Zakim and Boyer's Hepatology: A Textbook of Liver Disease

Zakim and Boyer's Hepatology: A Textbook of Liver Disease

Seventh Edition

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Philadelphia, PA 19103-2899

ZAKIM AND BOYER'S HEPATOLOGY: A TEXTBOOK OF LIVER DISEASE ISBN: 978-0-323-37591-7
SEVENTH EDITION

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Library of Congress Cataloging-in-Publication Data

Names: Sanyal, Arun J., editor. | Boyer, Thomas D., editor. | Terrault, Norah, editor. | Lindor, Keith D., editor.
Title: Zakim and Boyer's hepatology : a textbook of liver disease / [edited by] Arun J. Sanyal, Thomas D. Boyer, Norah A. Terrault, Keith D. Lindor.
Other titles: Hepatology
Description: 7th edition. | Philadelphia, PA : Elsevier, [2018] | Preceded by Zakim and Boyer's hepatology : a textbook of liver disease / [edited by] Thomas D. Boyer, Michael P. Manns, Arun J. Sanyal. 6th ed. c2012. | Includes bibliographical references and index.
Identifiers: LCCN 2016053023 | ISBN 9780323375917 (hardcover : alk. paper)
Subjects: | MESH: Liver Diseases
Classification: LCC RC845 | NLM WI 700 | DDC 616.3/62–dc23
LC record available at https://lccn.loc.gov/2016053023

Senior Content Strategist: Sarah Barth
Senior Content Development Specialist: Jennifer Ehlers
Publishing Services Manager: Catherine Albright Jackson
Senior Project Manager: Doug Turner
Designer: Paula Catalano

Printed in China

Last digit is the print number: 9 8 7 6 5 4 3 2 1

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Preface

In 1982 the first edition of *Hepatology: A Textbook of Liver Disease* was published. The book was based on pathophysiologic principles in the belief that these "principles form the basis for interpreting concepts about disease and for evaluating the validity of research." The first 12 chapters focused on normal hepatic function. The next 10 chapters focused on alterations of normal function leading to clinical illnesses, such as hepatic encephalopathy, and the last 24 chapters focused on specific disease states. There were only two chapters on viral hepatitis: one on the biology of hepatitis viruses and the second on clinical features of viral hepatitis. A single chapter on liver transplantation was included in the first edition. Over the next several decades, the book has changed dramatically, largely as a reflection of the tremendous progress that has been achieved in the field of hepatology.

As in the last edition, the seventh edition focuses more on abnormal, rather than normal, physiology. We hope that this new edition of *Zakim and Boyer's Hepatology: A Textbook of Liver Disease* will serve practicing hepatologists and gastroenterologists; general physicians, such as internists and general practitioners; and basic scientists who want to know more about the clinical spectrum, therapeutic approaches, and unmet needs of the diseases they investigate and treat.

The seventh edition is organized into 11 sections and addresses basics of liver function, management and assessment of liver disease, consequences of chronic liver disease, specific liver diseases, infections, immune diseases, vascular diseases, tumors, transplantation, pediatric and inherited liver diseases, and other conditions that affect the liver (e.g., pregnancy). As in the past, we invited new authors to write approximately one third of the chapters to provide fresh perspectives on subjects that appeared in previous editions.

To keep the book at a reasonable size and reduce cost, cited references are available on Elsevier's Expert Consult website, which will keep the tradition of the book being well referenced without being too large. The format that was used in the previous edition, including the placement of color photomicrographs and photos within each chapter, is used again here, and the art program has been thoroughly updated.

Hepatology, like medicine in general and infectious diseases in particular, does not respect any border. Liver diseases are a significant global health burden. Over the past 50 years, the discipline of hepatology has provided examples of discoveries in basic science that are immediately transformed into novel diagnostic procedures, molecular-based therapies, antitoxins, and vaccines. The discovery of the five major hepatotropic viruses is an example of success for translational medicine. Effective treatment for acetaminophen overdose has been developed from basic studies of drug metabolism and toxicity. Liver transplantation has evolved into a routine, life-saving procedure. We have seen the global success of hepatitis A and B vaccination programs, and we are following the clinical development of new generations of direct-acting anti-HCV drugs that hopefully will provide more effective and less toxic anti-HCV therapies. Finally, hepatocellular carcinoma, which is one of the top killers globally and one of the most important complications of end-stage liver disease, has become an area for innovative, molecularly targeted, anticancer therapies. We will be very pleased if this seventh edition is regarded as an international textbook for a global readership helping to combat a global health burden.

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Anatomy and Cellular Functions of the Liver

LIHUI QIN AND JAMES M. CRAWFORD

ABBREVIATIONS

AGE advanced glycation end-product
APC antigen-presenting cell
CGRP calcitonin gene-related peptide
GERL Golgi-SER-lysosome
HD high density
HSC hepatic stellate cell
ICAM-1 intercellular adhesion molecule-1
LAL liver-associated lymphocyte
LD low density
LFA-1 lymphocyte associated antigen-1
LGL large granular lymphocyte
LPS lipopolysaccharide
MDSC myeloid-derived suppressor cell
NK natural killer
nNOS neuronal nitric oxide syndrome
NPY neuropeptide Y
PG prostaglandin
RER rough endoplasmic reticulum
SDF stromal-derived factor
SER smooth endoplasmic reticulum
SMA smooth muscle actin
SOM somatostatin
SP substance P
TGFβ transforming growth factor β
TLR toll-like receptor
TNFα tumor necrosis factor α
Treg regulatory T cell
TXA_2 thromboxane A_2
VCAM-1 vascular cell adhesion molecule-1
VIP vasoactive intestinal peptide

Introduction

The liver is the largest organ in the human body. With the exception of the daily secretion of several liters of bile, there are no "moving parts" in the liver. This deceptive anatomic simplicity belies the extraordinary complexity of the biosynthetic and biodegradative pathways within the liver, serving as major elements of systemic metabolic and physiologic homeostasis (Table 1-1). In the process, the liver generates enough metabolic heat to be a prime source of core homeostatic temperature maintenance.

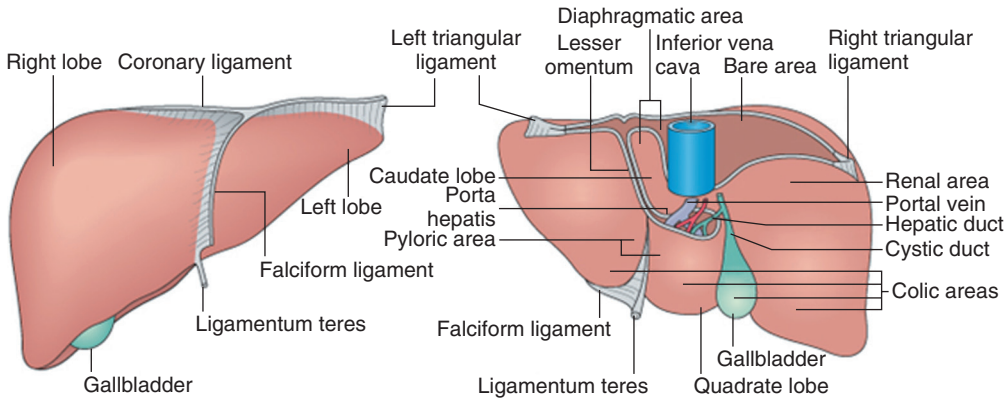
Gross Anatomy

The mature liver lies mainly in the right hypochondriac and epigastric regions of the abdominal cavity, below the diaphragm. The liver is attached to the diaphragm and protected by the ribs. In adults, the healthy liver weighs approximately 1400 g to 1600 g and extends along the midclavicular line from the right fifth intercostal space to just inferior to the costal margin. The anterior border of the liver then extends medially and crosses the midline just inferior to the xiphoid process. A small portion of the organ projects across the midline and lies in the upper left abdominal quadrant.

The liver is incompletely separated into lobes that are covered on their external surfaces by a thin connective tissue capsule. On the basis of external surface features, the liver is divided into right and left lobes by the falciform ligament, which is a peritoneal fold connecting the liver to the anterior abdominal wall and the diaphragm (Fig. 1-1). The right lobe is further subdivided inferiorly and posteriorly into two smaller lobes—the caudate and quadrate lobes.

The macroscopic functional divisions of the liver, however, are defined on the basis of vascular anatomy (Fig. 1-2). First is a plane that passes through the gallbladder and inferior vena cava, to the right of the midplane of the abdomen, that defines the halves of the liver supplied by the right and left branches of the portal vein and hepatic artery, together with biliary drainage into the right and left hepatic ducts. As a result, the quadrate lobe and a large portion of the caudate lobe are located to the right of the groove of the inferior vena cava but belong functionally to the left hemiliver. Further functional subdivision of the liver into eight segments having independent vascular and biliary supplies enables surgeons to resect segments of the liver while maintaining relative hemostasis.[1-3]

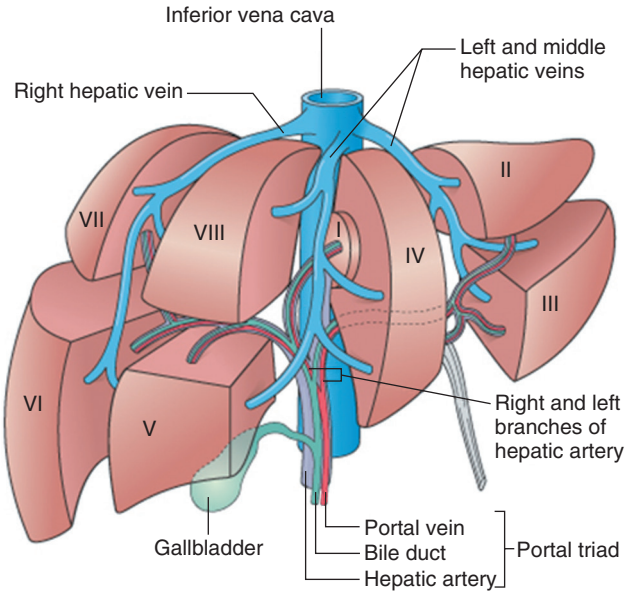
The liver is encapsulated by a thin connective tissue layer (Glisson's capsule) consisting mostly of regularly arranged type I collagen fibers, scattered type III fibers, fibroblasts, mast cells, and small blood vessels and containing sensory nerves. On the convex liver surface facing the abdominal cavity this connective tissue layer is covered by the simple squamous mesothelial cells of the peritoneal serosal lining. At the superior site of attachment of the



• **Fig. 1-1** Lobes, surfaces, and ligaments of the liver viewed anteriorly (left) and from a posteroinferior perspective (right). (Modified from Moore KL, Dalley AF. *Clinically oriented anatomy,* 4th ed. Philadelphia: Lippincott Williams & Wilkins, 1999: 264, ©1999, with permission from Lippincott Williams & Wilkins [http://lww.com].)

TABLE 1-1 Major Elements of Hepatic Function

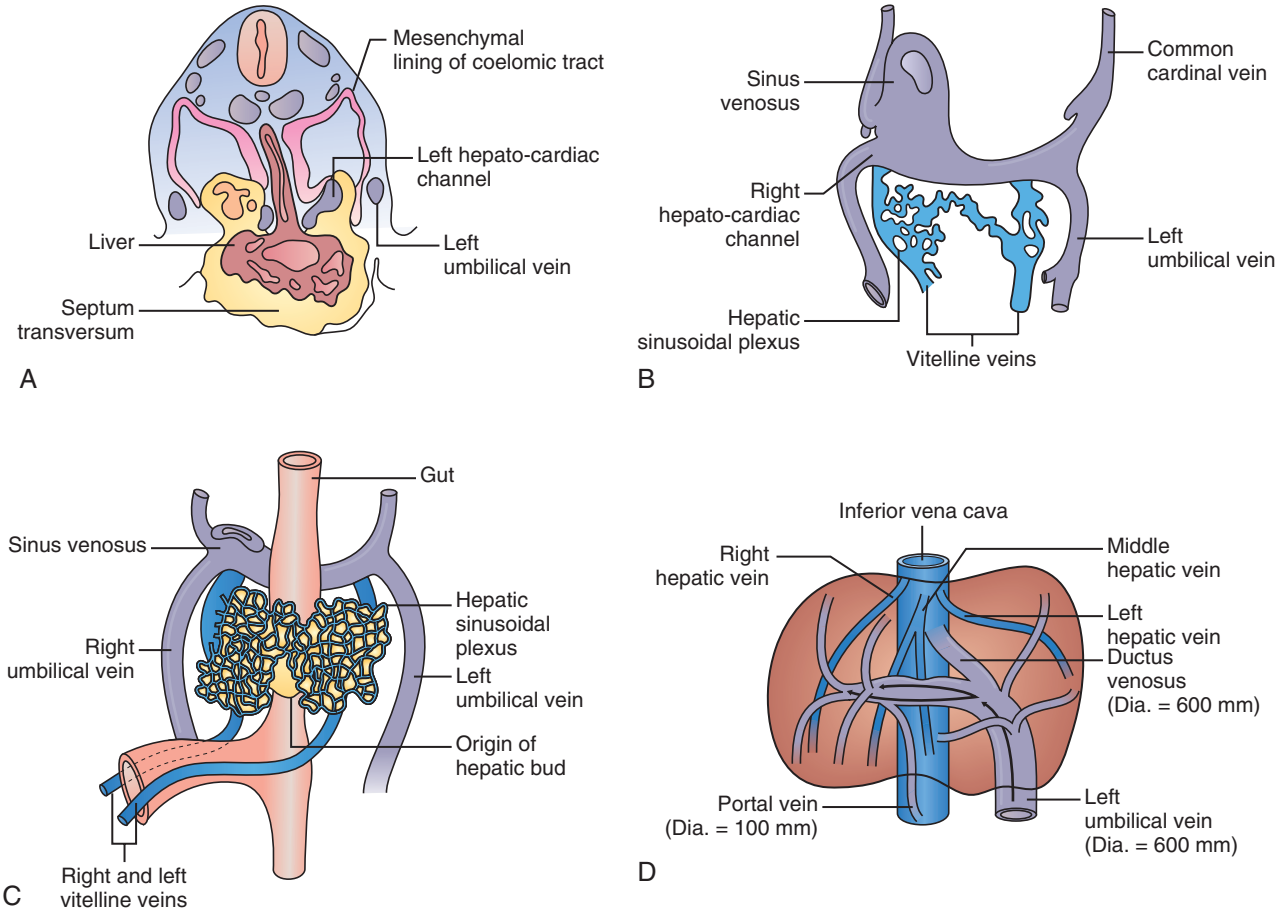
Cell Type	Function
Hepatocytes	Bile secretion Bile salt biosynthesis, conjugation, and secretion Bilirubin uptake, conjugation, and secretion Phospholipid and cholesterol secretion Plasma protein biosynthesis and secretion Plasma lipoproteins Plasma coagulation factors (prothrombin, fibrinogen, complement factors) Albumin Transferrin Plasma protein uptake and degradation Plasma lipoproteins Glucose homeostasis Metabolism and detoxification of drugs and toxins
Cholangiocytes	Secretion of bicarbonate-rich fluid into bile
Sinusoidal endothelial cells	Fenestrated barrier between sinusoidal blood and hepatocytes Endocytic processing of plasma proteins Lipoproteins Advanced glycosylation end products Immune complexes Immunoregulatory function Regulatory input into hepatic stellate cell, hepatocyte, and Kupffer cell function
Kupffer cells	Phagocytosis of particulate matter in sinusoidal blood Phagocytosis of apoptotic and necrotic hepatocellular debris Clearance of circulating microorganisms and endotoxin Immunoregulatory function Regulatory input into hepatic stellate cell, hepatocyte, and sinusoidal endothelial cell function
Hepatic stellate cells	Storage of vitamin A Control of microvascular tone of the sinusoid Production of extracellular matrix Regulatory input into hepatic regeneration Immunoregulatory function
Fibrocytes (portal tract)	Production of extracellular matrix



• **Fig. 1-2** Segmentation of the liver based on principal divisions of the portal vein and hepatic artery. (Modified from Moore KL, Dalley AF. *Clinically oriented anatomy,* 4th ed. Philadelphia: Lippincott Williams & Wilkins, 1999: 268, ©1999, with permission from Lippincott Williams & Wilkins [http://lww.com].)

falciform ligament to the liver, the two leaves of the ligament separate to form a large convex area devoid of peritoneum, the bare area, on the superior surface of the liver, directly apposed to the diaphragm. The right and left leaves of the falciform ligament then merge with reflections of the parietal peritoneum extending from the diaphragm forming, respectively, the triangular ligament over the left dome of the liver and the coronary ligament over the right dome of the liver. The posterior aspect of the liver is covered by peritoneal serosa, with reflections at the groove of the inferior vena cava into which the multiple hepatic veins empty.

The dual blood supply of the liver enters the organ at its hilus (porta hepatis) accompanied by the hepatic bile duct, lymphatics, and nerves. Approximately 80% of the blood entering the liver is poorly oxygenated and is supplied by the portal vein. This is the venous blood flowing from the intestines, pancreas, and spleen; venous blood from the gallbladder either drains into a cystic vein,



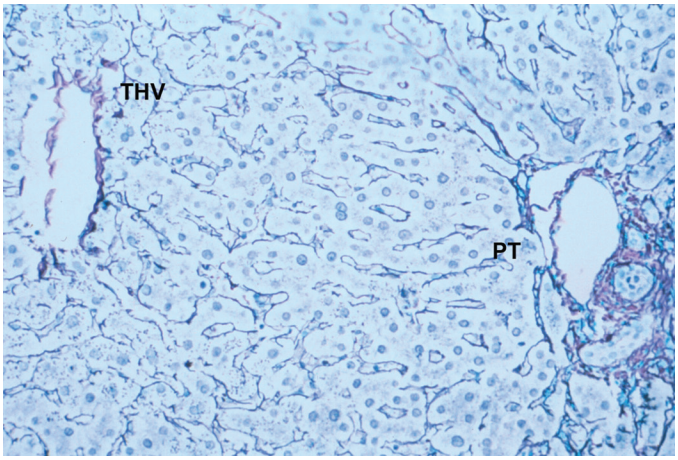
• **Fig. 1-3** Development of the liver. (**A**) Section through the region of the hepatic bud of a human embryo of 25 somites (26 days). (**B**) Vascular channels associated with the developing liver in a human embryo of 30 somites. (**C**) Vascular channels at a later stage showing development of the sinusoidal network. (**D**) Portal hepatic circulation in a human embryo of 17 mm (7 weeks). (Reproduced from MacSween RNM, et al. Functional morphology of the liver with emphasis on its microvasculature. In: MacSween RNM, et al., editors. *Pathology of the liver,* 4th ed. London: Churchill Livingstone, 2002: 4, ©2002, with permission of Elsevier.)

which empties into the right branch of the portal vein, or via small veins in the gallbladder bed directly into the parenchyma of the liver. The remaining 20% of the hepatic blood supply is well oxygenated and delivered by the hepatic artery.

Development of the Liver

The development of the liver has been extensively described,[1,2,4-6] and is illustrated in Fig. 1-3. Briefly, the liver primordium appears in human embryos during the third week of gestation as an endodermal bud from the ventral foregut just cranial to the yolk sac. This bud becomes the hepatic diverticulum as it enlarges, elongates in a cranio-ventral fashion, and develops a cavity contiguous with the foregut. The hepatic diverticulum consists of three portions: (1) the hepatic portion forms the hepatic corpus, including parenchymal cells and the elements of the portal tree; (2) the cystic portion forms the gallbladder; and (3) the most ventral portion forms the head of the pancreas. The hepatic portion grows into the septum transversum—a plate of mesenchyme that incompletely separates the thoracic and peritoneal cavities. During the fourth week of development, buds of epithelial cells extend from the hepatic diverticulum into the mesenchyme of the septum transversum as thick multicellular anastomosing cords. They become interspersed within the developing anastomotic network of capillaries arising from the symmetrically arranged vitelline veins returning from the abdominal cavity of the embryo, thus beginning to establish the close relationship of the hepatic parenchymal cells to the sinusoids. Blood draining from the parenchymal sinusoidal plexus within the nascent liver passes through the symmetric right and left hepatocardiac channels, to enter the sinus venosus.

This embryonic vascular pattern gives way to the definitive fetal vascular pattern by the seventh week: the paired vitelline veins unite to form a single portal vein, which divides into right and left branches upon entering the liver, and arterial sprouts from the hepatic arterial branch of the celiac axis course along the intrahepatic portal tree and become the organizing elements for formation of the intrahepatic biliary tree.[6,7] Within the parenchyma, the anastomotic pattern of both multicellular cords of parenchymal cells and sinusoids persists until several years after birth, by which time cords consisting of two or more parenchymal cells bounded on several sides by sinusoids have become plates consisting of single parenchymal cells bounded on at least two sides by sinusoids, particularly in the centrilobular region.



• **Fig. 1-4** Normal hepatic microarchitecture. A medium-power image of a reticulin-stained liver highlights architectural relationships in the parenchyma. A portal tract (PT, *right*) is separated by parenchyma from the terminal hepatic vein (THV, *left*).

Between the sixth week and birth the fetal liver serves as a hematopoietic organ and as the primary site for fetal blood formation until the third trimester, when most hematopoietic sites disappear as the bone marrow develops. Throughout the third trimester and well through childhood, the liver microanatomy matures from the hilum outwards, with the final maturation of the parenchyma not being completed until the adolescent years.

Microscopic Architecture

Fundamental understanding of liver function begins with knowledge of microscopic architecture (Fig. 1-4, Table 1-2). Portal tracts are the distribution network for the portal vein and hepatic arterial systems, and in turn the effluent collection network for the biliary tree. The parenchyma is home to hepatocytes, which account for 60% of the total cell population and 80% of the volume of the liver and are organized in an anastomosing system of plates that traverse the distance from the portal tract to the terminal hepatic venule. Between the plates of hepatocytes are the vascular spaces, termed *sinusoids*. This is a unique vascular bed, with large-bore fenestrated vascular channels that lack a basement membrane and allow free exchange of circulating macromolecules with hepatocytes (Fig. 1-5). So-called nonparenchymal cells lining the sinusoid include: sinusoidal endothelial cells, perisinusoidal hepatic stellate cells, and intraluminal Kupffer cells. Lastly, blood flowing through the sinusoids exits the liver through the branches of the hepatic venous system, the smallest of which is the terminal hepatic vein (also termed the *central vein,* as will be discussed).

Portal Tracts and the Biliary System

Both the portal vein and the hepatic artery, together with efferent autonomic nerves, enter the liver at the hilum. The hilum is also the site where bile ducts and lymphatics exit the liver. Branches of the hepatic artery, portal vein, bile duct, and lymphatic vessels ramify together in portal tracts through the liver parenchyma (Fig. 1-6). Portal tracts are sometimes referred to as portal triads owing to their three main elements (portal vein, hepatic artery, bile duct, Fig. 1-7). However, *portal tract* is the preferred terminology, because there is often a multiplicity of hepatic artery–bile duct pairs within any given portal tract.[8] The lymphatic channels

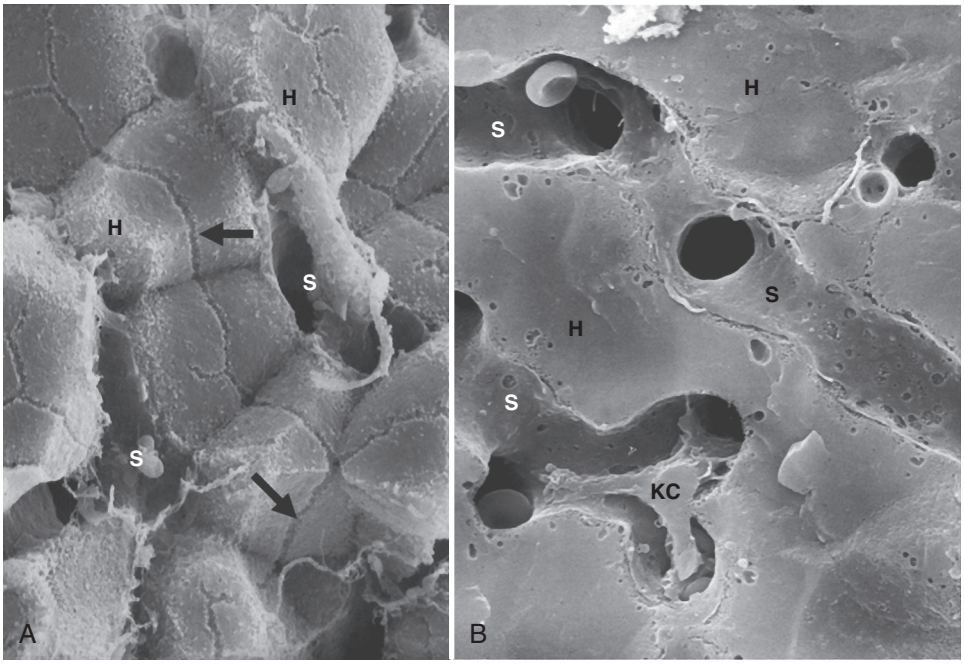
TABLE 1-2 Microscopic Structures of the Liver

Anatomic Compartment	Structural Element	Cell Types
Portal tract	Portal vein	Endothelial cells, smooth muscle cells
	Hepatic artery	Endothelial cells, smooth muscle cells
	Bile duct	Cholangiocytes
	Peribiliary glands (larger portal tracts only)	Glandular epithelial cells
	Bile ductule	Cholangiocytes
	Lymphatics	Endothelial cells
	Nerves	Terminal twigs of autonomic nervous system
	Canals of Hering (at the parenchymal-portal tract interface)	Cholangiocyte-hepatocyte channels
	Portal tract mesenchyme	Fibroblasts
Parenchyma	Liver cell plates	Hepatocytes
	Sinusoids	Nonparenchymal cells: Sinusoidal endothelial cells; Kupffer cells; Hepatic stellate cells; Pit cells (large granular lymphocytes)
Terminal hepatic vein	Terminal hepatic vein	Endothelial cells

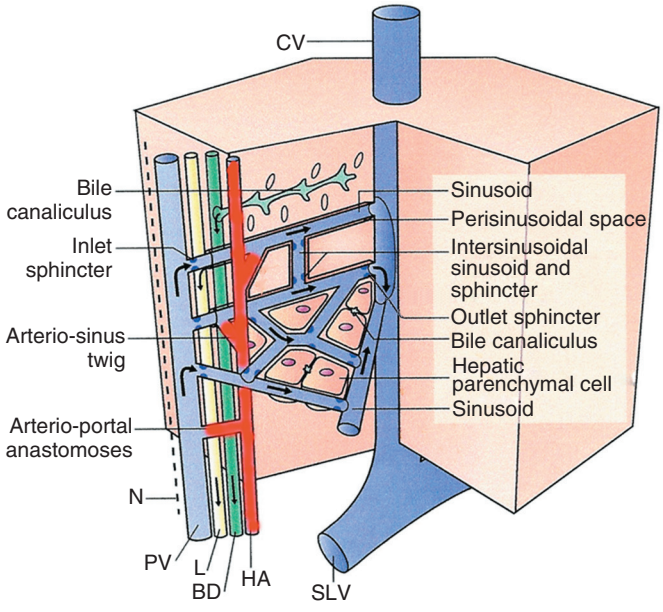
within portal tracts are usually collapsed and inconspicuous, as are the autonomic nerves, unless the latter are highlighted by special techniques (Fig. 1-8).

After repeated bifurcation, terminal branches of the portal veins and hepatic arteries supply blood to the sinusoids, extending roughly perpendicularly into the watershed regions between neighboring portal tracts (Fig. 1-9). Branches of hepatic arteries also supply the peribiliary plexus of capillaries nourishing the bile ducts. These capillary plexuses then drain into sinusoids (via arteriosinus twigs, Fig. 1-10), or occasionally into portal venules (arterioportal anastomoses). Because all these vessels have independently contractile periarteriolar sphincters, the sinusoids receive a varying mixture of portal venous and hepatic arterial blood.[9,10] After flowing through the sinusoids, blood is collected in small branches of hepatic veins (terminal hepatic veins, see Fig. 1-9). These veins course independently of the portal tracts and drain via the major hepatic veins, which emerge to join the inferior vena cava through separate orifices from the major liver lobes.

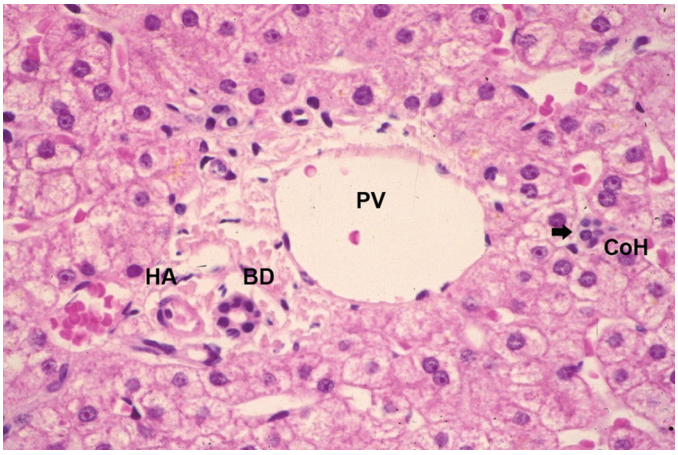
Lymphatic fluid originates from retrograde fluid flow in the space of Disse and is thereby collected into blind-ended lymphatic capillaries in the connective tissue spaces within the portal tracts.[11] The actual connection between the proximal end of the space of Disse at the portal tract–parenchymal interface and lymphatic channels is attributed to a proposed space of Mall,[12] the existence of which has never been proven. The fluid contained in these lymphatics moves toward the hepatic hilus and eventually into the cisternae chyli and thoracic duct. Lymph also leaves the liver in



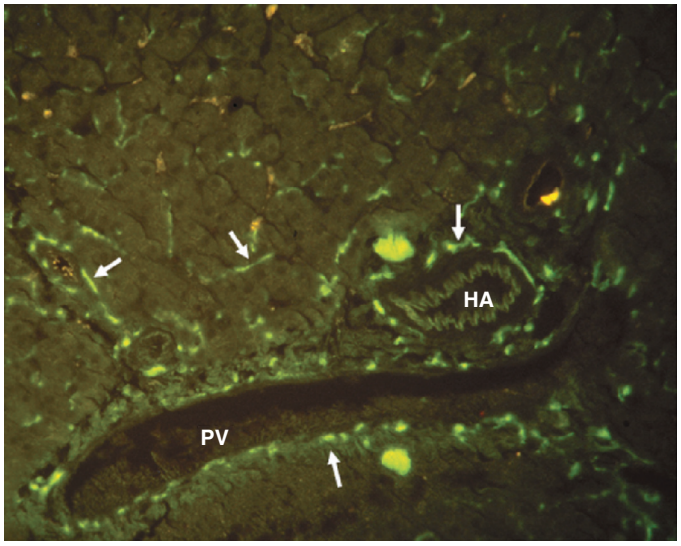
• **Fig. 1-5** Plates of hepatocytes (H) viewed by scanning electron microscopy. (**A**) The fracture plane highlights the lateral faces of hepatocytes (H), with bile canaliculi *(arrow)* in view. The labyrinth of sinusoidal spaces (S) is evident between hepatocytes plates. (**B**) A different image shows the sinusoidal spaces to better advantage. A Kupffer cell (KC) is present within the luminal space of the sinusoid. (Modified from McCuskey RS. Functional morphology of the liver with emphasis on its microvasculature. In: Tavoloni N, Berk PD, editors. *Hepatic transport and bile secretion.* New York: Raven Press, 1993: 2, ©1993, with permission from Lippincott Williams & Wilkins [http://lww.com].)



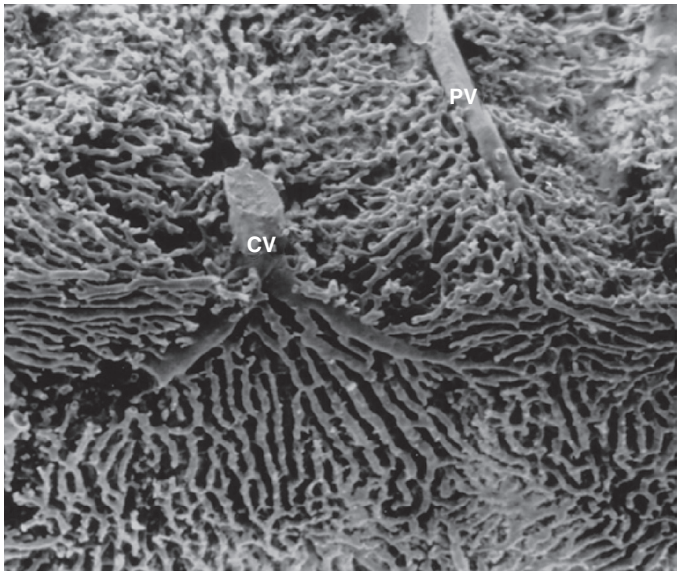
• **Fig. 1-6** Hepatic microvasculature as determined by in vivo microscopic studies. Smooth muscle cells at strategic points in the vascular network create functional sphincters that contribute to local blood flow regulation. Interconnections between the portal vein and hepatic artery network also are depicted. *Arrows* indicate direction of flow. *BD,* bile duct; *HA,* hepatic artery; *L,* lymphatic; *N,* nerve; *PV,* portal vein; *SLV,* sublobular hepatic vein; *THV,* terminal hepatic vein. (Modified from McCuskey RS. Functional morphology of the liver with emphasis on its microvasculature. In: Tavoloni N, Berk PD, editors. *Hepatic transport and bile secretion.* New York: Raven Press, 1993: 2, ©1993, with permission from Lippincott Williams & Wilkins [http://lww.com].)



• **Fig. 1-7** Normal portal tract. A high-power image shows a portal vein (PV) and a hepatic artery (HA)–bile duct (BA) pair, with a delicate investment of extracellular matrix. By routine light microscopy, nerves and lymphatic channels are not visible. An intraparenchymal segment of a canal of Hering *(arrow,* CoH*)* is visible to the right of the portal tract. Trichrome stain.



• **Fig. 1-8** Intrahepatic aminergic innervation in the dog. Brightly fluorescent nerve fibers are adjacent to the portal vein (PV), hepatic artery (HA), and bile duct (not visible in this section) and are also distributed intralobularly along the sinusoids *(arrows)*.

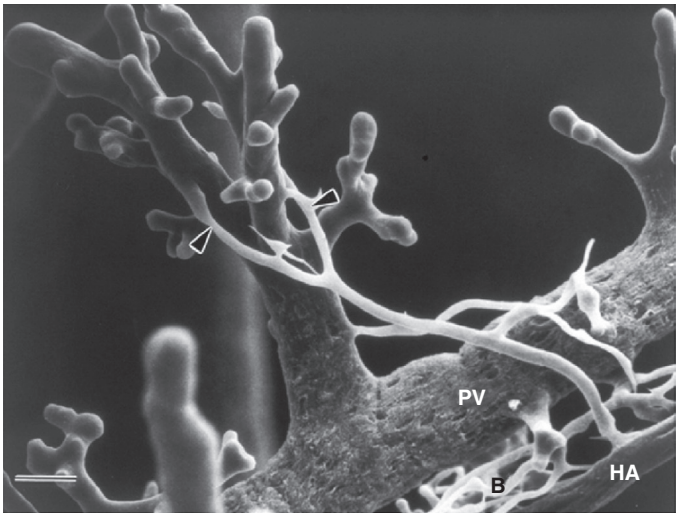


• **Fig. 1-9** Vascular cast of the hepatic microvasculature via the portal vein, demonstrating parenchymal vascular channels by scanning electron microscopy. Blood entering the liver via the portal vein (PV) ramifies into the tortuous sinusoidal network. The sinusoids converge upon the terminal hepatic vein (labeled *CV* for central vein), through which blood exits the liver. (Modified from McCuskey RS. The hepatic microvascular system. In: Tavoloni N, Berk PD, editors. *Hepatic transport and bile secretion.* New York: Raven Press, 1993: 4, ©1993, with permission from Lippincott Williams & Wilkins [http://lww.com].)

small lymphatics associated with the larger hepatic veins, which empty into larger lymphatics along the wall of the inferior vena cava. Lymphatics in the hepatic capsule drain to vessels either at the hilum or around the hepatic veins and inferior vena cava.[1]

The Biliary Tree

The biliary tree is the conduit by which fluid travels from the bile canaliculi between hepatocytes downstream to the lumen of the

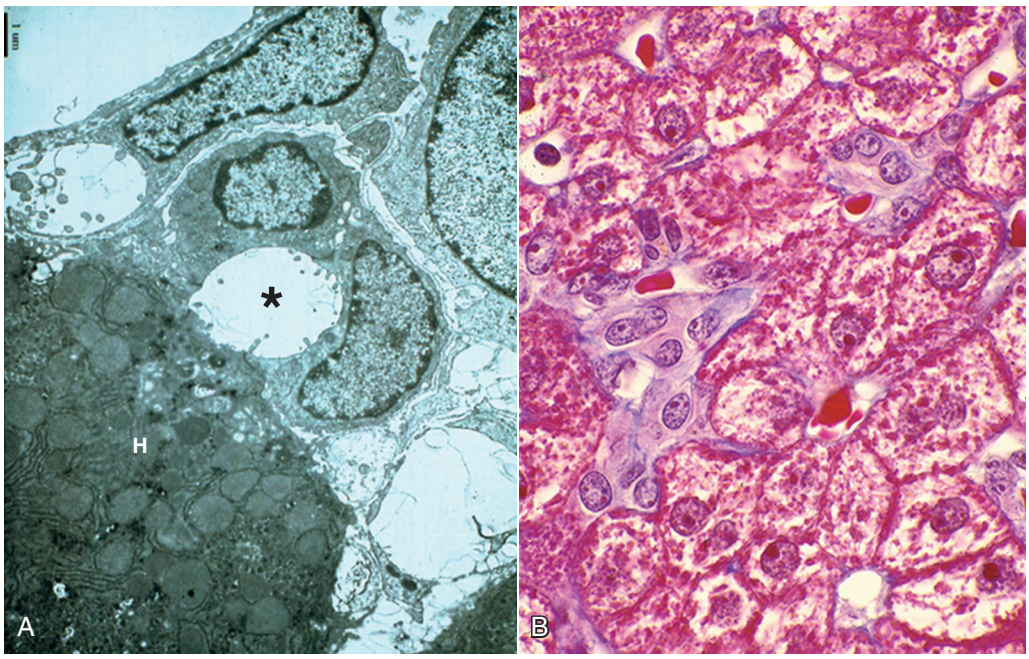


• **Fig. 1-10** Terminal ramifications of the portal vein (PV) and hepatic artery (HA). The portal vein gives rise to inlet venules that enter the parenchyma, from which sinusoids originate. Terminal branches *(arrowheads)* from hepatic artery frequently end in the inlet venules from the portal vein. The hepatic artery also gives rise to a peribiliary arteriolar plexus (**B**). (Modified from McCuskey RS. Functional morphology of the liver with emphasis on its microvasculature. In: Arias IM, et al., editors. *The liver: biology and pathobiology,* 3rd ed. New York: Raven Press, 1994: 1095, ©1994, with permission from Lippincott Williams & Wilkins [http://lww.com].)

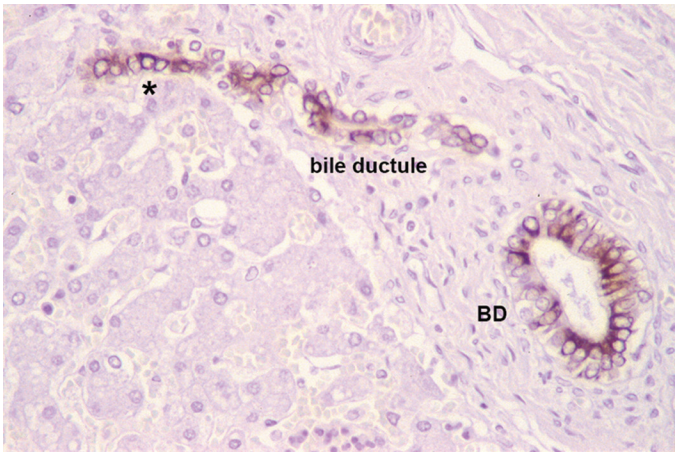
alimentary tract. The most proximal junction of the parenchymal canalicular network with the biliary tree is the *canal of Hering*, with half its circumference composed of one (or two) hepatocytes, and the other half composed of cholangiocytes—the polarized epithelial cells of the biliary tree (Fig. 1-11, *A*). These structures were originally thought to be visible only by electron microscopy.[13] However, careful three-dimensional reconstruction histologic studies demonstrated that canals of Hering were visible by light microscopy[14] and were shown to be present not just at the portal tract–parenchymal interface (as originally thought) but also extending into the hepatic parenchyma up to one third of the distance along the portal-to-central axis (see Fig. 1-11, *B*).[6] A further finding was that the canals of Hering are the anatomic compartment containing bipotential progenitor epithelial cells, which were capable of immense regenerative activity when the liver incurred severe damage—particularly if this damage was occurring at the portal-parenchymal interface.[15] Hence, canals of Hering are not only the most peripheral compartment of the biliary tree but also play a key role in the hepatic regenerative response to injury.

The biliary channel that traverses the short space between the portal-parenchymal interface and the bile duct is the bile ductule (Fig. 1-12). The bile ductule connects with the terminal bile duct at a roughly perpendicular angle—on three-dimensional step sections the angle of intersection is slightly off-perpendicular. The bile ducts converge down the length of the biliary tree, ultimately exiting the liver hilum as the right and left bile ducts to form the common hepatic bile duct just outside the liver corpus. Owing to normal variation in human bile duct anatomy, in 30% to 40% of individuals the confluence of the common hepatic bile duct is just internal to the liver.[16]

During hepatic morphogenesis, bile ductules and ultimately bile ducts arise from bipotential primordial cells at the interface of the embryonic portal tract mesenchyme and the



• **Fig. 1-11** Canal of Hering. (**A**). Electron micrograph demonstrating a channel *(asterisk)* lined partially by a larger hepatocyte (H, lower left) and partially by smaller cholangiocytes. (Courtesy of Donna Beer Stolz.) (**B**) High power light-micrograph of an H&E-stained tissue section, showing a linear array of cholangiocytes among hepatocytes, representing the extension of a canal of Hering into the most proximal portion of the periportal parenchyma.



• **Fig. 1-12** Biliary structures in the portal tract, highlighted by cytokeratin 19 immunostaining. A canal of Hering within the periportal parenchyma *(asterisk)* drains into a bile ductule, which traverses the parenchymal mesenchyme to empty into a bile duct (BD). As the bile ductule is angled slightly in relationship to the bile duct, the full length of the ductule is not visible in this one section. Tissue from an 18-week human fetus, so as to illustrate the nascent relationships of these three elements of the intrahepatic biliary tree. (Adapted from Crawford JM. Development of the intrahepatic biliary tree. *Semin Liver Dis* 2002; 22: 213–226, ©2002, with permission from Thieme-Stratton.)

parenchyma—termed the *ductal plate*.[6] As noted earlier, the penetration of hepatic artery branches into the embryonic portal tract mesenchyme appears to act as an organizing influence for ontogeny of the terminal bile ducts, and for creation of the arterial vascular supply for bile ducts. This in-growth of hepatic arterial elements and maturation of bile ducts begins at the hilum of the liver and extends outwards not only during the fetal period of liver development but also well into the growing years of childhood as the liver continues to enlarge to reach its adult size by early adolescence. This ontogeny is evident in the mature adult liver by the uniform 1 : 1 pairing of hepatic arteries and terminal bile ducts in cross-sections of terminal portal tracts (see Fig. 1-7).[6]

As regards the branching of the intrahepatic biliary tree, retrograde injection studies can demonstrate approximately 10 orders of dichotomous branches.[17] However, in three-dimensional studies of the human adult liver, one terminal bile duct is observed for every 2 mm^3 to 3 mm^3 of the adult liver, therefore representing the volume of a liver microarchitectural unit.[18,19] Based on the average size of the adult liver, between 17 and 20 orders of branching of the intrahepatic biliary tree would be expected. Given the limitations of retrograde filling techniques, and uncertainty in whether the terminal biliary tree branches symmetrically (dichotomously) or asymmetrically, the exact geometry of the intrahepatic biliary tree remains conjectural. Regardless, the adult liver must be supplied by 400,000 to 500,000 terminal bile ducts, corresponding to the estimated 440,000 microarchitectural units (defined as lobules or otherwise, see later) estimated to exist in the adult liver.[20]

Cholangiocytes

Cholangiocytes constitute 3% to 5% of the endogenous liver cell population,[21] beginning with the canals of Hering, bile ductules, and then lining the intrahepatic and extrahepatic bile duct system. Cholangiocytes are not inert; they modify the composition of bile during its transit through bile ducts by the secretion and absorption of water, electrolytes, and other organic solutes.[22,23] Indeed, in humans up to 40% of basal bile flow is produced by the ductal epithelium.[24] This secretion is driven by sodium and bicarbonate transport out of the cholangiocyte into the duct lumen, followed

by regulated passage of water through the intercellular tight junctions. Cholangiocyte secretion is under hormonal control by secretin and somatostatin.[25] The duct epithelium also secretes IgA and IgM (but not IgG).[26] Cholangiocytes reabsorb solutes from bile: glucose, glutamate, urate, and especially bile acids. Bile acids resorbed by the biliary epithelium are recirculated to hepatocytes via the peribiliary capillary plexus, creating a cholehepatic shunt pathway and promoting bile acid–dependent bile flow.[27]

Cholangiocytes display phenotypic heterogeneity along the length of the biliary tree,[28,29] including their becoming larger in basal-to-apical diameter with increasing diameter of the biliary lumen, concurrent with their acquiring increasing cytoplasmic complexity as evidenced by intracellular organelles such as the Golgi complex, intracytoplasmic vesicles, and mitochondria.[30] Cholangiocytes express receptors for epidermal growth factor, secretin, and somatostatin[31] and may secrete proinflammatory cytokines.[32] As would be expected for polarized epithelial cells residing on a basement membrane, cholangiocytes express cell-matrix adhesion molecules such as integrins in abundance.[33]

Peribiliary Glands

Extrahepatic bile ducts and the large bile ducts of the perihilar intrahepatic biliary tree have companion peribiliary glands, composed of branched tubule-alveolar seromucinous glands,[34] (Fig. 1-13). Along with mucin, these glands secrete substances such as lactoferrin and lysozyme.[35] They are reported to be stem cell niches of the biliary tree[36] capable of differentiating into hepatobiliary and pancreatic cells.[37,38] Thus, in addition to providing their seromucinous secretions to the flow of bile, cells of the peribiliary glands may play a key role in normal tissue turnover and injury repair.[38]

Portal Tract Mesenchyme

The liver has only a small amount of connective tissue in relation to its size: 5% to 10% of total protein and less than 3% of the relative area on a routine histologic section.[39] Extracellular matrix underlies the capsule of the liver (Glisson's capsule), ensheathes the intrahepatic venous system, and under normal conditions is found within the space of Disse only as delicate bundles of type IV collagen—the so-called reticulin network of the parenchyma. However, most of the extracellular matrix visible on histologic sections of the normal liver is within portal tracts, surrounding the normal vascular and biliary structures. This mesenchymal investment is minimal in the most terminal portal tract branches (see Fig. 1-7). The mesenchymal content is more prominent in the larger sublobular portal tracts.

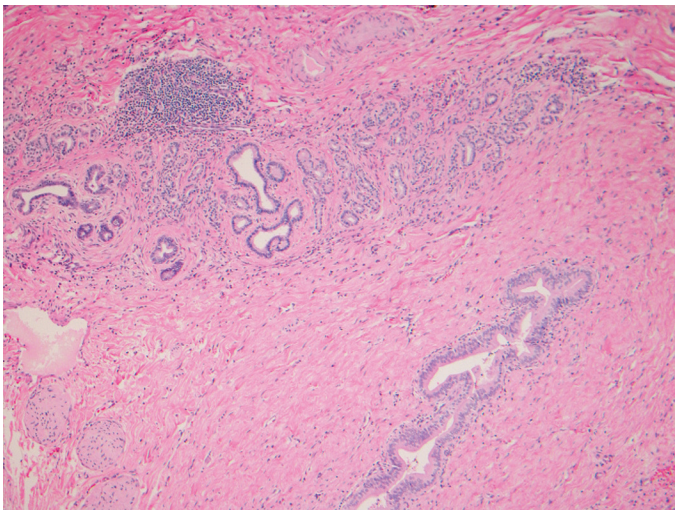
Portal tracts contain fibroblasts, which encircle bile ducts and bile ductules and may also be found elsewhere in portal tracts.[40-42] In the first instance, the basement upon which cholangiocytes reside is surrounded directly by peribiliary fibroblasts.[43] In the settings of bile duct inflammation and/or obstruction, these fibroblasts are rapidly activated with acquisition of smooth muscle actin, generating a myofibroblast phenotype.[41] In other settings, such as hepatitis, other populations of fibrogenic cells in portal tracts play a role. These include myofibroblasts loosely placed around the portal vein and hepatic artery, and fibrocytes in the loose connective tissue of the portal tract, especially at the interface between the portal tract and parenchyma.[44] Thus, in discussions of hepatic fibrogenesis, it is important to keep these particular cell types in mind.

Lobular and Acinar Functional Units

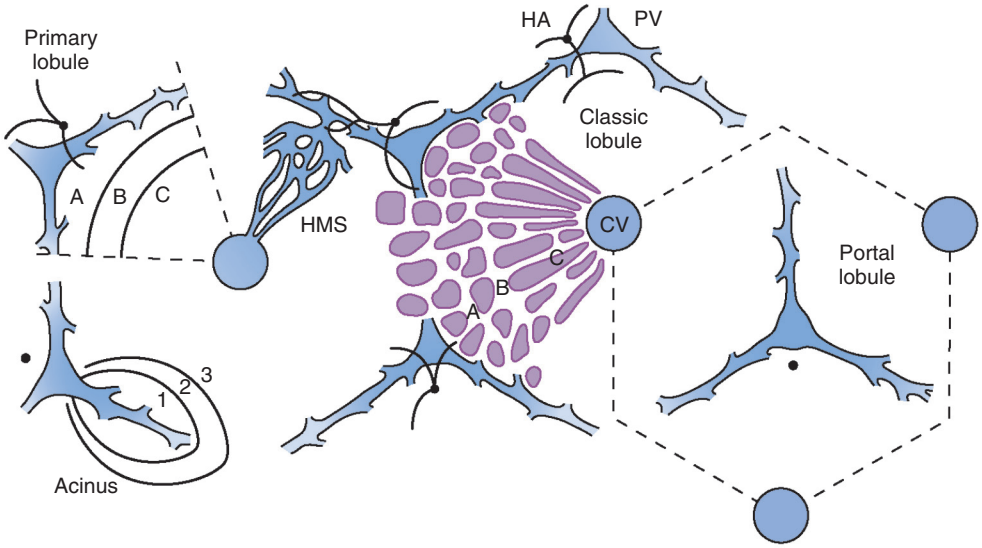
Models of hepatic parenchymal microarchitecture have been debated for the past century, and they are not mutually exclusive. The essential terms are *lobule* and *acinus* (Fig. 1-14). The classic hepatic lobule is a polygonal structure having as its central axis the terminal hepatic vein (in this model termed the *central vein*) with portal tracts distributed along its peripheral boundary.[45] The ideal formulation of the classic lobule is hexagonal with portal tracts at three of the six apices of the hexagon. In reality, the peripheral boundaries of lobules are poorly defined, and terminal hepatic veins may be draining sinusoidal blood supplied by a smaller or larger number of nearby portal tracts.[18] A key corollary of this terminology is that the region of the lobule around the terminal hepatic vein (or the central vein) is called *centrilobular*, and the region around the portal tract is called *periportal*.

An uncommonly used concept of lobular organization is the portal lobule, defined by bile drainage, with a portal tract at the center and terminal hepatic veins at the periphery.

The hepatic acinus[46] is a smaller triangular unit with the portal tract at one apex of the narrow base, a watershed region of the parenchyma at the other apex of the base, and the terminal hepatic vein at a more distant apex (see Fig. 1-14). The acinar concept aligns with the pattern of blood flow, in that there are penetrating portal venular channels that exit portal tracts and run along the base of the acinar triangle towards the watershed region.[47] In turn, terminal twigs of the hepatic arterial system also extend a short distance into the parenchyma. A broad front of blood thus travels through the sinusoids from the base of the acinar triangle towards the apex at the terminal hepatic vein, creating three zones. Zone 1 is the region of the parenchyma most proximate to vascular inflow, and thus is most oxygenated and has the most ready access to nutrients. Zone 2 is the midparenchymal region, and Zone 3 is the most distal from the vascular inflow. Zone 3 thus has the lowest oxygen content, and is most downstream from nutrient inflow. The effects of this lobular gradient (noting the interchanging of terms) will be examined in a subsequent section.



• **Fig. 1-13** Peribiliary glands. A cluster of peribiliary glands, with their conducting ducts (upper portion of the image), are present near the hepatic hilum. A major bile duct is present (lower right), along with an incidental aggregate of inflammatory cells (upper left).



• **Fig. 1-14** Microarchitecture of the liver. A primary lobule is formed from the inlet venules arising from portal veins (PV), giving rise to a sinusoidal "front" of blood that moves across equipotential lines A, B and C towards the terminal hepatic vein (THV). The acinus concept recognizes this front of blood as moving through Zones 1, 2 and 3 en route to the terminal hepatic vein. These three zones exhibit differing oxygenation and metabolism. The concept of the conical hepatic microcirculatory (HMS) unit recognizes an even smaller parenchymal unit, each being supplied by a single inlet venule. The classic lobule places the terminal hepatic vein (THV, also called a central vein) at the center, and portal tracts with their portal vein (PV) and hepatic artery (HA) at the periphery. The concept of a portal lobule, with a portal tract at its center, is essentially obsolete. (Modified from McCuskey RS. Functional morphology of the liver with emphasis on its microvasculature. In: Tavoloni N, Berk PD, editors. *Hepatic transport and bile secretion.* New York: Raven Press, 1993: 4, ©1993, with permission from Lippincott Williams & Wilkins [http://lww.com].)

Hepatocytes

Hepatocytes are polyhedral cells approximately 20 µm to 30 µm in diameter, with a volume of approximately 5000 μm^3. Their organization into anastomosing plates of the parenchyma is best illustrated by scanning electron microscopy (see Fig. 1-5).[48-51] The liver cell plates anastomose extensively in the periportal region, but become more simplified and radially oriented as they approach the terminal hepatic vein. Similar to other polarized epithelial cells, hepatocytes have distinct regions of the plasma membrane. The basal surfaces of hepatocytes face the sinusoid; with plasma membranes that have microvilli extending into the space of Disse (the space between hepatocytes and endothelial cells), increasing the surface area available for the exchange of materials between hepatocytes and blood plasma. Facing adjacent hepatocytes is the lateral surface of the hepatocyte. Because these two regions are topologically in continuity and are considered as one functional unit, the common term is the *basolateral plasma membrane.*

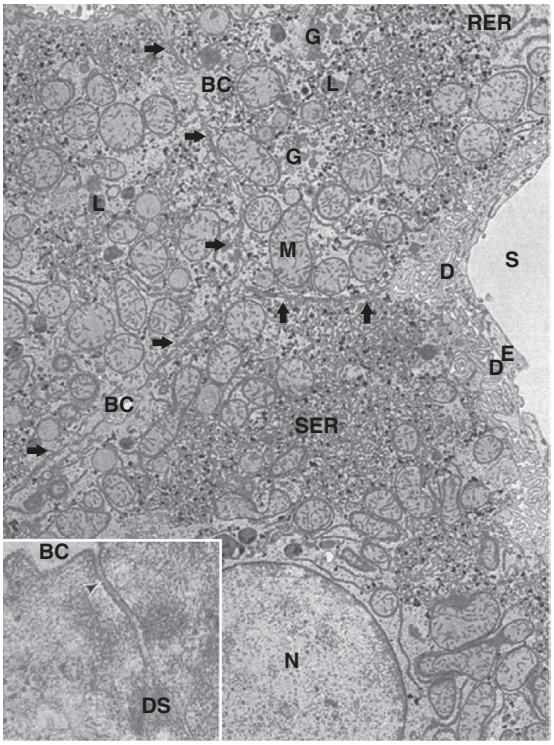
Directly between adjacent hepatocytes is an interconnecting network of intercellular channels between 1 µm and 2 µm in diameter termed *bile canaliculi*, constituting the apical or canalicular domain of the plasma membrane (see Fig. 1-5, *A*). The apical plasma membrane also forms microvilli to increase the surface area available for secretion. The apical domain is delineated from the basolateral plasma membrane by a continuous barrier of tight junctions (Figs. 1-15 and 1-16). Hepatocytes secrete bile into the canalicular space; the bile then drains toward portal tracts through the bile canalicular network (Fig. 1-17) for collection into the biliary system.

Plasma Membrane

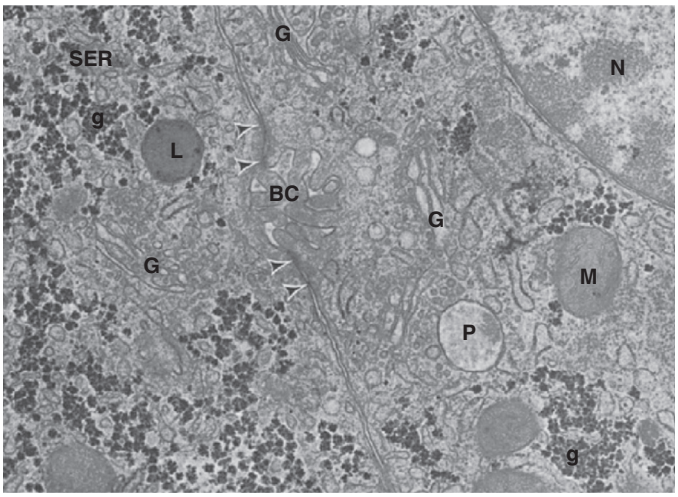
The hepatocyte basolateral plasma membrane is the site of uptake of blood-borne substances into hepatocytes, and secretion of hepatocellular products into the blood. This exchange of products is facilitated by the absence of a basal lamina between hepatocytes and the overlying sinusoidal endothelium, and by fenestrations in the endothelial cells. In addition to receptor proteins and a robust population of plasma membrane protein transporters for exchange of inorganic and organic solutes,[52] the basolateral membrane is a site of vigorous endocytic uptake and secretion of proteins.[53]

The lateral plasma membrane contains gap junctions that facilitate intercellular communication between adjacent hepatocytes along the length of the hepatocyte plate.[54] Gap junctions are an assemblage of connexons—membrane pores formed by the circular arrangement of six transmembrane proteins called connexins. Connexons in opposing plasma membranes are directly aligned and form aqueous channels that allow the passage of ions and small molecules, enabling the propagation of signals between adjacent cells. Desmosomes in the lateral membranes, as well as interdigitating undulations of adjacent plasma membranes, complete the stitching together of adjacent hepatocytes along the length of the hepatocyte plates.

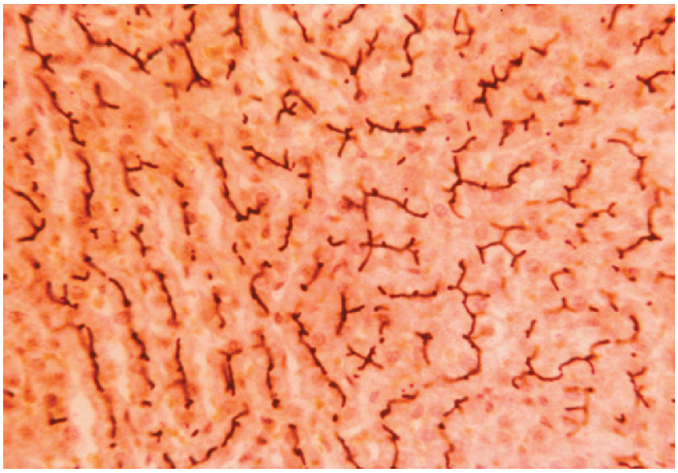
The apical surface of the hepatocyte plasma membrane is the site of secretion of electrolytes and organic solutes so as to form bile.[52] Transepithelial endocytic traffic of plasma solutes contributes to approximately 4% of the volume of secreted bile. Lysosomal contents are also discharged into the biliary space to a lesser extent.[55]



• **Fig. 1-15** Portions of three hepatocytes and a sinusoid. The lateral plasma membranes between hepatocytes are highlighted *(arrowheads);* two bile canaliculi are visible (BC). A sinusoid is present (S), with a sinusoidal endothelial cell (E) and the underlying space of Disse (D). A hepatocyte nucleus is present at the bottom (N), along with smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), Golgi (G), lysosomes (L), and mitochondria (M). The inset shows lateral hepatocyte plasma membranes immediately adjacent to a bile canaliculus (BC), with the tight junctional zone *(arrowhead)* and desmosomes (DS).



• **Fig. 1-16** Two adjacent hepatocytes and enclosed bile canaliculus (BC) and associated organelles. *G,* Golgi; *g,* glycogen; *L,* lysosome; *M,* mitochondria; *N,* nucleus; *P,* peroxisome; *SER,* smooth endoplasmic reticulum. *Arrowheads,* tight junctions.



• **Fig. 1-17** Bile canalicular network filled with dye injected retrograde into the bile duct.

Nucleus

Hepatocytes have one or two spherical nuclei containing one or more prominent nucleoli (see Figs. 1-15 and 1-16).[1,2,51] In human adults, 40% of hepatocytes are tetraploid rather than diploid, and hepatocyte ploidy number increases either as the cell ages or when the liver is stimulated to regenerate or respond to metabolic overload or oxidative stress.[56] Polyploid nuclei are characterized by their greater size, which is directly proportional to their ploidy. Multinucleated hepatocytes and polyploidy are consistent with high cellular function and demands and are mechanisms by which both nuclear and cytosomal biosynthetic machinery are increased to meet these functional demands. The high level of hepatocellular biosynthetic activity is also reflected in the high percentage of nuclei that are euchromatic, which indicates that transcription of most of the genome is occurring continuously. Thus, almost all of the deoxyribonucleic acid (DNA) is in the extended configuration, and little heterochromatin is observed. Hepatocytes engaged in the synthesis of many proteins have a large nucleolus (sometimes several) that can be recognized by light microscopy, and this characteristic is typical of hepatocytes.

The nucleolus is where ribosomal genes are located and ribosome biogenesis occurs.[57] Electron microscopy reveals the nucleolus to contain three main components: roundish fibrillar centers composed of thin loosely distributed fibrils, a dense fibrillar component containing tightly packed fibrils that surround the fibrillar centers, and a granular component constituted by granules that embed both fibrillar components. Ribosomal genes exist in an extended ready-to-be-transcribed configuration within the fibrillar centers and, in part, in the dense fibrillar component. Although the precise location of ribosomal gene transcription remains unclear, newly transcribed RNA molecules undergo early processing and maturation in the dense fibrillar component and are assembled into preribosomes in the granular component. Protein-rich ribosomal subunits then exit the nucleus through pores in the double membrane nuclear envelope.

Endoplasmic Reticulum, Ribosomes, and Golgi Apparatus

Rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), and Golgi complexes are abundant in mammalian

hepatocytes (see Figs. 1-15 and 1-16).[1,2,51,58] Their functions are related mainly to the synthesis and conjugation of proteins, metabolism of lipids and steroids, detoxification and metabolism of drugs, and breakdown of glycogen. The endoplasmic reticulum forms a continuous three-dimensional network of tubules, vesicles, and lamellae. Almost 60% of the endoplasmic reticulum has ribosomes attached to its cytoplasmic surface and is known as the RER. The remaining 40% constitutes the SER, which lacks a coating of ribosomes. The membranes of the endoplasmic reticulum are between 5 nm and 8 nm thick. The lumen of the RER is approximately 20 nm to 30 nm wide, whereas that of the SER is larger (30-60 nm). The morphologic characteristics and amount of the endoplasmic reticulum vary in the different zones of the liver lobule.

RER is arranged in aggregates of flat cisternae that may be found throughout the cytoplasm. It is more frequently distributed in the perinuclear, pericanalicular, and subbasilar regions of hepatocytes, and it is more abundant in periportal cells than in centrilobular cells.[59] The numerous attached membrane-bound ribosomes consist of a large and a small subunit, with the large subunit attached to the RER. Free ribosomes and polyribosomes are also present within the hepatocyte cytoplasm. Ribosomes contain RNA and ribosomal proteins and play a key role in the synthesis of proteins, particularly those destined for secretion or for delivery to intracellular membrane compartments or the plasma membrane. Vesicles containing these proteins are directed to the proximate (cis) cisternae of the Golgi apparatus, for further processing.

SER is less common and has a more complex arrangement than RER.[51] It is usually much more abundant in centrilobular than in periportal hepatocytes[59,60]; the high content of heme-containing cytochromes lends a darker pigmentation to the centrilobular region of the lobule, as is evident on visual inspection of the cut surface of the liver. The matrix within the SER tubules is usually slightly more electron-dense than the surrounding cytoplasm. SER membranes are irregular in size and present a tortuous course. They may be tubular or vesicular in structure with a width of 20 nm to 40 nm. SER is mainly distributed near the periphery of the cell. It is often in close relation to RER and Golgi membranes, as well as to glycogen inclusions.[51]

The ER is not the only site of protein synthesis in hepatocytes. Abundant free ribosomes in the cytoplasm participate in the synthesis of some proteins that will be secreted but synthesize essentially all of the structural proteins for the hepatocyte. Proteins that are to remain within the cytoplasm or are destined to enter the nucleus, peroxisomes, or mitochondria are completely synthesized by free ribosomes.

The Golgi complex is a three-dimensional structure in hepatocytes, characteristically consisting of a stack of four to six parallel cisternae, often with dilated bulbous ends containing electron-dense material.[10,51,58] Multiple Golgi complexes exist in each hepatic parenchymal cell, generally distributed near the nucleus. This structure shows a convex or proximal portion facing the nucleus and the endoplasmic reticulum (cis-Golgi), where small vesicles transfer proteins from the endoplasmic reticulum to the Golgi, and a concave part (trans-Golgi), which connects with a post-Golgi trans-Golgi network that directs proteins towards their final destinations: to organelle membranes of the cell, the plasma membrane, or for secretion. The cisternae may be up to 1 μm in diameter with a lumen that is 30 nm wide. The Golgi complex is capable of rapid and reversible structural reorganization into a tubuloglomerular network, while maintaining its biosynthetic capabilities.[61] With the SER, RER, lysosomes, other intermediate organelle compartments, and even the nuclear and mitochondrial envelope membranes, the Golgi is an integral part of the complex intracellular organelle network involving vesicular trafficking that enables uptake, sorting, degradation, biosynthesis, trafficking, and/or secretion of cellular proteins and lipids.[60-63]

Mitochondria

Mitochondria are large organelles and are very numerous in hepatocytes (1-2000 per cell; see Figs. 1-15 and 1-16), constituting approximately 18% to 20% of the cell volume.[64] They are the site of oxidative phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP), constituting the source of aerobic cellular metabolism.[51] Although the mitochondria are dispersed ubiquitously within hepatocytes, they are more concentrated near sites of ATP utilization and are often associated with the RER.

Mitochondria in hepatocytes may be round or elongated, with a width of between 0.4 μm and 0.6 μm and a length of between 0.7 μm and 1.0 μm. Longer (up to 4 μm) and larger (up to 1.5 μm in diameter) mitochondria are more numerous in periportal hepatocytes.[64] Mitochondria are bounded by an outer and an inner membrane, each 5 nm to 7 nm thick. The outer membrane possesses special pores that allow the passage of molecules smaller than approximately 2000 Da. The inner membrane's surface area is greatly increased by the presence of numerous cristae, which fold within the mitochondrial matrix. The space between inner and outer membranes presents a low-density matrix and ranges from approximately 7 nm to 10 nm in thickness. Mitochondria have a relatively low-density internal matrix in which lamellar or tubular cristae and a variable amount of small dense granules can be observed. The dense granules have a diameter of between 20 nm and 50 nm. In addition, filaments of circular mitochondrial DNA of approximately 3 nm to 5 nm in width and granules of approximately 12 nm in diameter containing mitochondrial RNA are also present. The DNA codes for some of the mitochondrial proteins that are synthesized in ribosomes within the organelle, but most of the mitochondrial protein is encoded by nuclear DNA. Mitochondria are self-replicating and have a half-life of approximately 10 days.

Lysosomes

Lysosomes in hepatocytes (see Figs. 1-15 and 1-16) consist of a heterogeneous population of organelles that are morphologically and functionally interrelated and contain hydrolytic enzymes.[51,65] These organelles form rounded single membrane–bound dense bodies, autophagic vacuoles, multivesicular bodies, and coated vesicles. In keeping with the earlier comments regarding the Golgi apparatus, lysosomes may be part of the intracellular membranous network known as the *GERL* (Golgi-SER-lysosome). The GERL is involved with endocytosis and exocytosis, serving as a site for sorting of secretory proteins for secretion and for trafficking of endocytosed proteins to lysosomes for degradation. Indeed, the GERL is the site where acid phosphatase makes its first appearance, most likely playing a role in formation of lysosomes.

Several classes of lysosomes can be identified within the hepatocyte cytoplasm:

- Primary lysosomes, small in size, are considered from a functional point of view to be in a resting phase.

- Secondary lysosomes are functionally activated.
- Autophagic vacuoles contain parts of degrading cytoplasmic organelles and are often delimited by a double membrane.
- Residual bodies are larger than primary and secondary lysosomes and are usually more numerous in older organisms.

The residual bodies contain the residues of nondigested material or pigments such as lipofuscins (which are considered undigestible permanent residues). Lipofuscin granules are the most numerous lysosomal bodies present in human hepatocytes.[59]

Lysosomes are frequently found near the plasma membrane proximal to the bile canaliculus, and are capable of discharging their contents into the biliary space.[55] The lysosomes in periportal hepatocytes are often larger and more positive for acid phosphatase than those in centrilobular hepatocytes.[59,60]

Peroxisomes

Peroxisomes are subcellular membrane-bound organelles that are usually rounded or slightly oval in shape (see Fig. 1-16), but may form dynamic elongated tubules several microns in length.[66,67] They participate mainly in oxidative processes, and play a key role in bile acid biosynthesis in the liver.[51,68] Each hepatocyte may contain between 300 and 600 peroxisomes, characteristically more numerous and larger than in other mammalian cells.[59] They contain a fine granular matrix and in some species (but not humans) a denser paracrystalline structure may be present. Peroxisomes may be more numerous in pericentral hepatocytes but they are generally homogeneously distributed within the hepatic lobule.[59,60] Peroxisomes are believed to originate as a focal protrusion of the RER.

Cytoplasmic Contents

The hepatocyte is extremely rich in non–membrane-bound cytoplasmic inclusions, including glycogen granules, lipid droplets, and pigments of various natures.[51] Glycogen granules are the most abundant inclusions in normal hepatocytes (see Figs. 1-15 and 1-16).[51,59] At the electron microscopy level they may occur either in the monoparticulate form (β particles, 15-30 nm in size) or, more frequently, as aggregates of smaller particles arranged to form "rosettes" (α particles). Glycogen granules are dispersed in the cytoplasm, but are often associated with the SER. Glycogen is depleted during fasting, disappearing first from periportal hepatocytes and then from centrilobular cells. Upon refeeding, the sequence reverses. In this fashion, hepatocytes constitute a major metabolic energy reserve during fasting, thus supporting systemic glucose homeostasis.

Lipid inclusions appear as empty vacuoles in histologic sections, or osmiophilic droplets by transmission electron microscopy, and are usually not surrounded by membranes. Lipid droplets consist of triglycerides in their interior, and are coated with a monolayer of phospholipids.[60] Small lipid droplets have a high surface/volume ratio and are accessible to cytoplasmic lipases that may degrade the retained triglyceride quickly.[69] Large lipid droplets have a low surface/volume ratio, and may reside in hepatocytes for long after the metabolic reasons for their deposition have subsided.

A variable amount of iron-containing granules are often present within the hepatocyte cytoplasm, which is heavily dependent upon the iron status of the host.[70] These are usually in the form of ferritin particles. With an approximately spherical shape, the iron-containing protein ferritin consists of a protein shell (apoferritin) 11 nm in diameter and an iron-containing central core approximately 5 nm in diameter. Hepatocyte iron deposits may also occur as single membrane-bound lysosomal bodies (residual bodies) forming aggregates of iron-containing electron-dense particles (siderosomes-hemosiderin granules). In addition to hepatocytes, liver endothelial cells and Kupffer cells[71] also accumulate intracellular iron under conditions of iron overload.

Cytoskeleton and Cytomatrix

The cytoskeleton is a structure that is considered to regulate the shape, subcellular organization, and movements of the cells. In the hepatocyte the cytoskeletal organization is dependent on the arrangement of the three main components of this structure: the microfilaments, the intermediate filaments, and the microtubules.[72,73] These filament types are regularly distributed in the cytoplasm and characterize the cytomatrix, which together with other finer filaments (microtrabeculae) is believed to contribute to the gel consistency of cytoplasm. Microfilaments, made of actin, and microtubules, consisting of tubulin, are both involved in intracellular motility. Microtubules are implicated in determining cell shape, completing mitosis, and regulating the intracellular transport of vesicles.[53] These structures assume a relevant role in the secretion of lipoproteins and albumin and the release of lipids into bile, especially in the liver. Microfilaments are more directly related to bile secretion. In fact, they are normally found around the bile canaliculi (pericanalicular web). Many experimental studies have shown that microfilaments play an active role in the dilatation and contraction of bile canaliculi,[74,75] thereby contributing to control of bile canalicular caliber and bile flow.

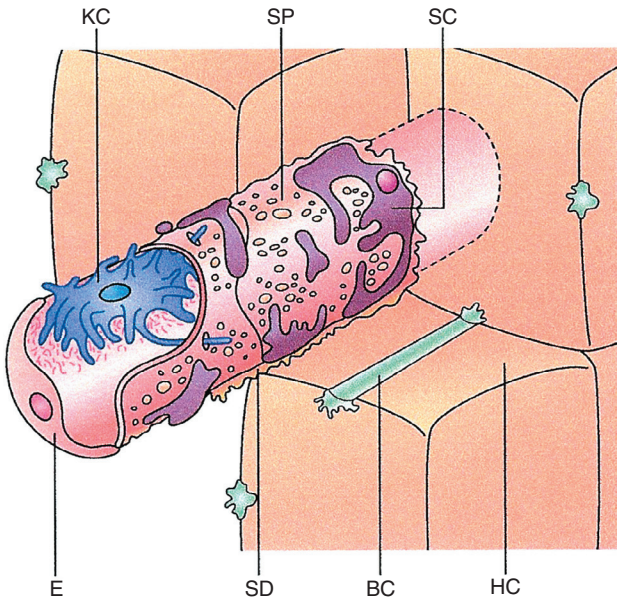
Intermediate filaments show a more complex architecture. They correspond to the epithelial cell tonofilaments of the old nomenclature. In the liver they show a relationship with Mallory bodies (the structural marker of human alcoholic liver disease). They are located around the nucleus, near the cell border, in the cytoplasmic network, and around the bile canaliculi.

The Hepatic Sinusoid

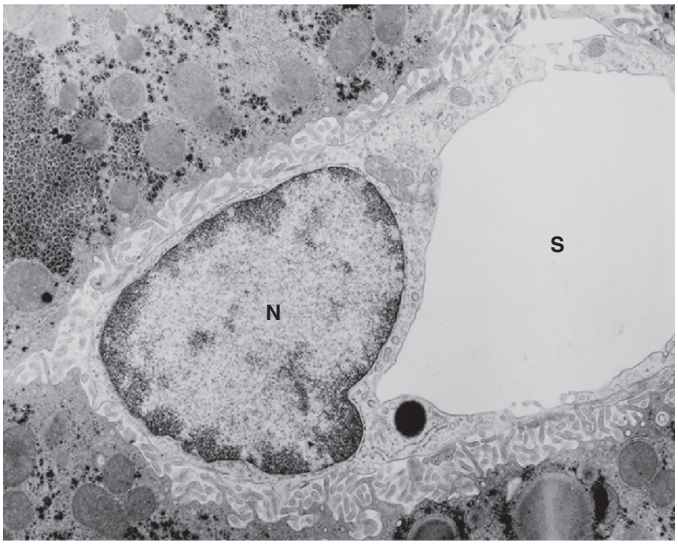
The hepatic sinusoid is a unique, dynamic, microvascular structure that serves as the principal site of exchange between the blood and the perisinusoidal space (i.e., space of Disse).[9] The sinusoid is the home of nonparenchymal cells, of which there are four recognized types (Figs. 1-18 and 1-19; see Table 1-2)[9,76]:

1. Fenestrated sinusoidal endothelial cells, which form the sinusoid lining that is in contact with the blood
2. Phagocytic Kupffer cells, which adhere on the luminal aspect
3. Hepatic stellate cells, specialized pericytes that extend processes throughout the space of Disse and serve as myofibroblasts during times of hepatic injury and repair
4. Pit cells, which are immunoreactive natural killer (NK) cells that are attached to the abluminal surface of the sinusoid and are part of a population of liver-associated lymphocytes[77]

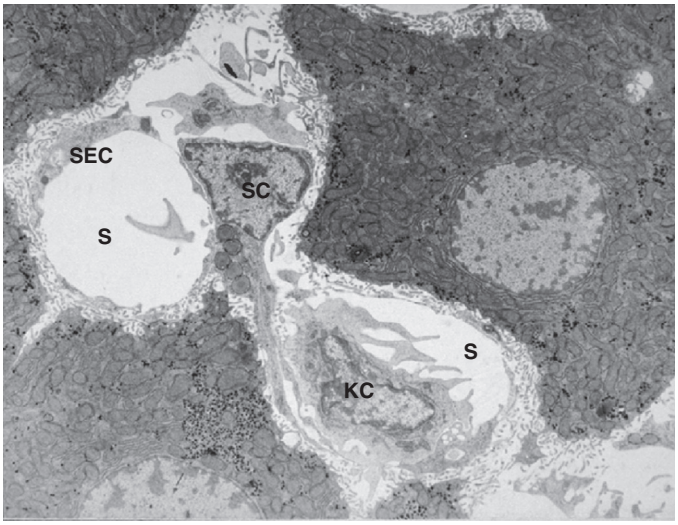
Together, the sinusoidal nonparenchymal cells represent approximately 6% of the total liver volume, but account for 30% to 35% of the total number of liver cells as measured by count of nuclei.[78,79] Whereas sinusoidal endothelial cells have the capacity to divide and proliferate, especially when stimulated by immune system modifiers,[80] sinusoidal macrophages and NK cells may also be increased in numbers by the respective recruitment and subsequent modification of monocytes and lymphocytes, principally of bone marrow origin.[81]



• **Fig. 1-18** Sinusoid wall and contiguous hepatocytes (HC). *BC,* Bile canaliculus; *E,* endothelium; *KC,* Kupffer cell; *SC,* stellate cell; *SD,* space of Disse; *SP,* sieve plate of fenestrae. (Modified from McCuskey RS. In: Tavoloni N, Berk PD, editors. *Hepatic transport and bile secretion.* New York: Raven Press, 1993: 6, ©1993, with permission from Lippincott Williams & Wilkins [http://lww.com].)



• **Fig. 1-20** Sinusoidal endothelial cell. The lumen of the sinusoid (S) is shown. The cell body of the sinusoidal endothelial cells contains a nucleus (N), and has limited perinuclear cytoplasm that contains a few organelles, such as mitochondria, a lysosome, and a few cisternae of endoplasmic reticulum. The endothelial cell rests on the hepatocyte microvilli filling the space of Disse. (Modified from Wisse E, et al. Structure and function of sinusoidal lining cells in the liver. *Toxicol Pathol* 1996;24:100–111, with permission.)

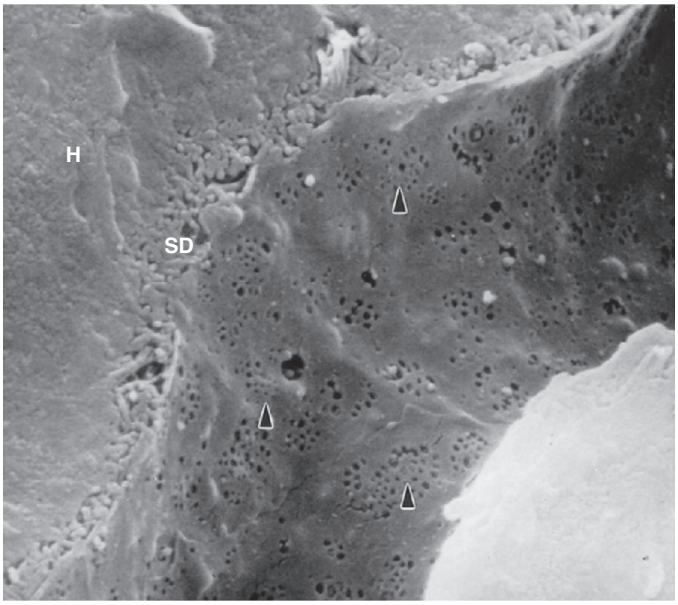


• **Fig. 1-19** Sinusoidal architecture. The sinusoidal lumen is shown. The sinusoidal endothelial cell (SEC) has an attenuated cell body that extends around the circumference of the lumen. A Kupffer cell (KC) is attached to the luminal surface and a stellate cell (SC) lies externally in the space of Disse. (Modified from McCuskey RS. Functional morphology of the liver with emphasis on its microvasculature. In: Tavoloni N, Berk PD, editors. *Hepatic transport and bile secretion.* New York: Raven Press, 1993: 6, ©1993, with permission from Lippincott Williams & Wilkins [http://lww.com].)

Sinusoidal Endothelial Cells

Ostensibly similar to endothelial cells found in capillaries elsewhere throughout the body, contiguous sinusoidal endothelial cells in the liver form the basic tubular vessel for conveyance of blood and its contents through the sinusoidal vascular channels coursing between the hepatocyte plates. The similarity ends there, because sinusoidal endothelial cells do not rest on a basement membrane but rather form an attenuated cytoplasmic sheet approximately 50 nm to 80 nm in maximum thickness, perforated by numerous holes (fenestrae). Also, unlike endothelial cells elsewhere, hepatic sinusoidal endothelial cells apparently do not form junctions with adjacent endothelial cells (Figs. 1-20 and 1-21). The sinusoidal endothelial cell fenestrae are so abundant that, on scanning electron microscopy, the greater part of the cell has a netlike appearance. Thus, the sinusoidal endothelium forms a porous barrier between the sinusoidal lumen and underlying hepatocytes, reinforced here and there where adjacent endothelial cells overlap one another (see Fig. 1-20).

The sinusoidal endothelial cell fenestrae vary greatly in size, but generally fall into two size categories: small fenestrae (0.1-0.2 μm in diameter) grouped in clusters, forming so-called sieve plates; and large fenestrae (up to 1 μm in diameter) which are more numerous at the distal end of the sinusoid. Thus, endothelial cell porosity is higher in the perivenular zone than in the periportal zone.[82] The smaller fenestrae traverse individual endothelial cells. The larger fenestrae lie between sinusoidal endothelial cells, and some workers consider that they may be artifacts due to fixation.[83] Regardless, there is evidence that fenestrae are labile structures, whose diameter is regulated and may change in response to endogenous mediators (e.g., serotonin) and exogenous agents, such as alcohol.[84] The extracellular matrix in the space of Disse also modulates the fenestrae. For example, lack of cell-matrix interaction results in loss of fenestrae in cultured sinusoidal endothelial cells, whereas cells plated on human amnion basement membrane retain their fenestrae.[85] The mechanism for active control of the diameters of these fenestrae appears to reside in actin-containing components of the sinusoidal endothelial cell cytoskeleton.[86-89] Additional cytoskeletal components form rings that delineate both the fenestrae and the sieve plates.[87,90] The



• **Fig. 1-21** Sinusoid illustrating fenestrae organized in clusters as sieve plates *(arrowheads)*. *H,* Hepatocyte; *SD,* space of Disse. (Reproduced from McCuskey RS. Functional morphology of the liver with emphasis on its microvasculature. In: Tavoloni N, Berk PD, editors. *Hepatic transport and bile secretion.* New York: Raven Press, 1993: 7, ©1993, with permission from Lippincott Williams & Wilkins [http://lww.com].)

number of fenestrae present in the hepatic sinusoid decreases as the individual ages.[91]

The unique porous structure of the hepatic sinusoid allows the endothelial cells to coarsely filter the sinusoidal blood, while permitting free passage of macromolecular solutes from the lumen into the space of Disse so as to come into contact with the basolateral plasma membrane of hepatocytes. However, large particles, such as newly generated chylomicrons, are excluded. Transport of particulates somewhat larger than the size of the fenestrae is postulated to be accomplished by the forced sieving and endothelial massage concomitant with the passage of blood cells, particularly leukocytes, through the sinusoids and the resulting interaction of these cells with the endothelial wall.[92]

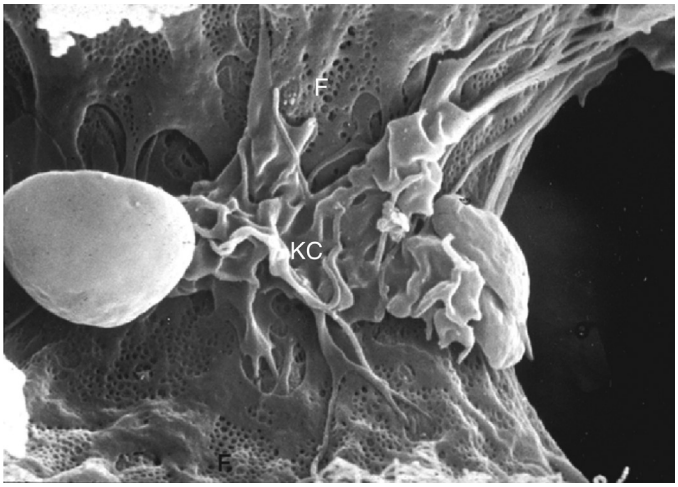
Sinusoidal endothelial cells show a number of phenotypic differences compared with vascular endothelium elsewhere.[93] They do not bind the lectin Ulex europaeus and, in most species, do not express factor VIII–related antigen (von Willebrand factor), although the cells assume these properties in chronic liver disease.[94] Furthermore, they contain absent to low levels of other molecules characteristically found in vascular endothelium, such as E-selectin, CD31, and CD34,[95] but do express Fcγ IgG receptors (CD16 and CDw32), CD4, CD14, and amino-peptidase N.[96] They also exhibit membrane immunoreactivity for ICAM-1.[96] The natural ligand for this adhesion molecule, LFA-1, is present on Kupffer cells; this receptor may therefore be involved in adhesion of Kupffer cells to the endothelial lining.[96] Up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression in sinusoidal endothelial cells may be important in "trapping" lymphocyte associated antigen-1 (LFA-1) positive lymphocytes in inflammatory liver diseases.[97]

Another unusual feature of sinusoidal endothelial cells is their high endocytotic activity.[98,99] Sinusoidal endothelial cells contain numerous cytoplasmic vacuoles and organelles associated with the uptake, transport, and degradation of material. These include bristle-coated pits (which are invaginations from the cell membrane), bristle-coated micropinocytotic vesicles, endosomes, transfer tubules, and lysosomes.[76,100] The variety of substances known to be endocytosed by sinusoidal endothelial cells includes proteins, glycoproteins, lipoproteins, glycosaminoglycans,[101-103] and, under certain conditions, larger particulates, which are phagocytosed in the absence of functional Kupffer cells.[104]

In addition, sinusoidal endothelial cells have been demonstrated to play a significant role in the processing and metabolism of lipoproteins and the removal of advanced glycation end-product (AGE) molecules.[102,105] This process appears to be directed towards uptake and lysosomal degradation of compounds rather than providing an alternative route for their transport from the sinusoidal lumen to the space of Disse. A large number of endogenous compounds may be endocytosed, some of which are effete molecules and are cleared from the circulation and others which are modified and appear to undergo transcytosis to hepatocytes, perhaps in a more selective fashion than macromolecular solutes passing only through the fenestrae.[99] Thus, the sinusoidal endothelial cells have a role in removing soluble immune complexes (similar to Kupffer cells) and have also been shown to store and metabolize serum immunoglobulin and to remove hyaluronic acid/chondroitin sulphate proteoglycans from the circulation.[106-108]

The sinusoidal endothelial cells also have synthetic activity and produce proinflammatory cytokines, such as interleukin-1, interleukin-6, and interferon,[109] nitrous oxide (NO), endothelins, eicosanoids, particularly prostaglandins PGI_2 and PGE_2, and thromboxane A_2 (TXA_2).[76,102] Thus, the sinusoidal endothelial cells participate in host innate immunity and regulation of sinusoidal blood flow in the liver. In addition, sinusoidal endothelial cells constitutively express the intercellular adhesion molecule ICAM-1, which along with vascular cell adhesion molecule-1 (VCAM-1) is up-regulated by inflammatory stimuli either in a direct manner or by mediators released from stimulated Kupffer cells, resulting in increased adhesion of leukocytes to the endothelial surfaces.[110] Under homeostasis, liver sinusoidal endothelial cells shift intrahepatic T-cell responses towards tolerance. Sinusoidal endothelial cells also act as antigen-presenting cells (APCs) that prime naive CD4+ T cells, induce CD4+ CD25+ Foxp3+ regulatory T cells (Tregs)[111] and immunosuppressive IL-10-producing Th1 cells,[112] and prime naive CD8+ T cells that leads to tolerance induction. Independent of their APC properties, sinusoidal endothelial cells express CD95 ligand and sinusoidal endothelial cell lectin (LSECtin, also known as *CLEC4G*), a ligand for CD44, that interact with activated but not resting T cells and inhibit T cell activation and proliferation or induce apoptosis. Sinusoidal endothelial cells can negatively regulate the APC function of neighboring dendritic cells via direct contact.[113,114]

Sinusoidal endothelial cells function as more than a living barrier between the blood and hepatocytes. They are a gatekeeper in the process of hepatic fibrogenesis. Capillarization of sinusoids with defenestration of sinusoidal endothelial cells in liver disease results in activation of hepatic stellate cells and induces deposition of extracellular matrix in the space of Disse. Maintenance/restoration of sinusoidal fenestrations and prevention of capillarization help preserve stellate cell quiescence and minimize/reverse fibrosis.[115,116] Sinusoidal endothelial cells contribute to the angiogenesis of liver regeneration through their effects on other liver cells[117,118] and serve as master regulators by linking the processes of regeneration and angiogenesis through angiopoietin-2.[119] Sinusoidal endothelial cells produce stromal-derived factor (SDF)-1 and its receptor CXCR4, and may constitute a fundamental niche



• **Fig. 1-22** Kupffer cell (KC) attached to luminal surface of sinusoidal endothelium. The fenestrae (F) of the sieve plates in underlying sinusoidal endothelial cells also are visible. (Modified from McCuskey RS. Functional morphology of the liver with emphasis on its microvasculature. In: Tavoloni N, Berk PD, editors. *Hepatic transport and bile secretion.* New York: Raven Press, 1993: 7, ©1993, with permission from Lippincott Williams & Wilkins [http://lww.com].)

for regulation of hematopoietic stem cell migration to the liver during extramedullary hematopoiesis.[120] Finally, LSECtin plays an important role in colorectal carcinoma metastasis to the liver.[121]

Kupffer Cells

Kupffer cells are hepatic macrophages and are present in the lumen of hepatic sinusoids (see Fig. 1-19), constituting the largest population of fixed macrophages in the body. They belong to the mononuclear phagocytic system, but manifest phenotypic differences that distinguish them from other macrophages. They are of considerable importance in host defense mechanisms and in addition have an important role in the pathogenesis of various liver diseases.[122] On scanning electron microscopy, Kupffer cells have an irregular stellate shape,[123] and within the sinusoidal lumen the cell body rests on the endothelial lining (Fig. 1-22). They are more numerous in the periportal sinusoids[124] and as noted earlier there is some evidence that, similar to hepatocytes, Kupffer cells also manifest functional heterogeneity in the lobule.[124,125] They do not form junctional complexes with endothelial cells but they may be found in gaps between adjacent endothelial cells and their protoplasmic processes may extend through the larger endothelial fenestrae into the perisinusoidal space of Disse. The luminal surface shows many of the structural features associated with macrophages: small microvilli and microplicae and sinuous invaginations of the plasma membrane.

Although Kupffer cells are considered to be fixed-tissue macrophages, they appear capable of actively migrating along the sinusoids, both with and against the blood flow, and can migrate into areas of liver injury and into regional lymph nodes.[126] They contain lysosomes and phagosomes, and the cisternae of their endoplasmic reticulum are rich in peroxidase. Their primary functions include the removal by ingestion and degradation of particulate and soluble material from the portal blood, and in this they discriminate between "self" and "nonself" particles. They act as scavengers of microorganisms and degenerated normal cells, such as effete erythrocytes, circulating tumor cells, and various macromolecules. These functions are in part carried out nonspecifically but they are also involved in the initiation of immunologic responses and the induction of tolerance to antigens absorbed from the gastrointestinal tract. The efficiency of this clearance function is shown by the fact that removal of particulate material is limited only by the magnitude of hepatic blood flow; removal of particles may approach single-pass efficiency. Kupffer cells also phagocytose dead hepatocytes, generated either through apoptosis or necrosis. Such phagocytosis occurs within hours of hepatocyte death,[127] so that the presence of apoptotic hepatocytes in a tissue section is indicative of recent cell death.

Kupffer cells play a major role in the clearance of gut-derived endotoxins from the portal blood and this is achieved without the induction of a local inflammatory response. It has been estimated that the portal blood concentration of endotoxin varies from 100 pg/mL to 1 ng/mL.[128] The precise mechanisms involved are not fully understood, but there appears to be finely balanced autoregulation between the release of proinflammatory and inflammatory mediators such as interleukins 1 and 6, tumor necrosis factor α (TNFα) and interferon γ, and mediators such as interleukin-10 which suppresses macrophage activation and inhibits their cytokine secretions.[129-131] Kupffer cells express TLR4, TLR2, TLR3, and TLR9 and respond to LPS. Kupffer cells act as both immune activating and immune regulatory cells depending on the specific situation.[132,133] Recent animal studies indicated Kupffer cells are comprised of diverse subsets with distinct ontogeny and functions, but this concept has yet to be fully explored in humans.[134]

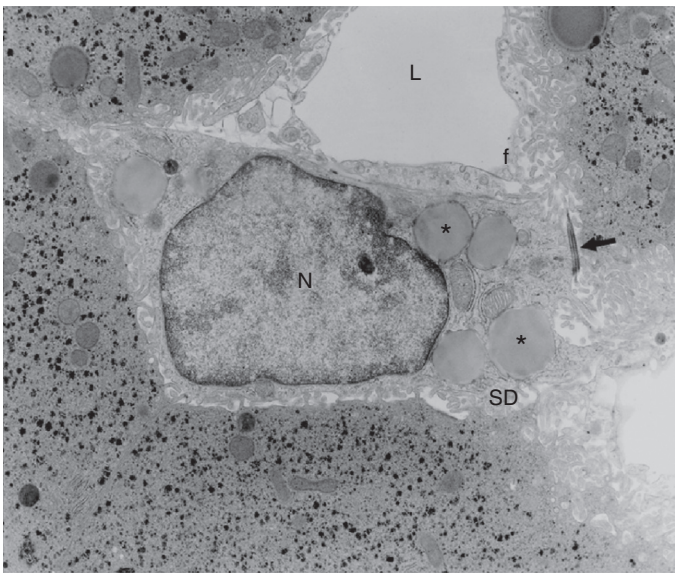
Several cytokines released by activated Kupffer cells are also thought to have local effects, modulating microvascular responses and the functions of hepatocytes and stellate cells.[135] Although Kupffer cells can express class II histocompatibility antigens[136] and can function in vitro as antigen-presenting cells, they appear to be considerably less efficient at this than macrophages at other sites.[137] Their principal roles in the immune response therefore appear to be antigen sequestration by phagocytosis and clearance of immune complexes.[138]

There is firm evidence from bone marrow transplant and liver transplant studies that Kupffer cells are derived, at least in part, from circulating monocytes.[139,140] However, Kupffer cells are capable of replication and their local proliferation accounts for a substantial part of the expansion of this cell population in response to liver injury.[141,142] Furthermore, Kupffer cells appear in the fetal liver of the mouse before there are circulating monocytes and there is evidence that they are derived from primitive macrophages that first appear in the yolk sac.[143] These data suggest that Kupffer cells may have a dual origin.

Stellate Cells

Within the space of Disse are stellate cells whose long cytoplasmic processes surround the sinusoids. Originally identified by Boll and von Kupffer in the 1870s, they were largely ignored until 1951 when Ito described their morphologic features on light microscopy.[144] They were subsequently referred to under a variety of terms—*Ito cells, hepatic lipocytes, fat storing cells, stellate cells,* and *parasinusoidal cells*.[145,146] The now accepted nomenclature for them is *hepatic stellate cells* (HSCs).[147]

Stellate cells comprise less than 10% of total resident liver cells under normal conditions and are regularly spaced along the sinusoids (approximately 40 μm from nucleus to nucleus).[148]



• **Fig. 1-23** Stellate cell lying within the space of Disse, which is covered by the endothelial lining. Fat droplets *(asterisks)* and cisternae of the endoplasmic reticulum are located in the cytoplasm. A small bundle of collagen fibers *(arrow)* is associated with the cell. *N,* Nucleus, *SD,* space of Disse; *S,* sinusoidal lumen; *F,* fenestrae. (Modified from Wisse E, et al. Structure and function of sinusoidal lining cells in the liver. *Toxicol Pathol* 1996;24:100–111, with permission.)

Despite their relative scarcity, their long cytoplasmic processes can cover the entire perisinusoidal area. It is notable that autonomic nerve endings running in the space of Disse come into contact with stellate cells, and the stellate cells respond to α-adrenergic stimulation.

Hepatic stellate cells are not readily visualized on light microscopy, but they may be readily seen in very thin histologic sections or by transmission electron microscopy (Fig. 1-23). The cells resemble pericytes and they establish close contacts with adjacent hepatocytes. The nucleus of a stellate cell is frequently located between hepatic parenchymal cells, whereas the thin, multiple cytoplasmic processes of these cells course through the perisinusoidal space and extensively embrace, like a cylindrical basket, the abluminal surfaces of the sinusoidal endothelium.[149] They contain many small lipid droplets that are rich in vitamin A. Rough endoplasmic reticulum and Golgi apparatus are well developed in these cells.

The biologic function of HSCs have been extensively reviewed.[150-153] Under noninflammatory conditions, HSCs have central roles in vitamin A storage and metabolism, hepatic organogenesis, regeneration and extracellular matrix homeostasis, drug metabolism and detoxification, and regulating blood flow through the sinusoids.[151] Once HSCs are activated under inflammatory conditions, they lose their perinuclear retinoid droplets, differentiate into myofibroblasts and assume a crucial role in liver angiogenesis,[151,153-155] regeneration, and development of hepatocellular carcinoma.[150,156] HSCs also take on the novel role of immune sentinels in the liver.[113,151,154]

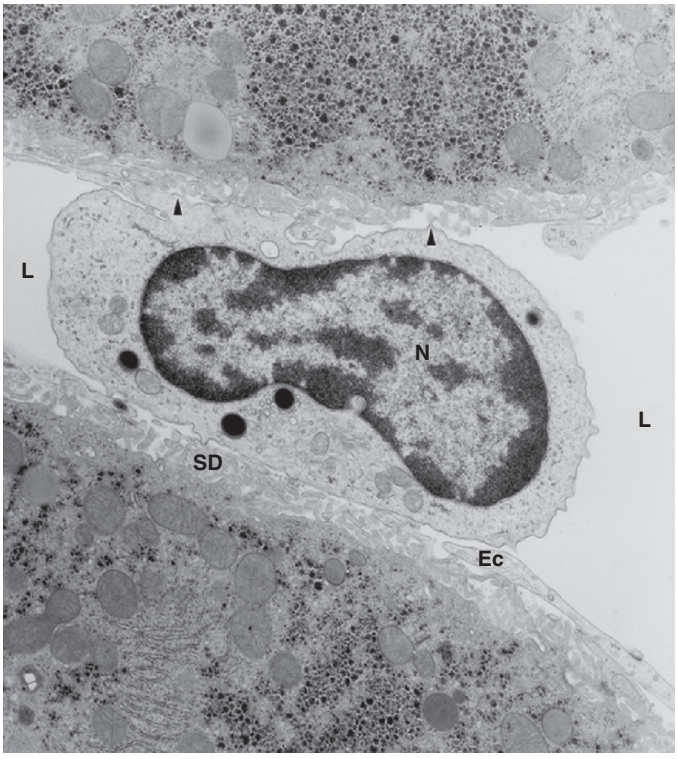
Hepatic stellate cells have four main functions in the liver:

1. They are a major site of storage for vitamin A.[157,158] Dietary retinyl esters reach the liver in chylomicron remnants. These pass from the sinusoidal lumen through the endothelial fenestrae and are taken up by hepatocytes. Most of the endocytosed retinol is rapidly transferred to the stellate cells for storage by an as yet poorly defined transport mechanism.[159] The cells contain a high concentration of cellular retinoid-binding protein and cellular retinol-acid binding protein.
2. They act in a pericyte-like manner around the sinusoids and may have a role in the control of microvascular tone in the normal liver.[158,160] Activated stellate cells have a definite contractile role in the injured liver owing to their up-regulation of α-SMA, and they respond to vasoactive agents such as endothelin 1 and nitric oxide.
3. They produce extracellular matrix proteins both in the normal liver and when activated in the process of liver fibrogenesis.[148,157] Healthy individuals have little or no basal lamina and collagen associated with the sinusoidal endothelium. As a result, the sinusoid wall is a highly permeable structure that permits continuity of plasma between the blood and the hepatocyte. However, with certain types of liver injury (e.g., cirrhosis) basement membrane material and collagen fibrils accumulate in the perisinusoidal space, resulting in capillarization of the sinusoid and impaired transvascular exchange.[161] Hepatic stellate cells are thought to be responsible for the synthesis of this material, following their transformation into myofibroblast-like cells having reduced numbers of fat droplets and vitamin A and an increased capacity to secrete extracellular matrix materials, including collagen types I and III to VI, fibronectin, laminin, tenascin, undulin, hyaluronic acid, biglycan, decorin, syndecan-containing chondroitin sulfate, heparan, and dermatan sulphate.[162]
4. They play a role in hepatic regeneration both in the normal liver and in response to liver injury.[163] They express hepatocyte growth factor[163,164] and this can be enhanced in human hepatic stellate cells in response to insulin-like growth factor-2.

In recent years, stellate cells have emerged as a prominent determinant of hepatic immunoregulation during injury. HSCs regulate immune function through four interrelated mechanisms:

- HSCs produce chemokines that facilitate inflammatory cell adhesion and migration into the liver.[151]
- HSCs can function as antigen-presenting cells to process protein antigens and present peptides to CD4+ and CD8+ T cells.
- HSCs express several pattern-recognition receptors, such as toll-like receptors (TLRs), and activated HSCs can induce NK cell activation, resulting in IFN-g production that play a role in the liver innate immunity.[165]
- They manifest immunosuppressive activities as bystander cells in the context of T cell immunity.

Overall, HSCs promote immune-suppressive responses in homeostasis, like induction of regulatory T cells (Tregs), T cell apoptosis (via B7-H1, PDL-1) or inhibition of cytotoxic CD8 T cells.[166] HSCs are a rich source of antiinflammatory mediators IL-10 and TGFβ, interfere with local T cell activation and cause induction and proliferation of Tregs. Because HSCs form a second layer of cells between the bloodstream and hepatocytes, the immune regulatory properties of HSCs can limit the effector function of those T cells that have extravasated from the sinusoidal lumen, preventing tissue damage and loss of organ function. This immune regulatory function is so strong that HSCs are even able to promote pancreatic islet allograft survival if they are cotransplanted. HSCs not only function as immune regulatory bystander cells during T cell activation and Treg induction, but also during contact with myeloid cells: HSCs also diminish the APC function of dendritic cells. During chronic inflammation in the liver, HSCs facilitate



• **Fig. 1-24** Liver-associated lymphocyte in close contact with the endothelial lining and in contact with microvilli of the underlying hepatocytes *(arrowhead)*. *N,* Nucleus; *SD,* space of Disse; *Ec,* endothelial cell; *F,* fenestrae; *S,* sinusoidal lumen. (Modified from Wisse E, et al. Structure and function of sinusoidal lining cells in the liver. *Toxicol Pathol* 1996;24:100–111, with permission.)

the differentiation of inflammatory monocytes into myeloid-derived suppressor cells (MDSCs) that impair T cell proliferation and effector function. The induction of multiple types of immune inhibitory cells by HSCs leads to a complex suppressive microenvironment.[113,114]

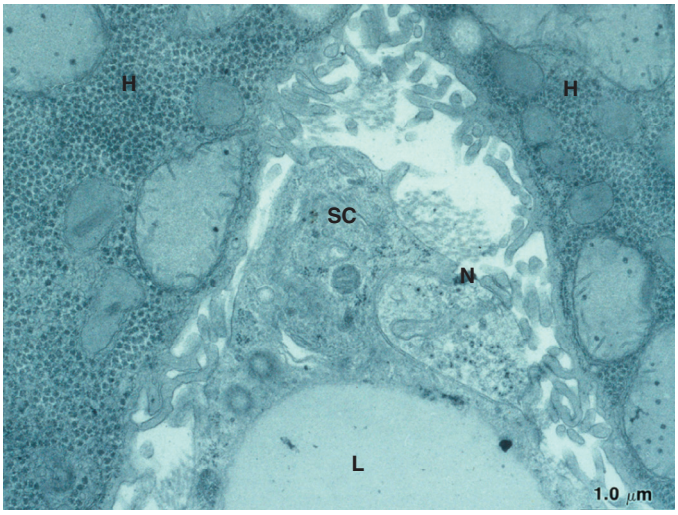
Liver-Associated Lymphocytes

Liver-associated lymphocytes (LALs) are derived from circulating large granular lymphocytes (LGL)[167] that become attached to the sinusoidal wall (Fig. 1-24); LGL possess natural killer (NK) activity and are part of a population of LAL.[77,168,169] Although the majority of LAL cell attachments to the sinusoidal wall are to endothelial cells, adhesion to Kupffer cells is not uncommon.

LALs also reside in the interstices between hepatocytes (pit cells). Pit cells have been shown to spontaneously kill tumor cells and produce a cytolytic factor that is up-regulated by biologic response modifiers such as zymosan, as well as by IL-2.[168] These substances also induce proliferation of pit cells, as does partial hepatectomy, perhaps through the activation of Kupffer cells. Finally, two types of pit cell have been recognized: high density (HD) and low density (LD). The LD pit cells have a greater number of smaller granules, as compared with the granules found in HD cells; in addition, LD cells exhibit more cytotoxicity.[170]

Innervation

Aminergic, peptidergic, and cholinergic nerves are contained in the portal tracts and affect both intrahepatic blood flow



• **Fig. 1-25** Nerve fiber (N) closely associated with a stellate cell (SC) in the space of Disse of a dog. *H,* Hepatocyte; *L,* lipid droplet.

and hepatic metabolism.[171,172] The role of neural elements in regulating blood flow through the hepatic sinusoids, solute exchange, and parenchymal function is incompletely understood.[173] This is due in part to limited investigation in only a few species, whose hepatic innervation may differ significantly from that of humans. For example, most experimental studies have used rats and mice, whose livers have little or no intralobular innervation. In contrast, most other mammals, including humans, have aminergic and peptidergic nerves extending from the perivascular plexus in the portal space into the lobule (see Fig. 1-8), where they course in the space of Disse in close relationship to stellate cells and hepatic parenchymal cells (Fig. 1-25). Although these fibers extend throughout the lobule, they predominate in the periportal region. Cholinergic innervation, however, appears to be restricted to structures in the portal space and immediately adjacent hepatic parenchymal cells. Neuropeptides have been co-localized with neurotransmitters in both adrenergic and cholinergic nerves. Neuropeptide Y (NPY) has been co-localized in aminergic nerves supplying all segments of the hepatic portal venous and the hepatic arterial and biliary systems. Nerve fibers immunoreactive for substance P (SP) and somatostatin (SOM) follow a similar pattern of distribution. Intralobular distribution of all of these nerve fibers is species dependent and similar to that reported for aminergic fibers. Vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) are reported to coexist in cholinergic and sensory afferent nerves innervating portal veins and hepatic arteries and their branches, but not the other vascular segments or the bile ducts. Nitrergic nerves immunoreactive for neuronal nitric oxide (nNOS) are located in the portal tract, where nNOS co-localizes with both NPY- and CGRP-containing fibers.

Heterogeneity

In completing this discussion of hepatic microanatomy—with occasional mention of structural variations of both hepatocytes and nonparenchymal cells from the periportal to the pericentral region, we must now consider the functional heterogeneity that occurs along the portal-to-central axis. Intralobular metabolic zonation creates variable cellular functions along the different

zones within each lobule.[174,175] For hepatocytes, the key enzymes involved in glucose uptake and release and in the formation of urea and glutamine are reciprocally located with glucogenic and urea cycle enzymes, principally in the periportal zone, and with glycolytic and glutaminogenic enzymes, in the centrilobular zone. Mixed-function oxidation and glucuronidation are mainly centrilobular functions, whereas sulfation is principally a periportal function. This zonation of enzymatic functions also is reflected ultrastructurally in differences in mitochondria and smooth endoplasmic reticulum among different zones. As a result of this zonation, as well as the portal-to-central oxygen gradient, most toxicologic and pathologic events in the liver show a considerable degree of zonal preference. An example of toxicants eliciting periportal injury is allyl alcohol; carbon tetrachloride and acetaminophen elicit centrilobular injury. Lastly, under normal conditions most bile formation occurs in the more proximate periportal regions of the lobule; with heavier bile acid loads entering the liver, bile acid uptake and bile secretion by hepatocytes can extend the length of the lobule.[176]

The lobular zonation of sinusoids is also manifested both structurally and functionally.[9,10] Near their origins from portal venules and hepatic arterioles, sinusoids are slightly narrower and are tortuous and anastomotic, forming interconnecting polygonal networks; farther away from the portal venules the sinusoids become organized as parallel vessels that converge on the terminal hepatic veins. Short intersinusoidal vessels connect adjacent parallel sinusoids. The volume of liver occupied by sinusoids in the periportal area is also greater than surrounding central venules. However, because of the smaller size and the anastomotic nature of the periportal sinusoids, the surface area available for exchange in the periportal sinusoids (surface area/volume ratio) is greater than that found in centrilobular sinusoids. As previously discussed, the size and pattern of distribution of endothelial fenestrae differ along the length of the sinusoid. At the portal end the fenestrae are larger but comprise less of the endothelial surface area than they do in the pericentral region. The functional significance of these regional differences is unclear.

Conclusion

Although the liver has no moving parts, the relationship of its microanatomy to its metabolic and physiologic function is exquisite. An understanding of liver function requires knowledge of hepatic microanatomy. Moreover, for physician and scientist alike, understanding the liver diseases discussed later in this volume is predicated on knowledge of how the different cellular and structural elements of the liver can become damaged and scarred, or repaired and regenerated. The current chapter has detailed both the long-documented gross, microscopic, and ultrastructural features of hepatic anatomy, and has aimed to provide insights into how the cells of the liver work together to perform its many functions. The text has included recent advances in this field, primarily at the cellular biologic level. These advances, and current key knowledge gaps, are highlighted later.

SUMMARY

Recent Progress

- portal tract: portal fibroblasts as separate from hepatic stellate cells
- biliary tree: canal of Hering as a regenerative compartment
- cholangiocytes: cholangiocyte physiology in bile formation
- hepatocytes: molecular regulation of hepatocyte cellular biology; intracellular molecular trafficking within the hepatocyte
- sinusoids: understanding of the hepatic stellate cell

Key Knowledge Gaps

- portal tracts: formation of hepatic lymph
- bile ducts and cholangiocytes: continued exploration of cholangiocyte biology
- interface: triggers of regeneration at the interface
- hepatocytes: continued exploration of hepatocyte biology
- sinusoids: molecular regulation of hepatic stellate cell biology
- immunology: molecular immunology of the sinusoid; continued exploration of liver as an immunologic organ
- nerves: neural regulation of hepatic regeneration and repair

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2

Bile Acids and Bile Flow: New Functions of Old Molecules

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ABBREVATIONS

ACOX acyl-CoA oxidase
ASBT apical sodium bile acid transporter
CTX cerebrotendinous xanthomatosis
FAB-MS fast atom bombardment ionization mass spectrometry
GC-MS gas chromatography–mass spectrometry
GGT γ-glutamyltranspeptidase
LDL low-density lipoprotein
LSIMS liquid secondary ionization mass spectrometry
PFIC progressive familial intrahepatic cholestasis
TUDCA taurine-conjugated form of ursodeoxycholic acid
UDCA ursodeoxycholic acid
VLCFA very long chain fatty acids
VSG vertical sleeve gastrectomy

Introduction

The importance of bile acid synthesis and metabolism to normal physiology and pathophysiologic states is well established. For a long time these small and relatively simple molecules, constructed on a steroid backbone, have been considered essential for cholesterol metabolism and bile flow and important for micelle formation for absorption of fats in the small intestine. More recently, bile acids have been further recognized as signaling molecules that regulate metabolism.[1-4] This chapter provides an overview of the pathways of bile acid synthesis and metabolism. It will focus on specific inborn errors in bile acid synthesis because these highlight the important role of bile acids in maintaining hepatic bile flow and as signaling integrators of metabolism.

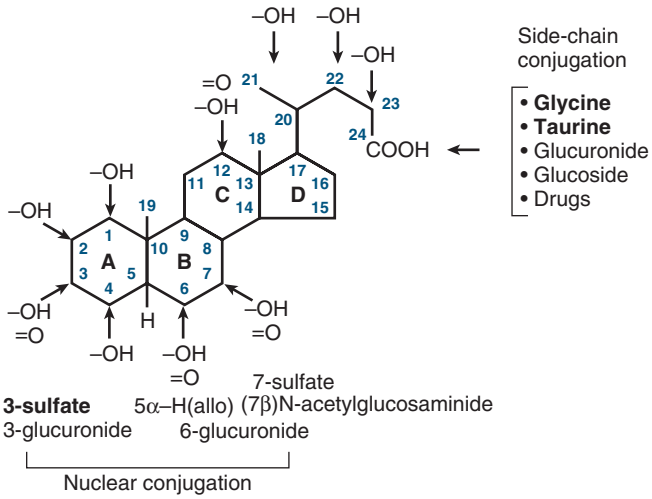
Pathways of Bile Acid Synthesis From Cholesterol

Structurally, bile acids possess a cyclopentanoperhydrophenanthrene (ABCD ring) nucleus and therefore belong to the chemical class of steroids.[5] They differ from steroid hormones and neutral sterols, such as cholesterol, in having a five-carbon-atom side chain with a terminal carboxylic acid (Fig. 2-1). Bile acids are synthesized in the liver from cholesterol by a complex series of reactions catalyzed by 17 different hepatic enzymes located in the endoplasmic reticulum, mitochondria, cytoplasm, and peroxisomes. Consequently there is considerable trafficking of intermediates between these subcellular compartments. Several of the enzymes are also found in extrahepatic tissues.[6,7] The enzymes involved in bile acid biosynthesis have all been isolated and well characterized in pioneering work performed in the late 1960s and the 1970s. More recently the role that each enzyme plays in the regulation of bile acid synthesis has been elucidated from studies of gene knockout animal models and humans with genetic defects in bile acid synthesis. Complementary (c)DNAs have now been described for these enzymes,[7] including the rate-limiting enzyme in the bile acid biosynthetic pathway, cholesterol 7α-hydroxylase,[8] and these have provided important tools to examine the regulation of bile acid synthesis and to confirm genetic defects in bile acid synthesis.

Conjugated (glycine and taurine) cholic and chenodeoxycholic acids are the two primary bile acids synthesized in humans, but there is considerable variability in the qualitative pattern of bile acid synthesis among animal species.[9] Rodents synthesize mostly cholic acid and the 6β-hydroxylated bile acid, β-muricholic (3α6β.7β-trihydroxy-5β-cholanoic acid), and these are predominantly taurine conjugated, whereas pigs synthesize a 6α-hydroxylated bile acid, hyodeoxycholic (3α,6α-dihydroxy-5β-cholanoic acid) and 6-oxo-lithocholic acid. Such differences need to be considered when working with different species and animal models of disease.

Although there is a tendency to illustrate the reactions in the bile acid synthetic pathway to occur in a linear fashion (Fig. 2-2), moving from initiation of changes to the steroid nucleus through modification of the side chain, in reality there is considerable substrate promiscuity for the 17 enzymes catalyzing the various reactions, which consequently results in vast number of different bile acids and intermediates being synthesized.[6] This is especially evident during early development, a period of physiologic cholestasis,[10,11] and in pathologic conditions that interfere with the integrity of the enterohepatic circulation. Furthermore, intestinal bacterial modifications, resulting in the formation of "secondary" bile acids, add a further level of complexity to the bile acid composition of biologic fluids.[12]

There are two main pathways leading to primary bile acid synthesis.[13-15] These are termed the *neutral* and *acidic* pathways, the former being the classic one that is initiated by the rate-limiting cytochrome P_{450} liver-specific enzyme, cholesterol 7α-hydroxylase (CYP7A1) leading to cholic acid synthesis,[16] and the latter being initiated by the action of cholesterol 27-hydroxylase



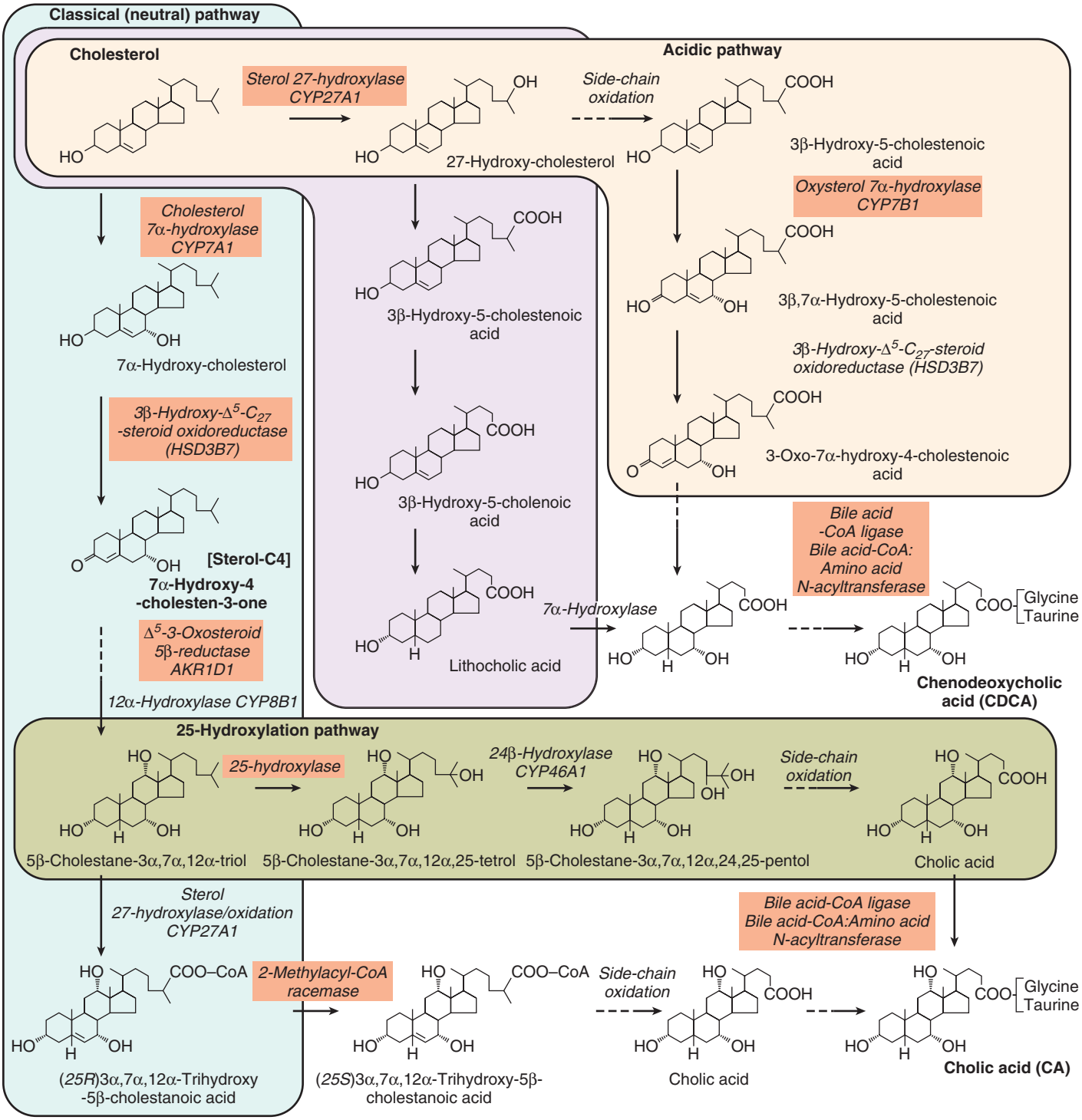
• **Fig. 2-1** The 5β-cholanoic acid nucleus that is the basic structure of C_{24}-bile acids of mammalian species. Shown are the cyclopentanoperhydrophenanthrene (ABCD) rings, the numbering system for the carbon atoms, and the metabolic sites of substitution of functional groups occurring under normal and pathophysiologic conditions. Unsaturation can also occur in the nucleus (mainly at positions Δ^4, Δ^5) and in the side chain. *The smaller font size* signifies the relative quantitative importance of the conjugation reactions.

(CYP27A1) on the side chain to yield chenodeoxycholic acid.[17] This acidic pathway leads to the formation of 3β-hydroxy-5-cholenoic and lithocholic acids as intermediates to chenodeoxycholic acid. These markedly hepatotoxic monohydroxy-bile acids are increased in early life and in cholestatic liver diseases. 27-Hydroxylation occurs in the liver and in many other tissues, including brain, alveolar macrophages, vascular endothelia, and fibroblasts,[18-20] and its extrahepatic role appears related to the cellular regulation of cholesterol homeostasis by its ability to generate oxysterols that are potent repressors of cholesterol synthesis.[21-23] It is now accepted that the acidic pathway contributes significantly to overall total bile acid synthesis, and especially to chenodeoxycholic acid synthesis.[14,21] Normal levels of bile acids are synthesized in mice even when the gene encoding cholesterol 7α-hydroxylase is knocked out, and bile acid synthesis is sustained in rats when cholesterol 7α-hydroxylase is inhibited by continuous infusion of squalestatin.[24] However, *Cyp7a1–/–* mice die within the first few weeks of life from liver failure and the consequences of fat-soluble vitamin malabsorption,[25,26] unless fat-soluble vitamins and cholic acid are fed to these animals immediately after birth.[27] Despite being deficient in cholesterol 7α-hydroxylase, primary bile acid synthesis occurs via the developmental expression of an oxysterol 7α-hydroxylase (CYP7B1) specific to the acidic pathway,[28] and this enzyme is essential in early human life to protect the liver from hepatotoxic monohydroxy-bile acids that are formed as intermediates in this pathway.[29] The aforementioned examples show that primary bile acid synthesis is not exclusively dependent on cholesterol 7α-hydroxylase, and under certain conditions, alternative pathways are induced. For some time it was evident that there were a number of different 7α-hydroxylases.[30-32] This was confirmed by Russell et al. following the isolation and characterization of the oxysterol 7α-hydroxylases, CYP7B1 and CYP39A1.[7,33] CYP7B1 has high activity in human liver and is also found in brain, kidney, and prostate, but its regulation is not fully understood.[34] It has broad substrate specificity, being active on the oxysterols, 27- and 25-hydroxycholesterol,[20,23,30,32] on the bile acids 3β-hydroxy-5-cholenoic and 3β-hydroxy-5-cholestenoic acids,[30] and also on C_{19} steroids.[35] The *CYP7B1* gene is localized to chromosome 8q21.3 and in close proximity to the *CYP7A1* gene. Genetically engineered *Cyp7b1 (–/–)* mice lacking this enzyme have elevated levels of 27- and 25-hydroxycholesterol, but not 24-hydroxycholesterol.[36] Similarly, extremely high levels of 27-hydroxycholesterol and the hepatotoxic monohydroxy bile acids hepatotoxic monohydroxy bile acid 3β-hydroxy-5-cholenoic and 3β-hydroxy-5-cholestenoic acids were found in an infant with a genetic defect in oxysterol 7α-hydroxylase.[29] A mutation in the *CYP7B1* gene will cause a phenotype of progressive and fatal liver disease[29,37,38] and indicates the quantitative importance of the acidic pathway in early human life.

Following the synthesis of 7α-hydroxycholesterol, modifications to the steroid nucleus take place; these result in oxidoreduction and C-12 hydroxylation, consequently preparing the sterol intermediates for direction into either the cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic) or chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic) pathways. According to convention, 7α-hydroxycholesterol is converted to 7α-hydroxy-4-cholesten-3-one, a reaction catalyzed by a microsomal NAD-dependent 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase (HSD3B7) enzyme (C_{27}3β-HSD), formerly referred to as a *3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase*.[39] This enzyme shows substrate specificity toward 7α-hydroxylated sterols and bile acids possessing a 3β-hydroxy-Δ^5 nucleus and is inactive on 7β-hydroxylated analogs. Comparable reactions occur in steroid hormone synthesis; however, the enzyme active on bile acid intermediates is a distinct single enzyme that shows absolute specificity toward C_{27}-sterols,[39] differing from the isozymes active on C_{19} and C_{21} neutral steroids. 3β-Hydroxy-Δ^5-C_{27}-steroid oxidoreductase is not exclusive to the liver but is also expressed in fibroblasts, which enables its activity to be determined in patients with a genetic defect in this enzyme.[40] Mutations in the gene encoding this enzyme are associated with progressive intrahepatic cholestasis,[41,42] and this is often the cause of late-onset chronic cholestasis.

12α-Hydroxylation of the product of the above reaction will direct the Δ^4-3-oxo intermediate into the cholic acid pathway. This reaction is catalyzed by a liver-specific microsomal cytochrome P_{450} 12α-hydroxylase (CYP8B1), which is highly expressed in rabbit and human liver, two species where deoxycholic acid is quantitatively important.[43] When the gene encoding this enzyme is knocked out in mice, there is loss of cholic acid and reduced cholesterol absorption.[7] The primary structures of the rabbit, mouse, and human enzymes have been established by molecular cloning of their cDNAs.[43,44] The activity of 12α-hydroxylase enzyme determines the relative proportion and synthetic rate of cholic relative to chenodeoxycholic acids and appears in humans to be up-regulated by interruption of the enterohepatic circulation and in animals by starvation.[45] It is possible that in utero there may be reduced activity of this enzyme because fetal bile has a predominance of chenodeoxycholic acid.[46] In contrast, the ratio of cholic acid to chenodeoxycholic acid is very high in neonatal bile[47] compared with adult bile.[48] The neonatal period of life is associated with a phase of physiologic cholestasis,[10] which may lead to an up-regulation in 12α-hydroxylase activity with a consequent increase in cholic acid synthesis.

7α-Hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one both undergo reduction with formation of a 3-oxo-5β(H)-structure, and this generates the basic *trans* configuration of the A/B-rings of the steroid nucleus that is common



• **Fig. 2-2** Metabolic pathways leading to the formation of the primary bile acids of cholic and chenodeoxycholic acids from cholesterol. Enzymes catalyzing the individual reactions are indicated by italics. Those enzymes where inborn errors in bile acids synthesis have been identified are highlighted. The intermediate sterol-C4 is highlighted as it provides a surrogate marker for bile acid synthesis. The *broken arrows* indicate multiple steps in the conversion. *Shaded areas* are the classic (neutral) pathway, the acidic pathway, and several alternative pathways.

to the majority of bile acids in most mammalian species. Allo(5α-H)-bile acids[49] are often major bile acid species of lower vertebrates but are found in small proportions in biologic fluids from humans. These are formed by an analogous reaction but catalyzed by a hepatic 5α-reductase. The K_m of 5α-reductase is high, and consequently under normal conditions 5β-reduction is favored. The Δ^4-3-oxosteroid 5β-reductase, a cytosolic aldo-keto reductase (AKR1D1), is a protein of approximately 38 kDa comprising 326 amino acids.[50] It differs significantly in structure from the 5α-reductase and has broad substrate specificity. Its crystal

structure,[51] and the effect of a number of point mutations on the substrate binding sites and enzyme activity, was recently reported.[52,53] Although under normal conditions this enzyme does not appear to be of regulatory importance for bile acid synthesis, its activity parallels the activity of cholesterol 7α-hydroxylase, and therefore measurement of the plasma concentration of 7α-hydroxy-4-cholesten-3-one (often referred to as *C4*, or *sterol C4*, see Fig. 2-2) can be used as an indirect assessment of hepatic cholesterol 7α-hydroxylase activity.[54] The finding of elevated proportions of 3-oxo-Δ^4 bile acids in biologic fluids during early life[55] and in advanced cholestatic liver disease[56] suggests that under pathologic conditions it is this enzyme that becomes rate limiting for bile acid synthesis, rather than cholesterol 7α-hydroxylase. Mutations in the gene encoding AKR1D1 are clinically manifest as progressive intrahepatic cholestasis and biochemically by the production of large amounts of C_{24}-3-oxo-Δ^4-bile acids and allo-bile acids.[52,57-59]

The enzyme catalyzing the conversion of the 3-oxo-5β(H)-sterols to the corresponding 3α-hydroxy-5β(H) intermediates is a soluble 3α-hydroxysteroid dehydrogenase (AKR1C4).[60] This enzyme catalyzes the oxidoreduction of a number of substrates, and several cDNA clones with sequence similarity to other aldo-keto reductases have been described, which suggests the existence of multiple isozymes. This final step in modification of the steroid nucleus results in the formation of the key intermediates, 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol (bile alcohols), which then undergo a sequence of reactions leading to side chain oxidation and consequent shortening by three carbon atoms (see Fig. 2-2).

The initial step in side chain oxidation of the bile alcohols involves hydroxylation of the C-27 carbon atom,[61] a reaction that is catalyzed by a mitochondrial cytochrome P_{450} 27-hydroxylase (CYP27A1)[62] and leads to the formation of 5β-cholestane-3α,7α,12α,27-tetrol. It is now known that CYP27A1 is also responsible for the complete oxidation reaction, which yields directly 3α,7α,12α-trihydroxy-5β-cholestanoic acid.[63] 5β-Cholestane-3α,7α,12α,27-tetrol may also undergo oxidation by the combined actions of soluble or mitochondrial alcohol and aldehyde dehydrogenases, but the relative importance of these reactions compared with the complete 27-hydroxylase-catalyzed reaction is not known.[61] cDNAs encoding the rat, rabbit, and human sterol 27-hydroxylase have been isolated. This enzyme is expressed in many extrahepatic tissues, and its function appears to be important in facilitating the removal of cellular cholesterol. It shows substrate specificity toward many sterols, including cholesterol and vitamin D, and is the same enzyme that catalyzes the formation of 27-hydroxycholesterol, the first step in the acidic pathway. When the sterol 27-hydroxylase gene is disrupted in the mouse,[64] bile acid synthesis is markedly reduced; however, mutations in this gene that cause the rare lipid storage disease of cerebrotendinous xanthomatosis (CTX) have only a modest effect on bile acid synthesis, partly because alternative pathways for bile acid synthesis support the production of compensatory levels of cholic acid.[65,66]

Studies using radiolabeled precursors have shown that 5β-cholestane-3α,7α,12α-triol can be first 25-hydroxylated in the microsomal fraction, then 24β-hydroxylated, and finally oxidized to cholic acid.[13,67] This pathway is specific for cholic acid because little or no hydroxylation of 5β-cholestane-3α,7α-diol has been demonstrated. Based on studies of patients with CTX, it was proposed that the C-25 hydroxylation pathway may be a major pathway for cholic acid synthesis in humans.[68] The quantitative importance of this pathway was later reevaluated in vivo by measuring the production of [^{14}C]acetone after labeling the cholesterol pool with [26-^{14}C]cholesterol.[69] This approach showed that the C-25 hydroxylation pathway accounted for less than 5% of the total bile acids synthesized in healthy adults and less than 2% in adult rats. Hydroxylation of cholesterol also occurs at the C-24 and C-25 positions in addition to the aforementioned cholesterol 27-hydroxylation to yield oxysterols, which are potent repressors of cholesterol synthesis. Cholesterol 24-hydroxylase (CYP46A1) is expressed in the brain to a greater extent than in the liver where it is considered to play a role in cholesterol secretion. In gene knockout mouse models of cholesterol 24- and 25-hydroxylases, bile acid synthesis is unaffected.[7]

The cholestanoic acids are next converted to CoA esters by the action of a bile acid–CoA ligase (synthetase) of which two forms have been identified: one activates newly synthesized C_{27} cholestanoic acids, and the other activates cholanoic acids formed as secondary bile acids returning to the liver for reconjugation.[70] The product of this reaction is the formation of the CoA esters of *(25R)*-3α,7α-dihydroxy-cholestanoic and *(25R)*-3α,7α,12α-trihydroxy-cholestanoic acids. The *(25R)*-diastereoisomers must be racemized to their *(25S)*-forms in order to penetrate the peroxisome for subsequent oxidation. This reaction is catalyzed by a 2-methylacyl coenzyme A racemase enzyme, the same enzyme that is also active on branched-chain fatty acids such as phytanic acids. A mutation in the gene encoding this enzyme leads to the accumulation of *(25R)*-cholestanoic acids and phytanic acids and presents with neurologic and liver disease.[71,72]

The final stage in modification of the side chain involves the β-oxidation of the cholestanoic acids, which occurs by a multiple-step reaction within peroxisomes.[61] The sequence of these reactions is analogous to the β-oxidation of fatty acids. The CoA esters of the cholestanoic acid are acted on by a specific peroxisomal acyl-CoA oxidase (ACOX2). This reaction is rate limiting, and the enzyme has been partially purified from rat liver and found to differ from the analogous acyl-CoA oxidase (ACOX1) utilizing fatty acids as substrates.[73] The situation in humans is somewhat different in that a single peroxisomal oxidase acts on both branched-chain fatty acids and bile acid intermediates.[74] Formation of a C-24 hydroxylated derivative occurs by the action of a bifunctional enoyl-CoA hydratase/β-hydroxyacyl-CoA dehydrogenase, a reaction that goes through a Δ^{24}-intermediate. Photoaffinity labeling experiments have shown that this enzyme is the same one that is involved in the peroxisomal β-oxidation of fatty acids. The dehydrogenase activity of the bifunctional enzyme yields a 24-oxo derivative that, following thiolytic cleavage by peroxisomal thiolase 2, releases three carbon atoms in the form of propionic acid.[75] This results in the formation of the C_{24} bile acid CoA end product. With the exception of the acyl-CoA oxidase, defects in any of the other enzymes responsible for the β-oxidation of very-long-chain fatty acids (VLCFAs) exhibit abnormalities in primary bile acid biosynthesis.[6]

Some mention of allo (5α-reduced)-bile acids is warranted even though under physiologic conditions they account for a relatively small proportion of the total bile acids in human biologic fluids. These are major bile acid species of many lower vertebrates.[9,49] In humans, 5α-reduced bile acids are usually formed by the action of intestinal microflora on 3-oxo-5β-bile acids during their enterohepatic circulation and consequently are found in significant amounts in feces.[12] In rodents, these 5α-reduced bile acids can be formed in the liver from 5α-cholestanol.[76] This pathway begins with 7α-hydroxylation of 5α-cholestanol and the product is then converted to 5α-cholestane-3α, 7α-diol

via the intermediate 7α-hydroxy-5α-cholestan-3-one. Hepatic 12α-hydroxylation of 5α-sterols is very efficient in the rat and readily leads to formation of allo-cholic acid.[76,77] A further pathway for allo-bile acid formation involves the hepatic 5α-reduction of 7α-hydroxy- and 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acids, a reaction catalyzed by a Δ^4-3-oxosteroid 5α-reductase, and the finding of large proportions of allo-bile acids in infants with severe cholestatic liver disease due to a AKR1D1 deficiency indicates these to be primary bile acids of hepatic origin in humans.[57] Both 5α-reductase isozymes are expressed in the liver beginning from birth.[78]

A striking feature of bile acid synthesis and metabolism during early life is the relatively large proportion of polyhydroxylated, unsaturated, and oxo-bile acids that are synthesized and not typically found in the biologic fluids of healthy adults.[46,79] Although frequently referred to as *atypical*, this moniker is a misnomer because they are in fact very typical of the developmental phase of hepatic metabolism. Interestingly, the qualitative and quantitative bile acid composition of biologic fluids in early life closely resembles that of adults with severe cholestatic liver disease, suggesting that in the diseased liver there is a reversion to more primitive pathways of synthesis and metabolism.[46,79] The most notable distinction in ontogeny is the prevalence of cytochrome P450 hydroxylation pathways[33] that rapidly decline in importance over the first year of life. The most important hydroxylation reactions are 1β-, 4β-, and 6α-hydroxylation that are of hepatic origin.[80-82] The concentrations of several of the metabolites, in particular hyocholic (3α,6α,7α-trihydroxy-5β-cholanoic) and 3α,4β,7α-trihydroxy-5β-cholanoic acids, exceed that of cholic acid in fetal bile.[46] The role of these hydroxylation pathways is uncertain, but additional hydroxylation of the bile acid nucleus will increase the polarity of the bile acid and facilitate its renal clearance, while also decreasing its membrane-damaging potential. In early life, and particularly in the fetus, an immaturity in canalicular and ileal bile acid transport processes leads to a sluggish enterohepatic circulation[11] and hydroxylation serves as a hepatoprotective mechanism.

Bile Acid Conjugation

Irrespective of the pathway by which cholic and chenodeoxycholic acids are synthesized, the CoA thioesters of these primary bile acids are ultimately conjugated to the amino acids glycine and taurine.[48] This two-step reaction is catalyzed by a rate-limiting bile acid-CoA ligase enzyme[83-85] followed by a bile acid CoA:amino acid N-acyltransferase (EC 2.3.1.65).[86,87] The genes encoding both enzymes, *SLC27A5* and *BAAT,* were cloned decades ago.[88,89] The conjugation reaction was originally believed to take place in the cytosol, but the highest activity of conjugating enzymes was later found to be in peroxisomes.[90,91]

Genetic defects in the bile acid amidation have been associated with fat-soluble vitamin malabsorption states with variable degrees of liver disease.[92-95] Recently, we identified and treated five patients (one male, four females) from four families with defective bile acid amidation caused by a genetically confirmed deficiency in *BAAT* with the conjugated bile acid, glycocholic acid.[96] The bile acid CoA:amino acid N-acyltransferase enzyme utilizes glycine, taurine, and interestingly β-fluoroalanine, but not alanine, as substrates.[86] It will also conjugate VLCFAs to glycine. The specificity of the enzyme has been examined in detail and found to be influenced by the length of the side chain of the bile acid; bile acids having a four-carbon-atom side chain, that is, nor(C_{23})–bile acids[97] and homo(C_{25})–bile acids, are both poor substrates for amidation.[87,97,98] In contrast, cholestanoic (C_{27}) acids are predominantly taurine conjugated. Significant species differences in substrate specificity are observed and should be considered when working with animal models. The human bile acid-CoA:amino acid N-acyltransferase conjugates cholic acid with both glycine and taurine; whereas the mouse enzyme shows selectivity only toward taurine. This is consistent with the mouse being an obligate taurine conjugator of bile acids, as is the rat and the dog. In humans, the final products of this complex multistep pathway are the two conjugated primary bile acids of cholic and chenodeoxycholic acids (see Fig. 2-2), and these are then secreted in canalicular bile and stored in gallbladder bile. In humans, glycine conjugation predominates with a ratio of glycine to taurine conjugates of 3.1 : 1 for normal adults.[48] In early human life, more than 80% of the bile acids in bile are taurine conjugated due to the abundance of hepatic stores of taurine.[46]

Although the principal bile acids of humans and most mammalian species are amidated, other conjugates occur naturally and these include sulfates,[99] glucuronide ethers and esters,[100,101] glucosides,[102] N-acetylglucosaminides,[103] and conjugates of some drugs.[104] These conjugates account for a relatively large proportion of the total urinary bile acids. Conjugation significantly alters the physicochemical characteristics of the bile acid,[94,105] and it serves an important function by increasing the polarity of the molecule, thereby facilitating its renal excretion, and by minimizing the membrane-damaging potential of the more hydrophobic unconjugated species.[106] Under physiologic conditions, these alternative conjugation pathways are quantitatively less important. However, in cholestatic liver disease, or when the liver is subjected to a bile acid load, as in ursodeoxycholic acid (UDCA) therapy, the concentrations of these conjugates in biologic fluids may change. Detailed knowledge of these metabolic pathways is limited, but it is evident that there is significant localization of bile acid-conjugating enzymes in the kidneys.[107]

Sulfation of bile acids, most commonly at the C-3 position but also at C-7, is catalyzed by a bile acid sulfotransferase,[108,109] an enzyme that in the rat, not in the human, exhibits sex-dependent differences in activity. Although much has been written about the potential importance of sulfation in early life, it is evident from the finding of a relatively small proportion of bile acid sulfates in fetal bile that hepatic sulfation is negligible in the fetus and neonate.[46] Indeed, it is most probable that urinary bile acid sulfates originate mainly by renal sulfation[107]; 60% to 80% of urinary bile acids are sulfated and their excretion increases in cholestasis. Only traces of bile acid sulfates are found in bile despite efficient canalicular transport of perfused bile acid sulfates.

A number of glucuronosyltransferases catalyze the formation of glucuronide ethers and esters.[100,101] The enzymes show substrate selectivity in that bile acids possessing a 6α-hydroxyl group are preferentially conjugated at the C-6 position forming 6-O-ether glucuronides,[101] whereas short-chain bile acids form mainly glucuronides.[100] Purification of the hyodeoxycholic acid-specific human UDP-glucuronosyltransferase and subsequent cloning of a cDNA[110] indicate that this enzyme is highly specific toward hyodeoxycholic (3α,6α-dihydroxy-5β-cholanoic) acid; no glucuronidation of hyocholic (3α,6α,7α-trihydroxy-5β-cholanoic) acid could be detected. It is probable that there is a family of isozymes that catalyze the glucuronidation of different bile acids.

Glucosides and N-acetylglucosaminides of nonamidated and amidated bile acids have been identified in normal human urine[111] with quantitative excretion comparable to that of glucuronide

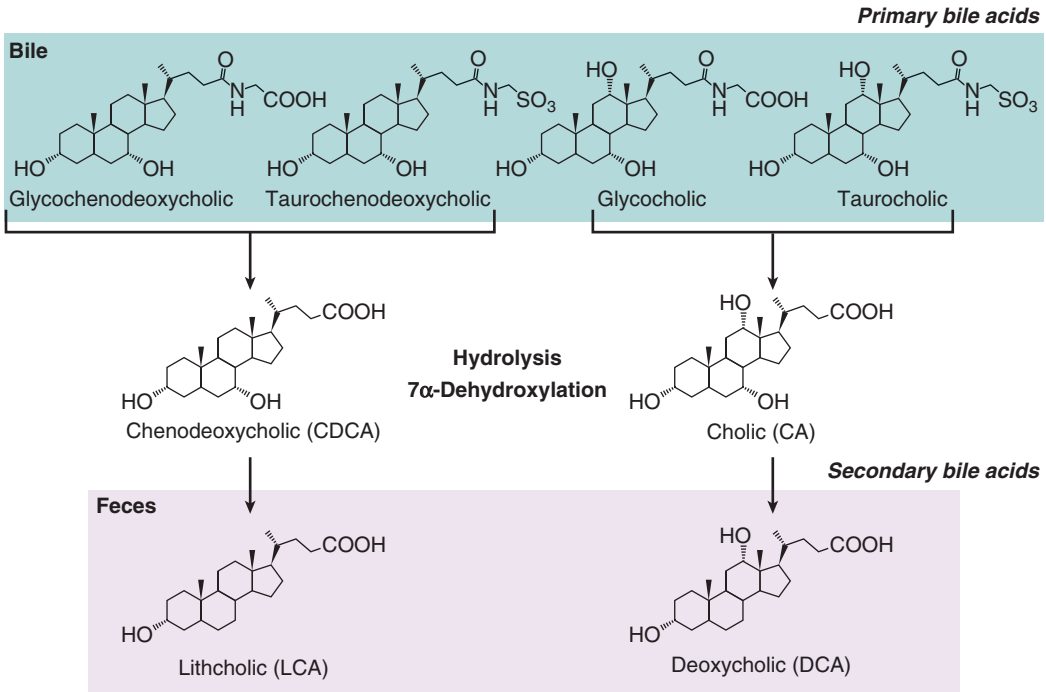
conjugates.[112] A microsomal glucosyltransferase has been isolated and purified from human liver[102] but is also present in extrahepatic tissues. The enzyme responsible for N-acetylglucosaminide formation exhibits remarkable substrate specificity in that it preferentially catalyzes the conjugation of bile acids having a 7β-hydroxyl group and consequently these conjugates account for more than 20% of the urinary metabolites of patients administered ursodeoxycholic acid.[113] Finally, the full extent to which drugs may compete for the conjugating enzymes is not known, although bile acid conjugates of 5-fluorouracil have been identified. The 2-fluoro-β-alanine conjugate of cholic acid was found to be a major metabolite in bile following administration of this therapeutic agent.[104]

Formation of Secondary Bile Acids

Intestinal microflora play an important role in bile acid synthesis and metabolism. Bacterial enzymes metabolize primary bile acids, altering significantly their physicochemical characteristics and influencing their physiologic actions during enterohepatic recycling. The result is the formation of a spectrum of secondary bile acids that are mainly excreted in feces[12] (Fig. 2-3). Deconjugation of conjugated bile acids, followed by 7α-dehydroxylation, are quantitatively the most important reactions, but bacterial oxidoreduction and epimerization at various positions of the bile acid nucleus also take place along the intestinal tract.[114] This is evident from bile acid profiles along the entire length of the human intestine obtained at autopsy that show relatively high proportions of secondary bile acids in the proximal jejunum, mid-small bowel, ileum, and cecum.[115,116] The enzymes that catalyze these reactions are found in a variety of organisms, such as *Bacteroides, Clostridia, Bifidobacteria, Escherichia coli,* and some of these reactions occur in the proximal small intestine.[117,118] Deconjugation precedes 7α-dehydroxylation, and the bacterial peptidases responsible for this reaction exhibit remarkable substrate specificity in that the length of the side chain is a crucial factor influencing this reaction. The enzyme kinetics, factors influencing the reactions, and molecular biology of a number of the bacterial enzymes have been extensively studied by Stellwag et al[118a] and Coleman et al.[118b] 7α-Dehydroxylation of cholic and chenodeoxycholic acids, a reaction that proceeds via a 3-oxo-Δ^4-intermediate, results in the formation of deoxycholic and lithocholic acids, respectively, and these secondary bile acids make up the largest proportion of total fecal bile acids.[12] Lithocholic and deoxycholic acids are relatively insoluble and consequently poorly absorbed. However, both bile acids are returned to the liver to regulate bile acid synthesis. It should be noted that in rats, deoxycholic acid is very efficiently 7α-hydroxylated in the liver and converted back to cholic acid, but this reaction does not take place in humans. Serum concentrations of deoxycholic acid therefore provide a useful means of assessing the extent of impairment of the enterohepatic circulation in cholestatic liver diseases.[119] Recent interest in gut dysbiosis with respect to gastrointestinal and systemic disease has further linked the dysmetabolism of bile acids to changes in the so-called gut-microbiome.[120,121]

Regulation of Bile Acid Synthesis

The major factor influencing bile acid synthesis is negative feedback by bile acids returning to the liver via the portal vein during their enterohepatic recycling. There are marked differences in the ability of different bile acids to regulate cholesterol 7α-hydroxylase.[122] For example, whereas the primary bile acids cholic and chenodeoxycholic acids down-regulate synthesis, bile acids possessing a 7β-hydroxy group, such as ursodeoxycholic acid, do not, and the latter may actually increase synthesis rates. This observation has relevance to the treatment of inborn errors of bile acid synthesis. Interruption of the enterohepatic circulation



• **Fig. 2-3** The main reactions of intestinal microbiome in the metabolism of bile acids. Conversion of primary bile acids secreted in bile into secondary bile acids excreted in feces.

by biliary diversion[123] or the feeding of anion exchange resins that bind bile acids in the intestinal lumen[124] results in an up-regulation of cholesterol 7α-hydroxylase activity. In general, factors that influence cholesterol 7α-hydroxylase activity cause concomitant changes in the activity of HMG-CoA reductase, the rate-limiting enzyme for cholesterol synthesis, and this serves to regulate cholesterol synthesis and maintain a constant cholesterol pool size. Interestingly, cholesterol 7α-hydroxylase exhibits a diurnal rhythm that is synchronous with the activity of HMG-CoA reductase and is reflected by diurnal changes in bile acid synthesis rates.[125] A significant nocturnal rise in bile acid synthesis takes place that may be regulated by glucocorticoids because this regulation can be abolished by adrenalectomy or hypophysectomy.

The mechanism involved in regulating cholesterol 7α-hydroxylase activity and therefore bile acid synthesis is complex and mediated through an ever-increasing discovery of nuclear receptors and transcription factors that have specificity for bile acids and oxysterols.[1,7,126-128] Bile acids have been shown to enter the nucleus of the hepatocyte, and nuclear concentrations increase with bile acid feeding.[129] These nuclear receptors include the farnesoid X receptor (FXR, NHR1H4), short heterodimer partner (SHP, NR0B2), liver receptor homolog-1 (LRH-1, NR5A2), hepatocyte nuclear factor 4α (HNF-4α), liver X receptor α (LXRα, NR1H3), pregnane X receptor (PXR, NR112), constitutive androgen receptor (CAR, NR13), and fibroblast growth factor-19 (FGF19) and its receptor FGFR4,[130] and the G protein–coupled receptor TGR5.[131] Much has been learned about the regulation of cholesterol and bile acid synthesis from gene knockout models of these nuclear receptors. For instance we now understand that a novel enterocyte protein, Diet1, is a regulator of FGF19 production at the posttranscriptional level. The Diet1 and Fgf15 (the mouse homologue of FGF19) appear to have overlapping subcellular localization in murine enterocytes. Diet1-deficient mice constitutively convert cholesterol to bile acids and are resistant to diet-induced hypercholesterolemia and atherosclerosis. Thus, Diet1 appears to be a control point for the production of FGF15/19 in enterocytes and a key regulator of bile acid and lipid homeostasis.[132] New bile acid molecules have been recently synthesized as specific agonists for these receptors in order to devise ways of regulating cholesterol homeostasis and glucose metabolism, and these are now in clinical trials.[133] In addition to influencing the transcriptional regulation of cholesterol 7α-hydroxylase in the liver, these receptors also induce transcription of IBABP, the ileal bile acid binding protein that is involved in the ileal uptake and conservation of the bile acid pool.

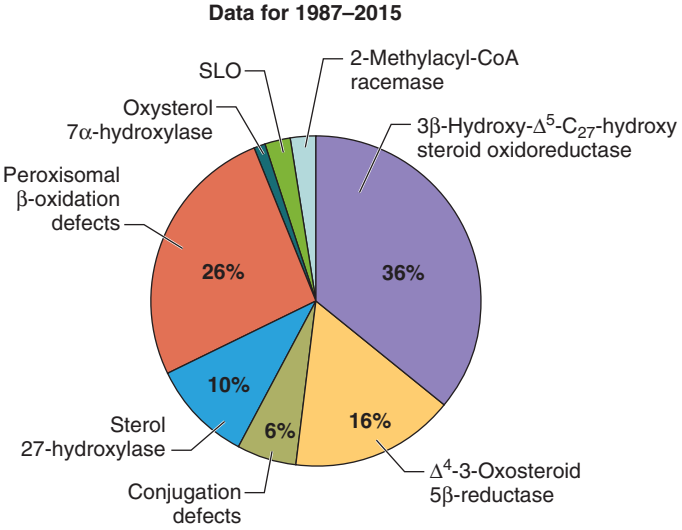
Defects in Bile Acid Synthesis Causing Metabolic Liver Disease and Syndromes of Fat-Soluble Vitamin Malabsorption

Defects in bile acid synthesis have profound effects on hepatic and gastrointestinal function and on cholesterol homeostasis, especially when the cause is a genetic mutation encoding the enzymes responsible for primary bile acid synthesis. Such defects lead to an overproduction of hepatotoxic atypical bile acids that are synthesized from intermediates accumulating in the pathway proximal to the inactive enzyme and a progressive cholestasis exacerbated by the lack of primary bile acids that are critical for promoting bile flow. Marked alterations in urinary, serum, and biliary bile acids are found in all infants and children with liver disease, and it can be difficult to determine whether such changes are primary or secondary to the liver dysfunction. The first bile acid synthetic defect causing liver disease was discovered as a result of applying the liquid secondary ionization mass spectrometry (LSIMS) technique of fast atom bombardment ionization mass spectrometry (FAB-MS).[134] This permitted the direct analysis of bile acids from a small drop of urine. Whereas FAB-MS is still a definitive technique for diagnosing bile acid synthetic defects, newer mass spectrometric approaches have since been used, including electrospray ionization tandem mass spectrometry[135,136] and gene sequencing techniques. However, mass spectrometry continues to offer the fastest and most accurate method of screening for these disorders because the mass spectra generated permit accurate identification of the lack of primary bile acids and presence of atypical bile acids specific to each defect.[134] To date, nine defects in bile acid synthesis have been described[i] (see Fig. 2-2), and all have a highly variable phenotypic expression of familial and progressive infantile or late-onset cholestasis, of syndromes of fat-soluble vitamin malabsorption, and of variable degrees of neurologic involvement (Fig. 2-4). An international screening program at Cincinnati Children's Hospital Medical Center found bile acid synthesis defects to account for 2% of 13,500 screened cases of idiopathic cholestatic liver disease in infants and children. Broadly, these defects can be categorized as deficiencies in the activity of enzymes responsible for catalyzing reactions to the steroid nucleus or to the side chain.

Defects Involving Reactions to the Steroid Nucleus

Three defects involving enzyme-catalyzing reactions that modify the ring structure of the steroid nucleus have been identified. Two reports of a 12α-hydroxylase (CYP8B1) defect have been proposed, but neither has been definitively confirmed.[139] The clinical presentations of the 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase (HSD3B7), Δ^5-3-oxosteroid 5β-reductase (AKR1D1), and

[i]See references 29, 57, 66, 72, 93, 95, 137, 138.

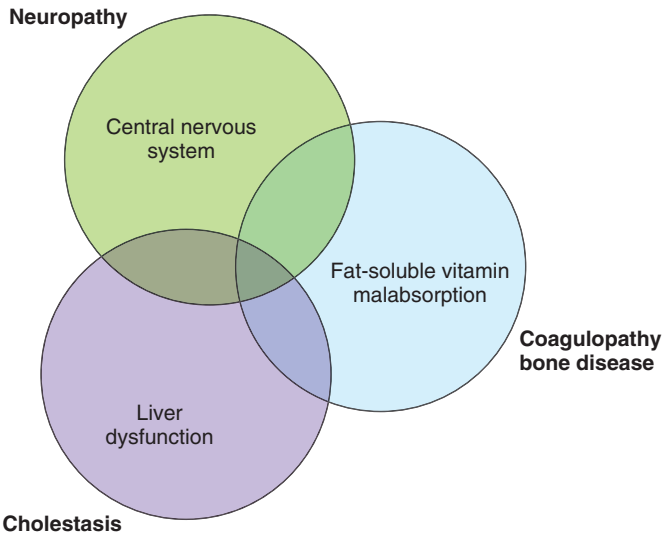


• **Fig. 2-4** The relative distribution of the different bile acid synthesis disorders identified in urine samples from 13,500 patients with unexplained liver disease screened at Cincinnati Children's Hospital Medical Center between 1987 and 2015.

oxysterol 7α-hydroxylase (CYP7B1) deficiencies are of progressive cholestatic liver disease. Typical biochemical abnormalities include elevations in serum liver enzymes, conjugated hyperbilirubinemia, and evidence of fat-soluble vitamin malabsorption. A normal γ-glutamyltranspeptidase (GGT) is highly associated with, although not an exclusive feature of, all of the bile acid synthetic defects. Serum cholesterol concentrations are generally normal. The early clinical history of these patients often shows fat-soluble vitamin malabsorption, and in some cases rickets precedes any evidence of liver dysfunction.[140] These abnormalities are usually responsive to oral vitamin supplementation, but these patients eventually present later in life with hepatosplenomegaly and elevated serum liver enzymes. The 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase deficiency is the most common of the bile acid synthetic defects, frequently accounting for cases of late-onset chronic cholestasis (Fig. 2-5).

Cholesterol 7α-Hydroxylase Deficiency

Although not presenting as cholestatic liver disease, a deficiency in CYP7A1 was found to be responsible for hypertriglyceridemia and gallstone disease in three related adults.[138] This finding followed a screening of the *CYP7A1* gene for mutations in patients presenting with elevated low-density lipoprotein (LDL)-cholesterol who were resistant to HMG-CoA reductase inhibitors. A 2-bp deletion (1302-1303delTT) was observed in exon 6 of the gene, resulting in a frameshift mutation and causing a Leu→Arg substitution that, when transfected into HEK 293 cells, led to an inactive protein product. All three patients were homozygous for this mutation and had serum cholesterol concentrations greater than 300 mg/dL, LDL-cholesterol greater than 180 mg/dL, and elevated triglycerides.[138] The heterozygous relatives of the two patients described also had elevated cholesterol levels. There was no evidence for cholestasis, fibrosis, or inflammation, but fatty changes in the liver were reported following biopsy. Fecal bile acid analysis revealed markedly reduced total bile acid output (6% of normal) and a high [chenodeoxycholic + lithocholic]/[cholic + deoxycholic] acid ratio, consistent with preferential synthesis of chenodeoxycholic acid via the acidic pathway for bile acid synthesis. This clinical phenotype differs from that of the *CYP7A1*−/− mouse knockout model, which has normal cholesterol levels.[27]



• **Fig. 2-5** The broad spectrum of overlapping clinical features of patients with bile acid synthesis disorders.

Oxysterol 7α-Hydroxylase Deficiency

The discovery of a genetic defect in CYP7B1[29] emphasizes the quantitative importance of the acidic pathway for bile acid synthesis in early life. Unlike the mouse, where this enzyme appears to be developmentally regulated,[25] or the rat, where it is induced when there is suppression in CYP7A1 activity,[24] it appears that in the neonatal period of humans CYP7B1 may be more important than CYP7A1 for bile acid synthesis. This genetic defect presents as severe progressive cholestatic liver disease. It was first described in a 10-week-old boy of parents who were first cousins[29] and has more recently been identified in three patients in early infancy.[37,38,141]

The index patient had severe cholestasis, cirrhosis, and liver synthetic failure from early infancy. He had progressive jaundice by 8 weeks of age, hepatosplenomegaly, and markedly elevated serum transaminases, but normal serum GGT. The liver biopsy revealed cholestasis, bridging fibrosis, extensive giant-cell transformation, and bile duct proliferation. A similar clinical picture and liver histology were reported for a 5-month-old Taiwanese infant[37] and a 6-month-old Japanese infant.[38] Oral UDCA therapy proved ineffective or led to deterioration in liver function tests in these patients. Oral cholic acid therapy was also ineffective in the index case, and that patient underwent orthotopic liver transplantation at 4½ months of age but died 3 weeks later from disseminated Epstein-Barr virus–related lymphoproliferative disease. The Taiwanese infant died at 11 months of age before transplantation could be performed, but the Japanese infant underwent living donor transplantation with a graft from the mother who had a compound heterozygous mutation (R112X/R417C) in the *CYP7B1* gene[38] and was reportedly still alive 2 years after transplantation. These examples highlight the severity of this bile acid synthetic defect. It is possible that this cause of idiopathic liver disease may go unrecognized due to its rapid downhill course in the early months of life. The therapeutic strategy for patients with this defect should in the future target down-regulation of sterol 27-hydroxylase. Recently an infant with CYP7B1 deficiency was reported to have worsened when given UDCA, consistent with previous findings, but showed clinical improvement when treated with oral chenodeoxycholic acid (CDCA) (10 to 15 mg/kg of body weight/day).[141]

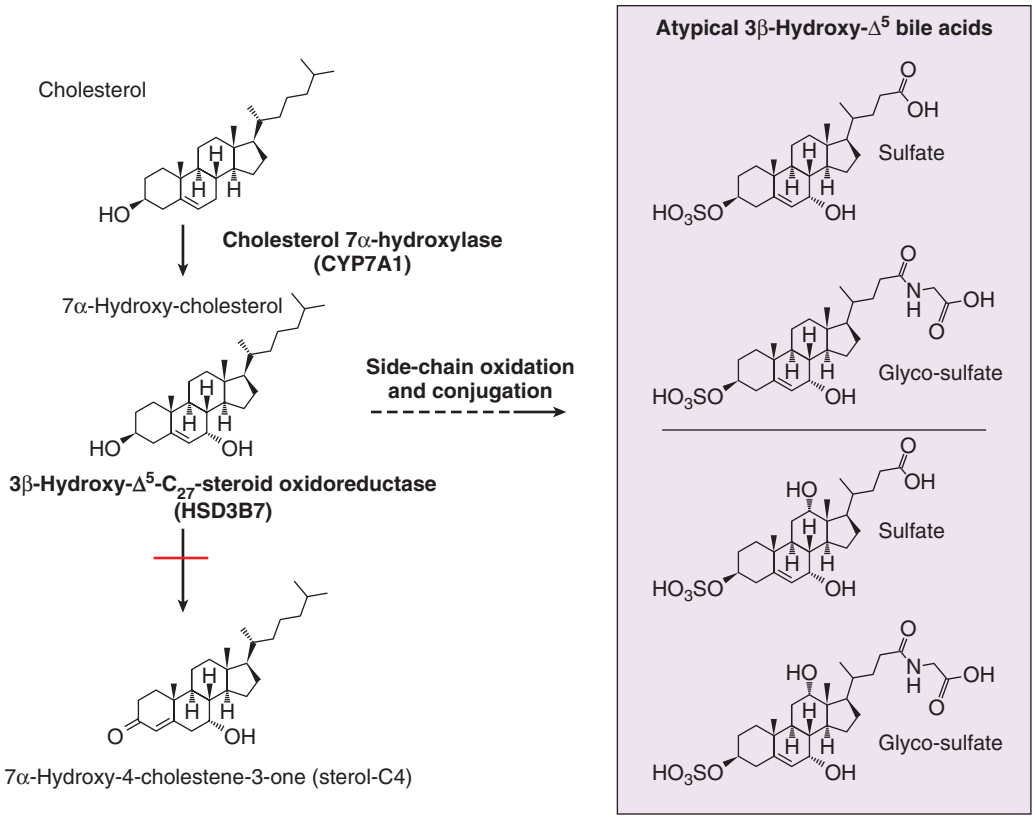
All these cases were diagnosed by mass spectrometry. FAB-MS analysis of the urine from the index case revealed an absence of primary bile acids and in their place large concentrations of unsaturated monohydroxy-C_{24} bile acids as sulfate and glycosulfate conjugates.[29] Gas chromatography-mass spectrometry (GC-MS) confirmed that these atypical bile acids were the unsaturated monohydroxy-bile acids, 3β-hydroxy-5-cholenoic and 3β-hydroxy-5-cholestenoic acids, which accounted for 97% and 86%, respectively, of the total serum and urinary bile acids. Additionally, 27-hydroxycholesterol concentrations in serum and urine were greater than 4500 times normal and no 7α-hydroxylated sterols were detected.[29] Similar GC-MS profiles were reported for the two Asian infants. The formation of 3β-hydroxy-5-cholenoic and 3β-hydroxy-5-cholestenoic acids occurs exclusively via the acidic pathway, and the mass spectrometry findings definitively establish a CYP7B1 deficiency while illustrating how important the acidic pathway is for bile acid synthesis in early life. Monohydroxy-bile acids with the 3β-hydroxy-Δ^5 structure and oxysterols are good ligands for FXR, which would suppress CYP7A1 preventing bile

acids being synthesized by the classic pathway. In the original case, molecular studies of the liver tissue confirmed the *CYP7A1* gene to be normal but there was no measurable enzyme activity or mRNA.[29] Furthermore, these unsaturated bile acids are extremely cholestatic[142] and the hepatotoxicity in these patients would be exacerbated by the lack of primary bile acids necessary to maintain bile flow. Oxysterol 7α-hydroxylase is essential for protecting the liver from hepatotoxic and cholestatic 3β-hydroxy-Δ^5 monohydroxy bile acids that otherwise would accumulate in the acidic pathway (see Fig. 2-2).

Molecular studies on liver tissue from the first patient showed no CYP7B1 activity or mRNA, and gene sequencing revealed a C to T transition mutation at position 388 in exon 5, providing unambiguous confirmation of the genetic defect in the oxysterol 7α-hydroxylase.[29] This patient was homozygous for this nonsense mutation, whereas both parents were heterozygous. When human embryonic 293 or Chinese hamster ovary cells were transfected with cDNA having the R388* mutation, there was no detectable CYP7B1 activity, and immunoblot analysis confirmed that the mutated gene encoded a truncated protein unable to catalyze 7α-hydroxylation.[29] In the Taiwanese infant a single substitution of C to T at position 538 of exon 3 of the *CYP7B1* gene that caused an amino acid transition from arginine to a stop codon at position 112 (R112->Stop) was identified,[37] whereas the Japanese infant had a compound heterozygous mutation (R112X/R417C) in exons 3 and 6 of the *CYP7B1* gene.[38] All patients were homozygous for their respective mutations and the parents were heterozygous.

3β-Hydroxy-Δ^5 C_{27}-Steroid Oxidoreductase Deficiency

This is the most common of the bile acid disorders and involves the second step in pathway—the conversion of 7α-hydroxycholesterol into 7α-hydroxy-4-cholesten-3-one, a reaction catalyzed by a microsomal 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase.[39] A deficiency of this sterol-specific enzyme[137] results in the accumulation of 7α-hydroxycholesterol within the hepatocyte. The other enzymes involved in bile acid synthesis catalyze the remaining transformations, including side chain oxidation so that in place of the normal primary bile acids, C_{24}-bile acids are synthesized retaining the 3β-hydroxy-Δ^5- structure characteristic of the substrate for the enzyme (Fig. 2-6). This defect was first described in a Saudi Arabian patient, the third infant of five to be affected by progressive idiopathic neonatal cholestasis; the two previous infants had died following a similar clinical history and were products of a consanguineous marriage.[137] All of the affected infants had progressive jaundice, elevated transaminases, and conjugated hyperbilirubinemia, and this generalized clinical presentation is common to all cases thus far recognized.[143-146] Upon clinical examination, patients with a 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase deficiency usually present with hepatomegaly, fat-soluble vitamin malabsorption, and mild steatorrhea. Pruritus is usually not a symptom. This inborn error is highly associated with elevated serum bilirubin and transaminases and a normal GGT concentration and this biochemical presentation is a useful clinical marker for a suspected defect. Furthermore, serum bile acid concentrations, if measured by enzymatic or immunoassay methods, can be expected to be normal and seemingly



• **Fig. 2-6** The metabolic pathway for primary bile acid synthesis from cholesterol depicting the biochemical presentation of a deficiency in 3β-hydroxy-Δ^5-C27-steroid oxidoreductase (HSD3B7), which presents in patients as early- or late-onset chronic cholestasis.

incompatible with the extent of cholestasis. Therefore, inclusion of serum bile acid determination in the clinical evaluation may provide a further clue to this defect. Histologic examination of these patients' livers shows hepatitis with the presence of giant cells and is consistent with cholestasis as evidenced by canalicular plugs, bile stasis, and inflammatory changes.[137,144,146-149]

As with most of the inborn errors involving the reactions responsible for nuclear modification, the 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase deficiency is progressive and familial in nature and is fatal if untreated. Age at onset and diagnosis is variable, ranging from 3 months to the adult years. Recently it was diagnosed in a 24-year-old woman with cirrhosis of unknown etiology; remarkably, her sister and a first cousin had died of cirrhosis at ages 19 and 6 years, respectively, and another 32-year-old first cousin was also affected.[150] Homozygosity mapping was used to identify a mutation in the *HSD3B7* gene, which established the diagnosis of a 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase deficiency as the cause of liver failure. This was subsequently confirmed by FAB-MS analysis of the serum from the living 32-year-old and deceased 24-year-old family members.[150] These cases indicate that a bile acid synthetic defect should be considered in cases of late-onset chronic cholestasis.[134]

Diagnosis of the 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase deficiency can be definitively established by FAB-MS or electrospray ionization mass spectrometry (EMI-MS) analysis of the urine,[134,136] which reveals an absence of the normal glycine- and taurine-conjugated primary bile acids and, instead, the presence of the sulfate and glyco-sulfate conjugates 3β,7α-dihydroxy- and 3β,7α,12α-trihydroxy-5-cholenoic acids (see Fig. 2-6). These atypical bile acids are recognized by their respective negative ions of m/z 469, 485, 526, and 542.[134] Some differences are observed between FAB-MS and ESI-MS mass spectra as evident from a report of a 26-year-old patient with a genetically confirmed mutation in the *HSD3B7* gene that showed in the urine a single dominant ion at m/z 462 by ESI-MS and an absence of the ions at m/z 469, 485, 526, and 542 typically observed with FAB-MS.[145] When the same urine sample was later analyzed by FAB-MS, this ion was of minor intensity and the characteristic ions at m/z 469, 485, 526, and 542 served to identify this defect (K. Setchell, unpublished observations). Such difference in ionization between these mass spectrometric techniques requires consideration to avoid misdiagnosis. Tetrahydroxy- and pentahydroxy-bile alcohols with a 3β,7α-dihydroxy-Δ^5 and 3β,7α,12α-trihydroxy-Δ^5 nucleus are also found in greatly increased amounts in urine, plasma, and bile.[151] These bile alcohols are mainly sulfated, in contrast to the glucuronide conjugates of saturated bile alcohols observed in CTX.[134,152] Although primary bile acids are not detectable in the urine, the bile may contain small proportions of cholic acid resulting from intestinal bacterial metabolism of the 3β-hydroxy-Δ^5 bile acids during enterohepatic recycling. It is possible that this may facilitate bile secretion and explain the delay in onset of cholestasis and longer survival of these patients.

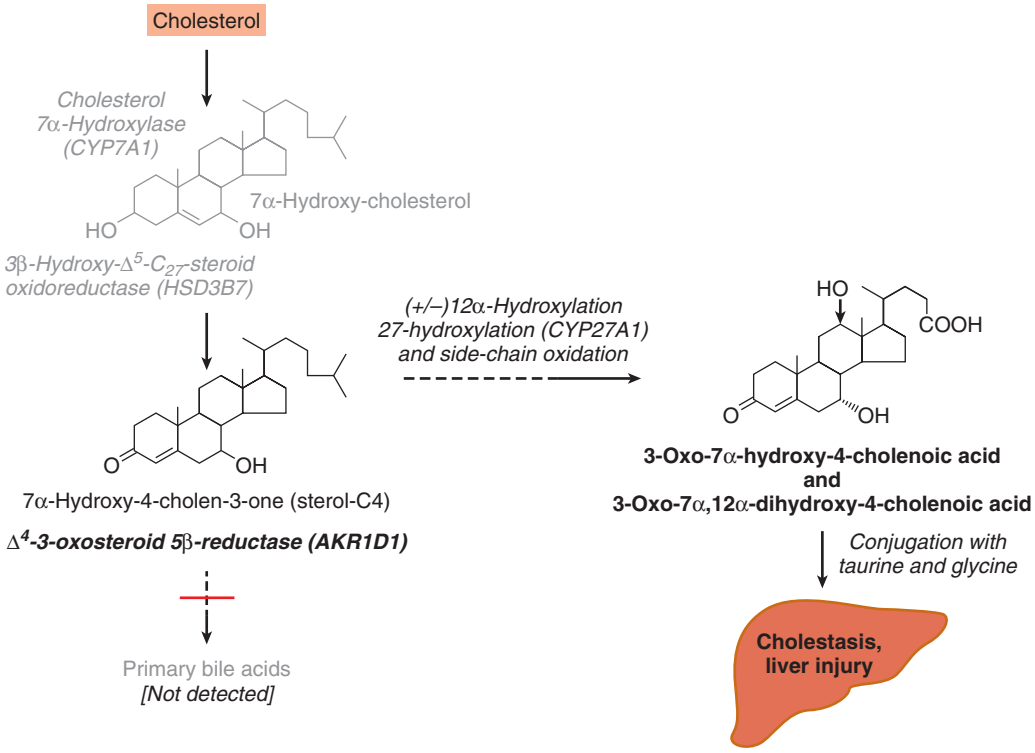
3β-Hydroxy-Δ^5-C_{27}-steroid oxidoreductase is expressed in fibroblasts, which means its activity can be measured in cultured fibroblasts using 7α-hydroxy-cholesterol as substrate. In contrast to healthy control subjects, patients with this defect have no detectable enzyme activity in fibroblasts[40,136] and the parents have a low but measurable activity consistent with a heterozygous phenotype. Sequencing of the *HSD3B7* gene localized to chromosome 16p11.2-12 has failed to find a common mutation for this disorder. In 16 patients, 12 different mutations were identified, including point mutations, small insertions, and deletions,[42] illustrating the diverse nature of the genetics. In an earlier report, a 2-bp deletion in exon 6 accounted for the inactivity of this enzyme in the index case with this bile acid defect.[41] In four cases, mutations were compound heterozygous, but most were inherited in homozygous form. When several of the identified mutations were expressed in HEK 293 cells, impaired synthesis of the normal protein could be demonstrated and this lacked enzyme activity.[42]

The mechanism of liver injury is considered to be by the accumulation of atypical bile acids concomitant with a lack of primary bile acids. In animal models, 3β-hydroxy-5-cholenoic acid produces cholestasis,[142] but this is not the case for 3β,7α-dihydroxy-5-cholenoic acid, which is rapidly metabolized to chenodeoxycholic acid in animals. This conversion does not occur in patients lacking the 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase enzyme. Studies using rat liver canalicular membrane vesicles have shown tauro-3β,7α-dihydroxy-5-cholenoic acid to be markedly cholestatic.[153]

Oral administration of the primary bile acid, cholic acid (5-15 mg/kg of body weight/day), is now the therapeutic approach for these patients and is expected to resolve the biochemical and histologic abnormalities and to improve growth.[134,146] Chenodeoxycholic acid has also been effective,[143,148,154,155] but this bile acid is more cathartic and may cause loose stools or even diarrhea in young infants. In some cases patients have been maintained temporarily on UDCA,[143,146] which is choleretic but does not inhibit bile acid synthesis, or a combination of UDCA and chenodeoxycholic acid.[155] Cholic acid, which was recently approved by the U.S. Food and Drug Administration (FDA) for the treatment of bile acid synthesis disorders, is a ligand for FXR, which downregulates hepatic CYP7A1 activity to limit production of hepatotoxic 3β-hydroxy-Δ^5 bile acids, while additionally providing the stimulus for bile flow. Concomitant with the disappearance of 3β-hydroxy-Δ^5 bile acids after initiating therapy, remarkable clinical and biochemical improvements occur with a normalization of liver function tests and resolution of jaundice in virtually all cases treated.[134,144] Furthermore, oral primary bile acid therapy in these patients avoids the need for liver transplantation, which is the only alternative therapy.

Δ^4-3-Oxosteroid 5β-Reductase (AKR1D1) Deficiency

A deficiency of the cytosolic Δ^4-3-oxosteroid 5β-reductase enzyme responsible for the catalytic conversion of 7α-hydroxy- and 7α,12α-dihydroxy-4-cholesten-3-one into the corresponding 3-oxo-5β(H) analogs was first described in monochorionic twins born with marked and progressive cholestasis[57] (Fig. 2-7). A previous sibling born with neonatal hepatitis had died of liver failure following a similar clinical course. Liver function tests revealed an elevation in serum transaminases, marked hyperbilirubinemia, and coagulopathy. Unlike the 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase deficiency, serum GGT concentrations are generally elevated. Liver biopsies showed marked lobular disarray as a result of giant-cell and pseudoacinar transformation of hepatocytes, hepatocellular and canalicular bile stasis, and extrahepatic medullary hematopoiesis. Electron micrographs showed small bile canaliculi that were slitlike in appearance, lacking the usual microvilli and containing variable amounts of electron-dense material.[57,144,149,156] An AKR1D1 deficiency was identified in both twins by urinary FAB-MS analysis, which indicated an elevated bile acid excretion and a predominance of bile acid conjugates with molecular weights consistent with unsaturated oxo-hydroxy- and oxo-dihydroxy-cholenoic acids. That these were 3-oxo-7α-hydroxy-4-cholenoic and 3-oxo-7α,12α-dihydroxy-4-cholenoic



• **Fig. 2-7** The metabolic pathway for primary bile acid synthesis from cholesterol depicting the biochemical presentation in patients with liver disease caused by a deficiency of Δ^4-3-oxosteroid 5b-reductase (AKR1D1).

acids was confirmed after extraction, hydrolysis, and derivatization of bile acids and GC-MS analysis.[57] Small proportions of allo(5α-H) isomers of cholic and chenodeoxycholic acids were also present and there was a lack of primary bile acids. These atypical bile acids accounted for up to 90% of the total urinary bile acids. There was a high concentration of allo-chenodeoxycholic, allo-cholic, and Δ^4-3-oxo bile acids in serum. Only traces (<2 μM) of bile acids were detected in bile. Studies using rat canalicular membrane vesicles show that Δ^4-3-oxo bile acids are poor substrates for the canalicular bile acid transporters,[153] presumably because of their poor solubility or low affinity for canalicular transporters. The presence of appreciable levels of allo-bile acids, normally minor metabolites, is explained by the accumulated substrates exceeding the K_m and V_{max} for the hepatic steroid 5α-reductase in the absence of AKR1D1 activity. Interestingly, steroid hormone studies of one patient with a 662C>T missense mutation in AKR1D1 deficiency found an almost total absence of 5β-reduced steroid hormone metabolites and a dominance of 5α-reduced metabolites, yet the patient had no obvious endocrine abnormalities.[157]

Δ^4-3-Oxosteroid 5β-reductase is not expressed in fibroblasts, but further evidence for a primary enzyme defect was established by immunoblot analysis of the cytosolic fraction from the liver using a monoclonal antibody raised against the rat AKR1D1. This monoclonal antibody recognized the 38KDa protein in the liver from patients with liver disease from other causes, but not from the patients with AKR1D1 deficiency. The cDNA for human Δ^4-3-oxosteroid 5β-reductase gene (*SRD5B, AKR1D1*) was reported,[158] and studies of three patients with high levels of Δ^4-3-oxo bile acids in urine and low or absent primary bile acids revealed three different mutations in this gene consistent with a primary enzyme defect in each case.[59] Two patients were homozygous for missense mutations (662C>T and 385C>T) and a third was homozygous for a single base deletion (511delT) in exon 5 leading to a premature stop codon. The liver biopsies of all three patients were characterized by giant-cell transformations, a common feature of many cases of inborn errors in bile acid synthesis.[144,149] Since these early reports, additional different mutations have been attributed to a deficiency in AKR1D1 activity in infants.[52,58,146] In a patient from Japan who met biochemical criteria for a deficiency in this enzyme, sequence analysis revealed a single silent mutation in the coding region of the gene, but immunoblot analysis of the liver homogenate using a monoclonal antibody revealed expression of the normal protein,[58] thus excluding a primary genetic defect. Increased production of Δ^4-3-oxo bile acids is not uncommon in patients with severe liver disease[56] and is a feature of immaturity in hepatic bile acid synthesis in infants during the first few weeks of life.[55] Several infants presenting with neonatal hemochromatosis were reported to have a Δ^4-3-oxosteroid 5β-reductase deficiency.[159] As primary bile acids are involved in the canalicular transport of iron, the question of whether the iron-storage defect may be secondary to the bile acid synthetic defect, or vice versa, has been raised.[159] In the case of a suspected AKR1D1 deficiency, it is important to repeat urinalysis because on some occasions liver disease resolves. These atypical bile acids spontaneously disappear, and the findings ideally should be supported by confirmation of a mutation in the gene.

The liver injury in this defect is the consequence of diminished primary bile acid synthesis and the hepatotoxicity of the accumulated Δ^4-3-oxo bile acids. The unique morphologic findings on electron microscopy (EM) of the liver of the first patients described[156] suggest that maturation of the canalicular membrane and the transport system for bile acid secretion may require a threshold concentration of primary bile acids in early

development. Primary bile acid therapy resulted in a normalization of the immature-appearing bile canalicular structures with a disappearance of the electron-dense material seen under EM in and around the canaliculi.

Treatment with oral cholic acid (5-15 mg/kg of body weight/day) in most patients has resulted in clinical and biochemical improvement, resolution of jaundice, and normalization of liver function tests, provided that therapy was initiated before significant liver damage occurred.[146,156] UDCA was reported to be not effective[160] or was used in combination with cholic or chenodeoxycholic acids in some patients.[146,160] Down-regulation in bile acid synthesis can be monitored by measurement of the concentrations of the Δ^4-3-oxo bile acids in urine, and dose can be titrated based on biochemical and clinical responses.[134] In a few patients cholic acid has failed to reverse the liver injury, but this was generally because diagnosis was established when there was cirrhosis and end-stage disease.

Defects Involving Reactions Leading to Side Chain Modification

Defects in the reactions involved in side chain hydroxylation and oxidation generally present as neurologic disturbances and/or syndromes of fat-soluble vitamin malabsorption. These manifestations emphasize the crucial role that bile acids play in the intestinal absorption of lipids. Liver disease is generally mild and may not necessarily be the primary clinical presentation, because low levels of primary bile acids are often made via alternative pathways of synthesis. CTX was the first defect in bile acid synthesis to be described[66,161] and shown conclusively to be due to mutations in the gene for sterol 27-hydroxylase (*CYP271A*). More recently, defects in bile acid conjugation and specific single enzyme defects in peroxisomal β-oxidation have been described. Generalized disorders in peroxisomal structure and function, distinct from single-enzyme defects in the fatty acid oxidation system, ultimately lead to progressive liver disease, but this is secondary to the underlying genetic disease.

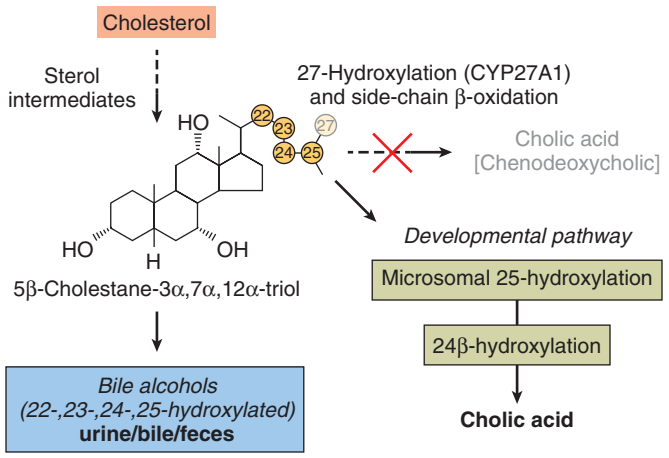
Sterol 27-Hydroxylase Deficiency: Cerebrotendinous Xanthomatosis

CTX is a rare, autosomal recessive lipid-storage disease that has an estimated prevalence of 1:70,000. Although CTX is usually not diagnosed until the second or third decade of life when patients become symptomatic, it has been detected in a few pediatric patients.[162,163] The clinical presentation includes symptoms of progressive neurologic dysfunction, dementia, ataxia, cataracts, and xanthomas in the brain and tendons.[164] It has been suggested that the presence of bilateral juvenile cataracts and a history of chronic diarrhea, although not specific for CTX, may represent an early clinical manifestation of the disease.[162,163] More recently CTX has been associated with a transient increase in serum liver enzymes in several infants, suggesting that the earliest clinical picture may be a mild cholestasis that ultimately resolves over the first few months of life.[165,166] In one case report CTX was associated with fatal cholestasis in infancy.[167] The main biochemical features of this disease are a significantly reduced synthesis of primary bile acids, elevated biliary, urinary, and fecal excretion of bile alcohol glucuronides, a normal or low plasma cholesterol concentration with excessive deposition of cholesterol and cholestanol in tissues, and a markedly elevated plasma cholestanol concentration.

More than 2 decades ago, Salen et al. demonstrated that the basic defect in this disorder was an impairment in side chain oxidation[161] and that chenodeoxycholic acid synthesis was affected to a greater extent than cholic acid synthesis. Conclusive evidence that the primary defect in CTX is a defect in sterol 27 hydroxylase (Fig. 2-8) comes from molecular studies, facilitated by the cloning of the human sterol 27-hydroxylase cDNA.[65] The gene is localized to the long arm of chromosome 2, and a large number of different mutations have been identified in CTX patients, including insertion, deletion, and point mutations.[66] Interestingly, the mitochondrial sterol 27-hydroxylase also catalyzes hepatic 25-hydroxylation of vitamin D, yet despite this, 25-hydroxy-vitamin D is not usually altered in CTX patients.

A striking feature is the accumulation of 5α-cholestan-3β-ol (cholestanol) in the nervous system and the markedly elevated concentrations of this sterol, but not cholesterol, in plasma.[66,168] The cholestanol/cholesterol ratio in plasma may be of diagnostic value,[169] although an elevation of this ratio and increased urinary excretion of bile alcohol glucuronides are often seen in patients with cholestatic liver diseases.[170,171] The most plausible explanation for the high cholestanol levels is that it arises from sterol intermediates that accumulate in the absence of an active sterol 27-hydroxylase. A pathway has been proposed involving 7α-hydroxylation of cholesterol, and conversion to 7α-hydroxy-4-cholesten-3-one, followed by 7α-dehydroxylation that is hepatic rather than intestinal, and yields cholest-4,6-dien-3-one as an intermediate. Radiolabeling studies confirmed this pathway, and CTX patients have elevated plasma 7α-hydroxy-4-cholesten-3-one and cholest-4,6-dien-3-one levels.[66] Furthermore, cholestyramine administration, which increases cholesterol 7α-hydroxylase activity, leads to increased plasma cholestanol concentrations, whereas chenodeoxycholic acid administration has the opposite effect.

Diagnosis of CTX at an early age is important to limit neurologic and cardiovascular complications resulting from the chronic and irreversible deposition of cholesterol and cholestanol in tissues. Diagnosis is generally based on a greatly increased plasma cholestanol/cholesterol ratio,[169] although in some cases this is not entirely reliable, and/or an elevated excretion of bile alcohols in urine.[134,152] These analyses are highly specialized, time-consuming,



• **Fig. 2-8** The metabolic pathways for primary bile acid synthesis from cholesterol depicting the biochemical presentation in patients with a sterol 27-hydroxylase deficiency (CYP27A1) causing cerebrotendinous xanthomatosis leading to diminished primary bile acid synthesis and excessive production of bile alcohols. Note that cholic acid is synthesized by the alternative 25-hydroxylation pathway (see also Fig. 2-2).

complex, and outside the scope of routine clinical laboratories. However, using mass spectrometry it is possible to rapidly and definitively diagnose CTX from an analysis of urine, which reveals the presence of increased levels of bile alcohol glucuronides.[134,152] The typical FAB-MS negative ion spectrum from a CTX patient reveals high levels of characteristic $[M-H]^-$ ions of bile alcohol glucuronides, thus permitting a diagnosis to be made. CTX can be further confirmed by complementing the mass spectrometry analysis with DNA sequencing of the *CYP27A1* gene and identifying the specific mutation.

CTX can be effectively treated by oral bile acid administration.[172-174] Chenodeoxycholic acid (750 mg/day), yet to be approved for CTX, and cholic acid, recently approved, both normalize plasma cholestanol and lead to a concomitant decrease in excretion of urinary bile alcohol glucuronides consistent with down-regulation in endogenous CYP7A1 activity. These biochemical changes are generally accompanied by an improvement in clinical symptoms, particularly the neurologic disturbances, and are most effective when initiated before onset of significant symptomology. Cholic and deoxycholic acids also decrease plasma cholestanol and cholic acid may be preferable in infants, but it should be stressed that ursodeoxycholic acid is ineffective.[175] Bile acid therapy may be more effective in reducing plasma cholestanol in patients with CTX if combined with an HMG-CoA reductase inhibitor, which additionally inhibits endogenous cholesterol synthesis.[176,177]

2-Methylacyl CoA Racemase Deficiency

A deficiency in 2-methylacyl CoA racemase, or alpha-methylacyl CoA racemase (AMACR), was reported in a 3-week-old female infant presenting with mildly elevated liver enzymes and low serum 25-hydroxy-vitamin D and vitamin E concentrations.[72] Identification of this defect was again based on mass spectrometric analysis of urine and serum, complemented by molecular studies. Molecular analysis of the gene encoding the AMACR showed a missense mutation (*S52P*) yielding an inactive protein in this patient. Interestingly, the same mutation was reported in two of three patients with an adult-onset sensory neuropathy characterized by elevated serum phytanic and pristanic acids, but neither fat-soluble vitamin malabsorption nor liver disease appeared to be features.[71] AMACR catalyzes the racemization of the (*25R*) diastereoisomers of THCA and DHCA to the respective (*25S*) isomers,[178] and this reaction is a prerequisite for the initiation of peroxisomal β-oxidation of the side chain of these C_{27} bile acid intermediates. It is also responsible for the racemization of (*2R*)-pristanoyl Co-A to its (*2S*)-diastereoisomer before peroxisomal β-oxidation, and this broad substrate specificity explains why in this genetic disease VLCFAs are normal while pristanic acid, a branch-chained fatty acid, is elevated. The urinary FAB-MS analysis yields a mass spectrum identical to that of patients with Zellweger syndrome; however, plasma VLCFAs and other peroxisomal enzyme markers are all normal. High performance liquid chromatography-electrospray ionization (HPLC-ESI) tandem mass spectrometry was used to separate the diastereoisomers of THCA and DHCA and to confirm the presence of exclusively (*25R*)-forms of THCA and DHCA in the patient's serum and bile.[72]

This infant responded successfully to fat-soluble vitamin supplementation and cholic acid therapy (15 mg/kg/day) with normalization of liver function tests, and she was neurologically and developmentally normal at age 3½ years. The patient's history was remarkable for a previous sibling who was healthy until 5½ months of age but died suddenly following an intracranial bleed that was secondary to vitamin K deficiency. The liver of this sibling, apparently having the same bile acid synthetic defect, was transplanted, and the recipient was alive 5 years later but receiving oral bile acid therapy.[72] Dietary restriction of phytanic acid should be implemented in AMACR deficiency to prevent longer-term neurologic damage caused by the accumulation of branched-chain fatty acids. An AMACR knockout mouse model confirms the importance of phytol restriction in preventing neurologic and hepatic manifestations.[179] Because the primary presentation in this infant and her deceased sibling was fat-soluble vitamin malabsorption, this discovery makes a strong case for screening for bile acid synthetic defects in cases of unexplained fat-soluble vitamin malabsorption or rickets to permit therapeutic intervention with primary bile acids as early as possible.

Side Chain Oxidation Defect in the 25-Hydroxylation Pathway

Clayton et al. proposed a defect in side chain oxidation in the 25-hydroxylation pathway for a 9-week-old infant presenting with familial giant-cell hepatitis and severe intrahepatic cholestasis.[180] The diagnosis was based on the findings of reduced cholic and chenodeoxycholic acid concentrations and elevated concentrations of bile alcohol glucuronides, specifically 5β-cholestane-3β,7α,12α,24-tetrol, 5β-cholest-24-ene-3β,7α,12α,24-tetrol, and 5β-cholestane-3β,7α,12α,25-tetrol in serum. These bile alcohols are not normally found in the plasma of infants with liver disease. Bile alcohol glucuronides were major metabolites in the urine.[180] Although this profile resembled that of CTX patients, it was concluded on the basis of the liver disease, which at that time had not been previously described as a feature of CTX, that this represented an oxidation defect downstream of the 25-hydroxylation step in this minor pathway for bile acid synthesis. The implications of the findings are that the 25-hydroxylation pathway, considered of negligible importance in adults, may be important in infants. The patient was treated with chenodeoxycholic and cholic acids, which led to normalization in serum transaminases and suppression of bile alcohol production.

Peroxisomal Disorders

Disorders involving peroxisomal assembly and function have a significant impact on bile acid synthesis. This is perhaps not surprising because the peroxisome packages at least 40 enzymes, including those required for the β-oxidation of fatty acids and bile acids, as well as the enzymes catalyzing bile acid conjugation. Most of the disorders in bile acid synthesis in peroxisomopathies are secondary to the primary defect of organelle dysfunction. The early diagnosis of a peroxisomopathy is interestingly often the result of the patient's referral to a gastroenterologist for evaluation of abnormal liver biochemistries.

Many of the peroxisomal disorders show similarities and overlap in clinical and biochemical presentation. Conditions in which there is a generalized impairment in peroxisomal function exhibit abnormalities in bile acid synthesis and metabolism, and these patients often have significant liver disease. Pinpointing the exact nature of the peroxisomopathy can be challenging and requires a battery of tests to examine the entire β-oxidation pathway of bile acids and VLCFAs, complemented by immunoblotting techniques to identify the presence and activity of other peroxisomal enzymes, and genetic screening to sequence the *PEX* gene exons for peroxisomal biogenesis disorders.

Mass spectrometry, both LSIMS (FAB-MS or ESI-MS) and GC-MS analysis of the urine and plasma/serum, permits accurate identification of abnormalities in peroxisomal β-oxidation of bile acids,[134,181] particularly when there is evidence of progressive liver disease. A typical FAB-MS of the urine reveals the presence of unconjugated THCA, taurine-conjugated THCA, and taurine-conjugated tetrahydroxylated cholestanoic acids. Elevated levels of DHCA, THCA, and a C_{29}-dicarboxylic bile acid in biologic fluids are a consistent feature of patients with Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease and pseudo-Zellweger syndrome,[182-187] and peroxisomal biogenesis disorders.[188-190] Of the single enzyme defects, X-linked adrenoleukodystrophy and pseudoneonatal adrenoleukodystrophy both show normal bile acid synthesis with no accumulation of cholestanoic acids. DHCA concentrations are in general much lower than THCA, particularly in younger patients, and this is explained by its preferential conversion to THCA by 12α-hydroxylation. The origin of a unique C_{29}-dicarboxylic acid found in serum of many Zellweger syndrome patients is presumed to be from side chain elongation in the endoplasmic reticulum.[182] Although bile acid synthetic rates are low in patients with Zellweger syndrome,[191] increased serum concentrations of primary bile acids are frequently found and are probably a consequence of impaired hepatic function. Additional metabolism of THCA by microsomal hydroxylation in the side chain (to produce C-24 hydroxylated, varanic acid isomers) and in the nucleus (to produce C-1 and C-6 tetrahydroxy-cholestanoic acids) gives rise to many tetrahydroxylated cholestanoic acids that are excreted in urine and present in plasma, and these are of diagnostic value.[181,186,187] The urine from parents of patients with Zellweger syndrome, who are heterozygous for this most severe form of peroxisomal defect, will have normal urinary bile acid excretion with negligible or no detectable cholestanoic acids.[187] In genetic counseling of affected families, prenatal diagnosis is possible by specific detection of elevated concentrations of DHCA and THCA in amniotic fluid.[192]

Whereas the diagnosis of Zellweger syndrome is often straightforward, characterizing and differentiating patients with less severe phenotypes and with single enzyme defects involving peroxisomal enzymes is more difficult. There have been several case reports of presumed THCA-CoA oxidase deficiencies, and phytanic and pristanic acids, when measured, have been elevated.[193-196] All patients presented with ataxia, and unlike patients with AMACR deficiency who share a similar biochemical profile,[72] there was no evidence for any neurologic disorder. With the more recent recognition of the complexity of peroxisomal biogenesis disorders, it is possible that some of these previously reported cases could have been due to mutations in *PEX* genes, of which there are 12 known.

Treatment of the peroxisomopathies is difficult because of their multiorgan pathophysiology and is to a large extent restricted to managing symptoms. Dietary restriction of VLCFAs and phytanic acid and administration of oleic acid have provided minimal to no benefit in patients with full-blown Zellweger syndrome. Clofibrate, which has been shown in rats to induce peroxisomal proliferation, has proven to be of no therapeutic value.[197] In general, the prognosis in most peroxisomal disorders is poor, and patients with Zellweger syndrome generally succumb to respiratory failure. The progressive liver disease that commonly develops in peroxisomal disorders may in part be due to increased synthesis and accumulation of C_{27}-bile acids and reduced primary bile acid synthesis. Infusion of tauro-THCA in rats induces red-cell hemolysis and produces a hepatic lesion showing mitochondrial disruption similar to that found in Zellweger patients.[198] In an attempt to limit the severity of liver injury in a Zellweger syndrome patient, primary bile acids were given orally, and biochemical markers of liver function and histology improved markedly, most notably by a decrease in the extent of bile-duct proliferation and inflammation.[187] Urinary and serum concentrations of cholestanoic acids also decreased. A striking and sustained increase in growth and significant improvement in neurologic symptoms were also noted. Based on these observations and the successful treatment of patients with primary enzyme defects in bile acid synthesis, cholic acid therapy has now been used in a number of patients with peroxisomal disorders with variable outcomes.[134] It was recently approved by the FDA for peroxisomal disorders where bile acid synthesis is impaired. In a study of patients with a variety of peroxisomopathies, including Refsum disease, neonatal adrenoleukodystrophy, and Zellweger syndrome, treatment with cholic acid (10-15 mg/kg of body weight/day) for periods ranging from 4.7 to 11 years had variable outcomes. Not surprisingly, most treatment failures were in patients with more severe Zellweger syndrome. Patients with single enzyme defects in peroxisomal function causing abnormal bile acid synthesis showed greater responsiveness and may benefit from oral cholic acid therapy.[134] Patients with the peroxisomal biogenesis defects may benefit as evidenced from successful treatment of a patient with a *PEX10* defect,[190] who thus far has been treated with oral cholic acid for more than 18 years.

Bile Acid-CoA Conjugation Defects

Hepatic conjugation in humans is extremely efficient and as a result negligible amounts of unconjugated bile acids (<2%) typically appear in bile under normal and most cholestatic conditions,[199] and even after therapeutic doses of the unconjugated bile acid, ursodeoxycholic acid, is administered.[200] The first case of defect in bile acid amidation was described in 1994 in a 14-year-old boy presenting with fat and fat-soluble vitamin malabsorption.[93,92] This child was of Laotian descent and in the first 3 months of life presented with conjugated hyperbilirubinemia, elevated serum transaminases, and normal GGT. Two other patients, a 5-year-old Saudi Arabian boy and his 8-year-old sister, who were products of a consanguineous marriage, were identified with the same bile acid defect soon after. Remarkably, the boy had undergone a Kasai procedure for a mistakenly diagnosed biliary atresia, whereas his sister was reportedly asymptomatic at time of diagnosis. Conjugation defects have since been identified in more than 10 additional patients[94,95,201] with a clinical history of normal or mildly elevated liver function tests but with severe fat-soluble vitamin malabsorption and rickets. In one patient, this resulted in bone fracture. All had subnormal levels of vitamin E, vitamin K, 25-hydroxy-vitamin D, and 1,25-dihydroxy-vitamin D. The phenotype of the amidation defect is quite variable, with severe cholestasis and liver failure requiring liver transplantation in one patient. The clinical presentation and biochemical features of defective amidation closely paralleled the predicted features hypothesized by Hofmann and Strandvik some years earlier.[202] This conjugation defect was also reported in a number of patients from an Amish kindred and was associated with mutations in the *BAAT* gene encoding the bile acid-CoA:amino acid N-acyltransferase. In some of the cases a mutation in the *TJP2* gene encoding tight junction protein 2 was reported.[94] More recently, the first confirmed defect associated with a mutation in the *SLC27A5* gene encoding the bile acid CoA ligase was

reported.[95] The patient, of Pakistani origin born to consanguineous parents, presented with cholestasis, elevated serum bilirubin and transaminases, normal serum GGT concentrations and low fat-soluble vitamins and had been receiving total parenteral nutrition. The liver biopsy showed extensive fibrosis. The patient was homozygous for a missense mutation C.1012C>T in the *SLC27A5* gene, and interestingly a second mutation was discovered in the gene encoding the bile salt export pump. No mutations were found in *BAAT.* Diagnosis of a bile acid amidation defect is readily achieved by mass spectrometry. FAB-MS and ESI-MS negative ion mass spectra of the urine, serum, and bile reveal a distinct profile in which there is a major ion at m/z 407 corresponding to unconjugated cholic acid. In addition, ions characterizing sulfate and glucuronide conjugates of dihydroxy- and trihydroxy-bile acids are usually present but those of glycine- and taurine-conjugated bile acids are absent.[93,95] Serum and urinary bile acids are markedly elevated in these patients and comprise predominantly cholic and deoxycholic acids. Discerning whether the defect resides in the bile acid CoA ligase or in the bile acid-CoA:amino acid N-acyltransferase requires the use of molecular techniques to sequence the *SLC27A5* and *BAAT* genes for mutations or immunostaining of a liver biopsy for the presence of the enzymes, because the mass spectrometric bile acid profiles of these two defects are indistinguishable.

Although inborn errors in bile acid synthesis usually present as well-defined progressive familial cholestatic liver disease, cholestasis is generally not a primary manifestation of a bile acid conjugation defect, presumably because synthesis of high levels of unconjugated cholic acid is sufficient to maintain bile flow. The main feature of fat-soluble vitamin malabsorption occurs because of reduced biliary secretion of bile acids and an inability to form mixed micelles because of rapid passive absorption of unconjugated cholic acid in the proximal small intestine. Although these patients conjugate bile acids with glucuronic and sulfuric acids, these conjugates do not promote lipid absorption. Treatment with oral glycocholic acid was effective in resolving the fat-soluble vitamin malabsorption of five patients with the amidation defect caused by mutations in *BAAT,*[201] whereas UDCA therapy was used in the one reported patient with a mutation in the gene encoding the bile acid-CoA ligase.[95] More recently we studied 10 pediatric patients with fat-soluble vitamin deficiency, some with growth failure or transient neonatal cholestatic hepatitis, and found increased urinary bile acids that were predominantly excreted in unconjugated forms as sulfates and glucuronides. Glycine or taurine conjugates were absent in the urine, bile, and serum of these patients. Thus, the biochemical profile was consistent with defective bile acid amidation, and molecular analysis of *BAAT* confirmed four different homozygous mutations in eight patients tested.[92] The recognition that genetic defects in bile acid synthesis are associated with unexplained fat-soluble vitamin malabsorption warrants a concerted effort to explore patients with this phenotype for defective bile acid synthesis.

Other Disorders Influencing Bile Acid Synthesis and Metabolism

In conditions that alter the integrity of the enterohepatic circulation, significant changes in bile acid synthesis and metabolism will occur. Because serum bile acid concentrations reflect a balance between intestinal input and hepatic extraction, it is evident that pathophysiologic changes to the intestinal tract will be reflected by secondary changes in bile acid synthesis and metabolism. Such examples include ileal resection and bacterial overgrowth,[119] whereas an inborn error in ileal bile acid transport has been shown to cause bile acid malabsorption.[203,204] Genetic defects in cholesterol synthesis, such as Smith-Lemli-Opitz syndrome,[205] also alter bile acid synthesis because of the reduced availability of cholesterol.

Increased concentrations and alterations in bile acid metabolism can be found in most cholestatic liver diseases, but discerning whether these are primary or secondary to the liver injury is often difficult. Primary bile acid synthetic defects, especially those involving the steroid nucleus, present as progressive familial intrahepatic cholestasis (PFIC), and represent a distinct entity of the PFIC syndromes separate from those recognized to arise from defects in the canalicular organic anion transporter proteins.[206-208] A shared feature of Byler disease, designated PFIC type 1 (Byler disease), PFIC type 2 (the bile salt export pump defect), and bile acid synthesis disorders, is a consistently low serum GGT level, but differential diagnosis is possible from performing bile acid analysis. PFIC type 1 and 2 patients present with high serum bile acid concentrations; whereas primary bile acids are lacking in the bile acid synthetic defects. PFIC type 3 patients also have a normal serum GGT level but defective phospholipid secretion. Patients with Byler disease have severe growth failure and usually die before 3 years of age without transplantation. For bile acid synthetic defects, except for the oxysterol 7α-hydroxylase deficiency,[29] the prognosis is excellent—provided oral primary bile acid therapy is initiated before there is significant loss of quantitative liver function[134,146] and the need for liver transplantation can be avoided. Cholic acid therapy (10-15 mg/kg of body weight/day) was recently approved for the treatment of bile acid synthetic disorders. It is therefore important in the clinical evaluation of patients with PFIC to screen for potential defects in bile acid synthesis as early as possible. All of these defects can be recognized by a combination of molecular and analytical studies, and bile acid and phospholipid analysis of bile is helpful in the differential diagnosis. Whereas only nine bile acid synthesis disorders have been described in the complex pathway for bile acid synthesis, it is likely that, with whole genome sequencing, mutations in the genes encoding the remaining eight enzymes will be revealed and the phenotypes described.

Bile Acids as Signaling Integrators of Metabolism

Earlier in this chapter, we introduced the concept of bile acids as signaling molecules in the context of bile acid synthesis regulation. Recent evidence suggests that bile acids signal to control a much wider spectrum of metabolic processes including satiety, energy expenditure, and hepatic lipogenesis.

Enterocyte Bile Acid Physiology

Bile acid reabsorption in the terminal ileum occurs in an active manner against its concentration gradient predominantly through the apical sodium bile acid transporter (ASBT).[209] We understand that FGF15/19 is released by the ileum after bile acid stimulation of the intestinal FXR-FGF15/19 system.[210] The Diet1 intestinal protein was recently highlighted to be a critical intermediary step in the ileal FXR-FGF signaling pathway.[3] The release of FGF15/19 from enterocytes then signals FGFR4 in the liver. FGF15/19 action in the liver has been said to be dependent on the integrity

of the receptor FGFR4 and its obligatory co-receptor βKlotho.[211] FGF15 directly inhibits Cyp7a1 through its liver receptor FGFR4 independent of the liver FXR-SHP pathway.[212,213] Further, the intestinal FXR-FGF pathway suppresses both Cyp7a1 and Cyp8b1 hepatic gene expression.[214] It is now understood that the intestinal FXR-FGF-FGFR4-mediated inhibition of bile acid synthesis impacts both Cyp7a1 (7α-hydroxylation) and Cyp8b1 (12α-hydroxylation) equally. FGF15 signaling also works subsequent to insulin as a postprandial inhibitor of hepatic gluconeogenesis through the inactivation of the transcription factor cAMP regulatory element-binding protein[215] and lipogenesis through Srebp1c.[216] A decrease in fasting FGF15/19 levels has been shown to be associated with the development of nonalcoholic fatty liver disease in obese adolescents[217] and more recently improvement in obesity has been ascribed in part to an increase in gut gluconeogenesis.[218]

Muscle and Brown Adipose Tissue Metabolism

Circulating bile acids bind to TGR5, a plasma membrane-bound G protein-coupled receptor that is present in distal small intestine, colon, pancreas, skeletal muscle, and brown adipose tissue.[77] The activation of this receptor TGR5 leads to a secretion of the incretin hormone glucagon-like peptide-1 from enterocytes,[1] which may influence insulin secretion from the pancreatic β cells.[219] Activation of the TGR5 receptor in skeletal muscle and brown adipose tissue also mediates the conversion of thyroxine to triiodothyronine, which could increase energy expenditure.[220] We have also reported that skeletal muscle gene expression of bile acid responsive targets, Kir6.2 and cyclooxygenase IV, are increased after bariatric surgery.[221]

Bile Acid Changes After Bariatric Surgery

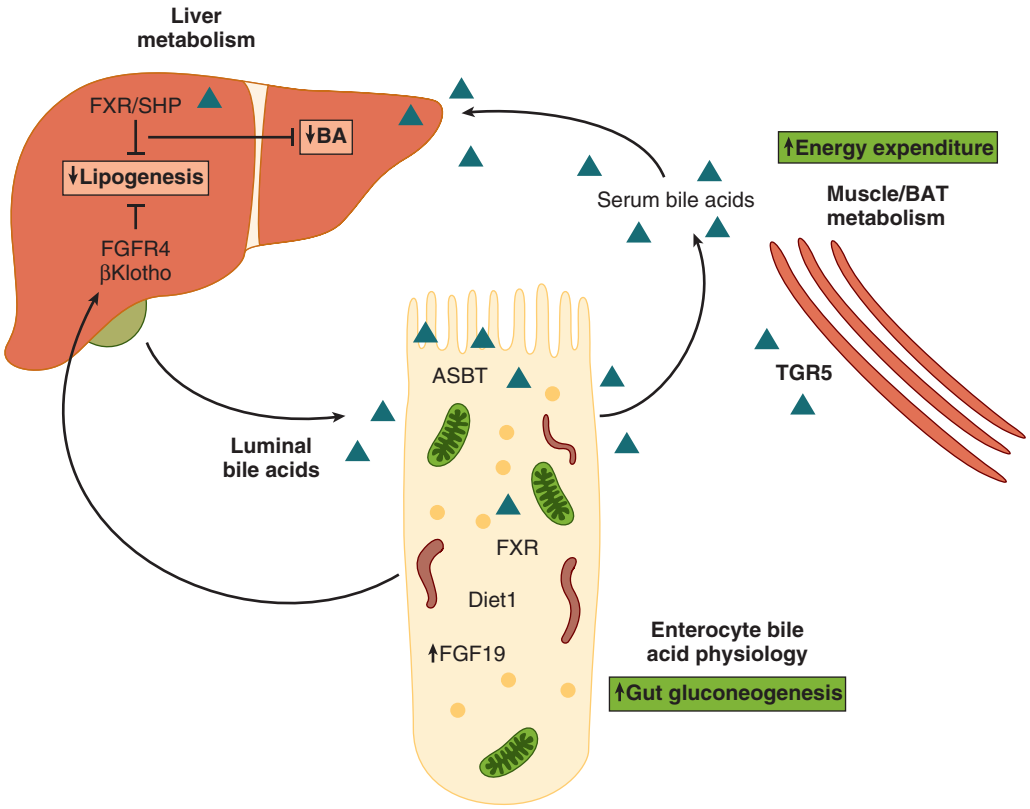
Many of the weight-loss or bariatric surgeries have been reported to result in increased levels of serum bile acids. Patti et al. reported elevated serum bile acid levels after bariatric surgery, wherein the bile acid subfractions taurochenodeoxycholic, taurodeoxycholic, glycocholic, glycochenodeoxycholic, and glycodeoxycholic acids were all significantly higher in the Roux-en-Y gastric bypass subjects compared with overweight and morbidly obese weight-matched control subjects.[222] Nakatani et al. also showed that restrictive procedures (9 of this group of 15 patients had vertical sleeve gastrectomy [VSG]) resulted in increased total serum bile acid levels as the subjects lost weight.[223] We have subsequently shown that this increase in serum bile acid levels is not ubiquitous to all bariatric procedures. Roux-en-Y gastric bypass results in an increase in serum bile acids, but we did not find a similar increase in individuals who lost weight after undergoing a gastric banding procedure.[221] These human data in conjunction with our in vivo experimental work with rat ileal interposition[224] and bile diversion surgeries[225] present compelling data that the bile acid enterohepatic circulation is important to the improvements in metabolism seen after bariatric procedures.

Why certain bariatric procedures increase serum bile acid levels is an open question. Multiple potential pathways involved in the rise of serum bile acids levels after VSG surgery include (1) ghrelin activation and its link to bile acid production,[226] (2) changes in intestinal pH after VSG that would impact bile acid reabsorption, (3) changes in the microbiome influencing bile acid circulation, (4) enhanced gastric emptying rates that shorten bile acid transit, and (5) bile acid-triggered distal gut anorexic hormone release such as glucagon-like peptide-1.[227-229] Bile acid reabsorption in the terminal ileum occurs in an active manner against a concentration gradient,[230] predominantly through active transport involving the ASBT.[209] Increased villi length and total surface area in addition to the greater ASBT-stained area have been observed after bariatric surgery and potentially explain the elevated serum levels of bile acids. This intestinal adaptive response is similar in many experimental bariatric and nonsurgical murine cohorts that have been shown to have higher serum bile acid levels.[210,224-226,231] Increased bile acid absorption coupled with the suppression of hepatocyte bile acid uptake transport mechanisms (Oatp/Ntcp) as we have observed[210] may together explain the elevated serum bile acid levels.

Rats that underwent bile diversion lost significantly more weight than rats that had sham surgeries. Further, bile-diverted rats had improved glucose tolerance, less liver steatosis, and a higher postprandial glucagon-like peptide-1 response, in addition to higher serum bile acids. The taurine-conjugated form of serum bile acid ursodeoxycholic acid (TUDCA) was specifically higher in the bile-diverted rats. Interestingly in a separate experiment we observed that bile acid gavage of TUDCA or UDCA in diet-induced obese rats reduced hepatic steatosis and endoplasmic reticulum stress. Using a murine model of VSG, we further reported that serum bile composition in the VSG-treated obese mice had increased cholic and TUDCA levels. These compositional changes in bile acids in VSG mice explained observed down-regulation of hepatic lipogenic and bile acid synthesis genes. We further reported that increases in serum bile acids in post-VSG mice correlate with postsurgery weight loss and that changes in serum bile composition could explain suppression of hepatic genes responsible for lipogenesis.[210] Specifically, performing VSG on mice lacking the nuclear receptor FXR, or NR1H4, we demonstrated that the therapeutic value of VSG does not result from mechanical restriction imposed by a smaller stomach. Rather, in the absence of FXR, the ability of VSG to reduce body weight and improve glucose tolerance is substantially reduced. VSG was also seen to be associated with changes to gut microbial communities.[232]

Bile Acid Signaling as a Treatment for Nonalcoholic Steatohepatitis

Recently, direct hepatic FXR activation has been shown to suppress lipogenesis through SREBP1c[233] and the bile acid production enzymes Cyp8b1 and Cyp7a.[214] We have also shown that steatosis improvement after bariatric surgery involves suppressed hepatic lipogenesis.[210] Further, FXR activation using 6-ethyl-chenodeoxycholic acid (obeticholic acid) has also been shown to protect against body weight gain and liver lipid accumulation in obese rats.[234] More recently a large clinical trial reported a significant improvement in nonalcoholic steatohepatitis when patients were treated with OCA, but there were also disproportionate lipid abnormalities in OCA- compared with placebo-treated patients.[235] Patients that received OCA had increased levels of plasma LDL–cholesterol and decreased levels of plasma high density lipoprotein–cholesterol. In a separate set of experiments we investigated a downstream target of FXR implicated in hepatic lipogenesis and bile acid production: the small heterodimer partner (SHP) pathway. We found that diet-induced obese mice genetically modified to overexpress SHP in the liver (SHP-Tg mice) lost weight after VSG and had decreased steatosis. On the other hand, diet-induced obese mice lacking SHP (SHP-knockout mice) had weight loss



• **Fig. 2-9** Bile acid (BA) enterohepatic circulation as a metabolic signaling integrator. *ASBT,* apical sodium bile acid transporter; *BAT,* brown adipose tissue; *FGF19,* fibroblast growth factor-19; *FGFR4,* fibroblast growth factor-19 receptor; *FXR,* farnesoid X receptor; *βKlotho,* FGFR4 co-receptor; *SHP,* short heterodimer partner; *TGR5,* G protein–coupled receptor.

independent of SHP status. In contrast, SHP-knockout mice that underwent VSG lost weight but developed hepatic inflammation and had increased liver injury.[236] Together these data demonstrate the ability of alterations in bile acids to recapitulate important metabolic improvements seen after bariatric surgery (Fig. 2-9). Furthermore, the intestinal microbiome is increasingly recognized to play an important role in disease and in obesity-related conditions, and not surprisingly, alterations in bile acid metabolism will be induced by changes in bacterial metabolism. The interplay between bacteria and bile acids is likely to be shown to be key to explaining some of the findings we have discussed.

Conclusion

This chapter describes the complex pathway for bile acid synthesis from cholesterol and illustrates how genetic defects in genes encoding the enzymes responsible for bile acid synthesis or their transport influence normal physiology. Impaired bile acid synthesis and transport leads to a broad spectrum of symptoms ranging from cholestasis, fat and fat-soluble vitamin malabsorption, to neuropathy consistent with the physiologic and physicochemical properties of bile acids. There is now a renaissance in interest in bile acids given the recognition that they regulate numerous biochemical pathways associated with energy, glucose, and fat metabolism. Consequently, novel drugs based on the bile acid backbone are likely to be developed to address the global problems of obesity and diabetes and the liver disease associated with them.

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