions to become hydrated and thermodynamically stable.

Conclusions

Using the above techniques and biliary systems, it has been possible to elucidate a multitude of steps in the process starting from bile secretion within the canalicular spaces in the liver and those occurring during bile flow in the biliary tree, and evidence in the gallbladder.

Table 1 depicts the various methods that have been used to provide microstructural data of bile. Based on these experiments the events leading to cholesterol gallstone formation have been outlined. Biliary phospholipid molecules are secreted by the hepatocytes into the bile canalicular lumina as unilamellar vesicles (Cohen et al., 1989; Marzolo et al., 1990; Crawford et al., 1995). During flow in the biliary tree vesicular cholesterol with some phospholipids are gradually taken up into bile salt rich micelles. Since mixed micelle formation requires the solubilization of more phospholipids than cholesterol, excess cholesterol remains to be solubilized in vesicles, which become gradually supersaturated and thermodynamically metastable (Halpern et al., 1986; Harvey et al., 1988; Peled et al., 1988, 1989). When the cholesterol carrying capacity of the lipid aggregates is exceeded cholesterol starts to precipitate and form cholesterol monohydrate crystals as documented by VELM (Halpern et al., 1986). During cholesterol crystallization, filaments, tubular and helical microstructures, and plate-like cholesterol monohydrate crystals as well as their transitions were noted primarily by light microscopy (Kaplun et al., 1997). Ultimately, within the gallbladder cholesterol crystals are agglomerated with an organic matrix to form cholesterol gallstones as could be seen using SEM (Osuga et al., 1974a,b; Wosiewitz, 1983).

Acknowledgements. This work was performed in partial fulfillment of the requirements for a Ph.D degree of Ronit Pakula, Sackler Faculty of Medicine, Tel Aviv University, Israel. We wish to thank Prof. Aliza Tietz-Devir for her helpful comments and critically reviewing this manuscript.

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Accepted December 22, 1999