CHARACTERIZATION OF HEPATIC GLYCOGEN AND FAT METABOLISM IN RATS AND HUMANS WITH LIVER DISEASE

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presented by

Corinne Bettina Lang
Eidg. dipl. Apothekerin
born the 6th April 1968
in Lucerne (LU)
citizen of Buchrain (LU)

Prof. Dr. G. Folkers, examiner
Prof. Dr. med. Dr. pharm. S. Krähenbühl, co-examiner

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To my parents and my sisters Gaby and Andrea
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SUMMARY

Long-term cholestasis in the rat leads to secondary biliary fibrosis and cirrhosis and is associated with alterations in hepatic energy metabolism. Glycogen as well as fatty acid metabolism in the liver are affected: first, rats with long-term cholestasis show reduced hepatic glycogen stores due to impaired glycogen synthesis, and second decreased plasma concentrations of β-hydroxybutyrate during starvation and impaired metabolism of short- and long-chain fatty acids in isolated hepatocytes or in isolated mitochondria. Hepatic energy metabolism was therefore studied in rats with short-term cholestasis, before the development of secondary biliary fibrosis and cirrhosis in the first two of the four projects studied.

In the first project, the study design was as follows: rats were bile duct ligated (BDL rats) for four or eight days and compared to either sham-operated control rats pair-fed to BDL rats (pair-fed CON rats) or sham-operated control rats fed ad libitum (ad libitum-fed CON rats).

All animals were investigated in the fed state. Four days after surgery, the hepatic glycogen content was 21.6±7.6 versus 21.2±8.5 mg/g liver in BDL and pair-fed CON rats, respectively, while ad libitum-fed CON rats had a glycogen content of 72.9±7.7 mg/g liver. Eight days after surgery, the hepatic glycogen content was significantly reduced in BDL compared to pair-fed (31.2±8.9 vs. 59.1±5.4 mg/g liver) or ad libitum-fed CON rats (58.3±4.7 mg/g liver). Similar patterns were observed with the hepatic glycogen content expressed per ml hepatocytes or per liver on both occasions examined.

Histological analysis of livers from BDL rats confirmed the decrease in the hepatic glycogen stores and showed that almost all hepatocytes were affected. At both time points, the total activities of glycogen synthase and glycogen phosphorylase were significantly reduced in BDL as compared to pair-fed or ad libitum-fed CON rats, while the active fractions of these enzymes were not affected. The mRNA levels normalized to GAPDH of both glycogen synthase and glycogen phosphorylase were significantly reduced in BDL as compared to pair-fed CON rats at both time points. The plasma glucagon concentrations were significantly increased in BDL versus pair-fed CON rats at both time
points, while the corresponding insulin concentrations were decreased in BDL rats at four, but not eight days after surgery. In skeletal muscle, the glycogen content and activities of glycogen synthase and phosphorylase remained unaffected at both time points.

In conclusion, acute cholestasis in rats leads to a rapid decrease in the hepatic glycogen content, expressed per ml hepatocytes, gram liver and per total liver. The activities and expression of glycogen synthase and phosphorylase were decreased at both time points studied. While reduced intake of food is sufficient to explain the decrease in the hepatic glycogen stores in BDL and pair-fed CON rats four days after surgery, reduced glycogen synthesis is the most probable cause of the decrease in hepatic glycogen content in BDL rats eight days after surgery.

In the second project, the hepatic fatty acid metabolism was investigated in rats with bile duct ligation for five or ten days (BDL rats) and the findings were compared to pair-fed control rats (CON rats). All animals were studied after starvation of 24 hours. The plasma β-hydroxybutyrate concentration was reduced in BDL rats (0.54±0.10 vs. 0.83±0.30 mmol/l at five and 0.59±0.24 vs. 0.88±0.09 mmol/l at ten days in BDL and control rats, respectively). In isolated liver mitochondria, state 3 oxidation rates for various substrates were not different between BDL and control rats. Production of ketone bodies from 14C-palmitate was reduced by 40% in mitochondria from BDL rats at both time points, whereas production of 14CO₂ was maintained. These findings indicated intact function of the respiratory chain, Krebs cycle and β-oxidation and suggested impaired ketogenesis (HMG-CoA pathway). Accordingly, the formation of acetoacetate from acetyl-CoA by disrupted mitochondria was reduced in BDL rats at five (2.1±1.0 vs. 4.8±1.9 nmol/min/mg protein) and at ten days (1.7±1.0 vs. 6.2±1.9 nmol/min/mg protein). The principle defect could be localized at the rate-limiting enzyme of the HMG-CoA pathway, HMG-CoA synthase, which revealed decreased activity, and reduced hepatic mRNA and protein levels.

In conclusion, hepatic fatty acid metabolism is impaired in rats with short-term cholestasis. The import of fatty acids into the mitochondria, the activity of the electron transport chain as well as the activity of the Krebs cycle and the β-
oxidation are normal in liver mitochondria from BDL rats. Reduced ketogenesis is the main factor for this impaired fatty acid metabolism in rats after short-term bile duct ligation. Ketogenesis is impaired due to decreased mRNA levels of HMG-CoA synthase, leading to reduced hepatic protein levels and to decreased activity of this key enzyme of ketogenesis.

The reversibility of the impaired hepatic fatty acid metabolism in rats with long-term cholestasis had been investigated in the third project. Rats after Roux-en-Y anastomosis showed decreased mitochondrial fatty acid metabolism which is probably due to alterations in mitochondrial β-oxidation. This study aimed to investigate the role of mitochondrial β-oxidation and ketogenesis in rats with long-term cholestasis and after reversal of biliary obstruction in more detail.

In this project, the hepatic fatty acid metabolism was investigated in rats with bile duct ligation for 4 weeks (BDL rats), and 3, 7, 14, 28 and 84 days after reversal of biliary obstruction by Roux-en-Y anastomosis (RY rats), and in sham-operated control rats (CON rats). All animals were studied after starvation of 24 hours.

In comparison to controls, BDL rats had reduced β-hydroxybutyrate concentrations in plasma (0.25±0.10 vs. 0.75±0.20 mmol/L) and in liver (2.57±0.20 vs. 4.63±0.61 μmol/g), which increased significantly after restoring bile flow. Hepatic expression of carnitine palmitoyltransferase (CPT) I was unaffected in BDL rats and increased in RY rats. In contrast, CPT II expression was reduced in BDL rats and increased in RY rats, without reaching control values.

Oxidative metabolism of L-glutamate, succinate, palmitate, palmitoyl-CoA and palmitoylcarnitine by isolated liver mitochondria was reduced in BDL rats and recovered 7 to 14 days after reversal of biliary obstruction. Formation of acid soluble products and 14CO2 from 1-14C-palmitate by intact mitochondria was decreased in BDL rats and recovered 7 days after restoring bile flow. Formation of acetoacetate from acetyl-CoA by disrupted mitochondria was decreased in BDL rats and recovered after 3 months. Both mRNA and protein expression of HMG-CoA synthase, the rate limiting enzyme of the HMG-CoA cycle, was reduced in livers of BDL rats and increased significantly after reversal of biliary obstruction. While impairment of hepatic fatty acid metabolism is multifactorial in
BDL rats, impaired expression and activity of HMG-CoA synthase is the major factor after reversal of biliary obstruction.

In the fourth project, hepatic glycogen metabolism was investigated in patients with different types of liver cirrhosis. Patients with alcohol-induced liver cirrhosis have reduced hepatic glycogen stores. Due to the importance of hepatic glycogen metabolism in cirrhosis and the small database currently available, this study aimed to assess the following questions: i) are the hepatic glycogen stores decreased in all types of liver cirrhosis, independently of etiology ii) can the expected reduction be explained by the reduced content of hepatocytes only and iii) what are the principle mechanism for the expected reduction in the hepatic glycogen stores? Patients with different types of liver cirrhosis (n=22, alcoholic, cholestatic, viral) and control patients undergoing liver surgery (n=14) were studied in the post-absorptive state. The hepatic glycogen content, activities and mRNA expression of glycogen synthase and phosphorylase, as well as liver morphology were determined by standard methods. Cirrhotic and control patients were comparable regarding age, body weight, serum glucose, β-hydroxybutyrate and transaminase levels.

Cirrhotic patients had a reduced glycogen content per g liver (26±19 vs. 45±17 mg/g), per ml hepatocytes (39±27 vs. 50±21 mg/ml) and per liver (33.1±21.8 vs. 61.3±20.7 g). Regarding subgroups, the hepatic glycogen content was reduced in patients with alcoholic or biliary, but not in patients with viral cirrhosis. This finding was confirmed histologically, revealing that the decrease in hepatic glycogen in patients with alcoholic or biliary cirrhosis was not diffuse but showed lobular differences. Activities and mRNA expression of glycogen synthase and phosphorylase were not different between cirrhotic and control patients.

In conclusion, patients with alcoholic and biliary cirrhosis show decreased hepatic glycogen stores whereas patients with viral cirrhosis are not affected by a loss of hepatic glycogen. Maintained activity and expression of glycogen synthase and phosphorylase and the focal pattern of glycogen loss suggest that local hepatic factors are responsible for these findings.
ZUSAMMENFASSUNG


Die erste Studie lief folgendermaßen ab: Ratten wurden für 4 oder 8 Tage gallengangsligiert (BDL-Ratten) und entweder mit schein-operierten Kontroll-Ratten, welche zu den BDL-Ratten paargefüttert wurden (paargefütterte CON-Ratten) oder mit schein-operierten Kontroll-Ratten, welche ad libitum gefüttert wurden (ad libitum gefütterte CON-Ratten), verglichen. Alle Tiere wurden in gesättigtem Zustand untersucht. 4 Tage nach der Operation war der Glykogen Gehalt in der Leber in BDL-Ratten und paargefütterten CON-Ratten 21.6±7.6 bzw. 21.2±8.5 mg/g Leber. Die ad libitum gefütterten CON-Ratten indessen hatten einen Glykogen Gehalt von 72.9±7.7 mg/g Leber. 8 Tage postoperativ war der hepatische Glykogen Gehalt in den BDL-Ratten gegenüber den paargefütterten oder den ad-libitum gefütterten CON-Ratten signifikant reduziert (31.2±8.9 vs. 59.1±5.4 mg/g Leber, bzw. vs. 58.3±4.7 mg/g Leber). Der Glykogen Gehalt berechnet pro ml Hepatozyten oder pro Leber entsprach jenem berechnet pro Gramm Leber.

Die histologische Analyse der Lebern der BDL-Ratten bestätigte die Abnahme der hepatischen Glykogen-Reserven und zeigte, dass fast alle Hepatozyten beeinträchtigt wurden.
Zum Zeitpunkt beider Messungen waren bei den BDL-Ratten, verglichen mit den paargefüttert oder ad libitum gefütterten CON-Ratten, die totalen Enzymaktivitäten der Glykogen Synthase und der Glykogen Phosphorylase deutlich reduziert, während die aktiven Anteile dieser Enzyme nicht beeinträchtigt wurden. Die mRNA-Werte normalisiert zu GAPDH sowohl der Glykogen Synthase, wie auch der Glykogen Phosphorylase, hatten bei den BDL-Ratten, verglichen mit den paargefüttert CON-Ratten, zu beiden Zeitpunkten deutlich abgenommen.

Die Glucagon Konzentrationen im Plasma waren bei den BDL-Ratten gegenüber den paargefütterten CON-Ratten bei beiden Messungen signifikant höher, während die Insulin-Konzentrationen in BDL-Ratten 4 Tage postoperativ abgenommen hatten, jedoch 8 Tage danach wieder Normalwerte erreicht hatten.

In den Skelettmuskeln blieben der Glykogen Gehalt und die Enzymaktivitäten der Glykogen Synthase und Phosphorylase zum Zeitpunkt beider Messungen unverändert.

Folglich führt die akute Cholestase in Ratten zu einem raschen Abbau des hepatischen Glykogens, berechnet pro ml Hepatozyten, pro Gramm Leber und pro Leber. Die Enyzmaktivitäten und die Expression der Glykogen Synthase und Phosphorylase waren bei beiden Messungen reduziert.

Während die geringere Nahrungsaufnahme die Reduktion des Glykogen Gehaltes in der Leber von BDL und paargefütterten Ratten 4 Tage postoperativ zu erklären vermag, ist eine reduzierte Glykogen Synthese die wahrscheinlichste Ursache für den tieferen hepatischen Glykogen Anteil in BDL-Ratten 8 Tage nach dem Eingriff.

In der zweiten Studie wurde der hepatische Fettsäure-Metabolismus in Ratten, die für 5 oder 10 Tage gallengangsligiert (BDL-Ratten) wurden, untersucht. Die Resultate wurden mit paargefütterten Kontroll-Ratten verglichen (Kontroll-Ratten).

Alle Tiere wurden nach 24-stündigem Fasten untersucht. Die Konzentration von β-Hydroxybutyrat im Plasma in den BDL-Ratten war reduziert (0.54±0.10 vs. 0.83±0.30 mmol/l nach 5 und 0.59±0.24 vs. 0.88±0.09 mmol/l nach 10 Tagen in BDL, bzw. Kontroll-Ratten).

In der Tat war die Bildung von Acetoacetat aus Acetyl-CoA von aufgebrochenen Mitochondrien in BDL-Ratten nach 5 (2.1±1.0 vs. 4.8±1.9 nmol/min/mg Protein) und nach 10 Tagen reduziert (1.7±1.0 vs. 6.2±1.9 nmol/min/mg Protein). Die Hauptursache, die zu diesem Resultat führte, konnte in einem Defekt des geschwindigkeitsbestimmenden Enzmys des HMG-CoA Zyklus, der HMG-CoA Synthase, gefunden werden. Die HMG-CoA Synthase wies eine verringerte Aktivität, eine reduzierte hepatische mRNA und eine geringere Protein-Expression auf.


Die Ketogenese wird durch die verringerten mRNA-Werte der HMG-CoA Synthase, welche zu reduzierten hepatischen Protein-Werten und zu geringerer Aktivität dieses entscheidenden Enzmys der Ketogenese führt, beeinträchtigt. Die Reversibilität des beeinträchtigten Fettsäure-Metabolismus in Ratten mit Langzeitcholestase war Gegenstand der dritten Studie. Ratten mit Roux-en-Y Anastomose zeigten einen verringerten mitochondrialen Fettsäure-Metabolismus, was wahrscheinlich auf Veränderungen in der mitochondrialen β-Oxidation zurückzuführen ist. Die aktuelle Studie zielte darauf ab, die Rolle der mitochondrialen β-Oxidation und der Ketogenese in Ratten mit
Langzeitcholestase und nach Behebung des biliären Verschlusses genauer zu untersuchen.

In diesem Projekt wurde der hepatische Fettsäure-Metabolismus in Ratten, die während 4 Wochen gallengangsligiert waren (BDL-Ratten) und 3,7,14,28 und 84 Tage nach der Behebung des biliären Verschlusses durch Roux-en-Y Anastomose (RY-Ratten) und in schein-operierten Kontroll-Ratten (CON-Ratten) analysiert. Alle Tiere wurden nach 24-stündigem Fasten untersucht. Verglichen mit den CON-Ratten hatten BDL-Ratten reduzierte β-Hydroxybutyrat Konzentrationen im Plasma (0.25±0.10 vs. 0.75±0.20 mmol/L) und in der Leber (2.57±0.20 vs. 4.63±0.61 μmol/g), welche nach der Wiederherstellung des Gallenflusses signifikant anstiegen.

Die Expression der Carnitin-Palmitoyltransferase (CPT) I in der Leber war bei BDL-Ratten unbeeinflusst und in RY-Ratten leicht erhöht. Die CPT II Expression hingegen war in BDL-Ratten reduziert und stieg in RY-Ratten, ohne aber Kontrollwerte zu erreichen.

Der oxidative Metabolismus von L-Glutamat, Succinat, Palmitat, Palmitoyl-CoA und Palmitoylcarnitin in isolierten Leber-Mitochondrien war in BDL-Ratten verringert und stabilisierte sich 7 bis 14 Tage nach der Wiederherstellung des Gallenflusses auf Normalwerte.


In der vierten Studie wurde der hepatische Glykogen Metabolismus in Patienten mit verschiedenen Typen von Leber-Zirrhose untersucht. Es ist bekannt, dass

Patienten mit verschiedenen Arten von Leberzirrhose (n=22, alkoholisch, cholestatisch, viral) und Kontroll-Patienten, an welchen einen Eingriff an der Leber vorgenommen wurde (n=14), wurden im post-absorptiven Stadium untersucht.


Zirrhotische Patienten hatten einen reduzierten Glykogen-Gehalt pro g Leber (26±19 vs. 45±17 mg/g), pro ml Hepatozyten (39±27 vs. 50±21 mg/ml) und pro Leber (33.1±21.8 vs. 61.3±20.7 g). In den Untergruppen war der hepatische Glykogen-Gehalt in Patienten mit alkoholbedingter und bilärer Zirrhose reduziert, nicht aber in Patienten mit viraler Zirrhose. Dieses Resultat wurde histologisch bestätigt und macht deutlich, dass die Abnahme von hepatischem Glykogen in Patienten mit alkoholischer oder bilärer Zirrhose nicht diffus war, sondern lobuläre Unterschiede zeigte. Bei den Enzymaktivitäten und der mRNA Expression der Glykogen Synthase und Phosphorylase konnte zwischen zirrhotischen und Kontroll-Patienten kein Unterschied festgestellt werden.

vermuten, dass lokale Faktoren in der Leber für diese Resultate verantwortlich sind.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>APS</td>
<td>ammoniaperoxo sulfate</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BDL</td>
<td>bile duct ligation</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCl₄</td>
<td>carbon tetrachloride</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CoASH</td>
<td>reduced form of coenzyme A</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CPT I/II</td>
<td>carnitine palmitoyltransferase I/II</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N,N' tetraacetic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide (oxidized form)</td>
</tr>
<tr>
<td>FADH₂</td>
<td>flavin adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glycerinaldehydephosphate dehydrogenase</td>
</tr>
<tr>
<td>GLUT 2</td>
<td>glucose transporter 2</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl-CoA</td>
</tr>
<tr>
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<td>interleukin-1 beta</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-morpholino-propanesulfonic acid</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide (oxidized form)</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
</tbody>
</table>
mRNA  messenger RNA
MW  molecular weight
PCA  perchloric acid
PCR  polymerase chain reaction
PI3-kinase  phosphatidylinositol 3-kinase
RT  reverse transcription
RY  Roux-en-Y anastomosis
SDS  sodium dodecylsulfate
SLS  N-laurosyl sarcosine
SSC  sodium chloride-sodium citrate
TMPD  N,N,N',N'-tetramethyl-p-phenylenediamine
tRNA,  transfer RNA
TEMED  N,N,N',N'-tetramethylethylene-diamine
Tris  tris(hydroxymethyl)aminomethane
U  units
UDPG  uridine diphosphate glucose
vol  volume
wt  weight
1 INTRODUCTION

1.1 Liver function

The liver is the largest gland and the second largest organ in the body, averaging about 1500 g, or 2% of the body weight in a normal adult. Residing at the crossroads between the digestive tract and the rest of the body, the liver has the important job of maintaining the body's metabolic homeostasis. This includes the processing of amino acids, carbohydrates, lipids, and vitamins; synthesis of serum proteins (albumin, prothrombin, fibrinogen and other clotting factors); biotransformation of circulating metabolites; phagocytosis of particulate material in the splanchnic circulation; and the formation and excretion of bile (500 to 1000 ml/each day). Processing and redistribution of metabolic fuels, such as glucose and fatty acid, are thus major responsibilities of the liver. Acute or chronic liver injury can reduce the metabolic and synthetic capabilities of the liver, resulting in diverse clinical disorders (Crawford et al., 1994).

1.2 Hepatic architecture

The hepatic architecture is ideally suited for the liver's various metabolic functions. The functional unit of the liver is the hepatic acinus (Fig. 1).

![Fig. 1 Acinar organization of the liver.](image)

A model of the structural and functional features of the liver acinus is shown. Portal regions are identified as triangles containing bile ducts (BD), hepatic artery (HA), and portal vein (PV). Blood flow (arrows) is perpendicular to the portal triad and drains into the terminal hepatic vein (thV). The periportal (1), midzonal (2), and pericentral zones (3) are illustrated within the hepatic acinus.
Hepatocytes count for 90% of the liver's mass, but only 70% of all liver cells. Functional differences exist in pericentral hepatocytes, as compared with periportal hepatocytes, which surround the hepatic portal triad referred to as the periportal zone (Stolz, 1998). The periportal hepatocytes are enriched for gluconeogenetic enzymes and they are mostly responsible for liver regeneration, whereas the pericentral hepatocytes serve primarily for the cytochrom P450 induced metabolism of chemicals such as drugs (Reichen, 2000). Hepatocytes are polarized epithelial cells that are surrounded by three distinct membrane domains: (1) the sinusoidal membrane, which forms the sinusoidal space, (2) the canalicular membrane, which circumscribes the canaliculus and (3) the lateral hepatic membrane, which lies between adjacent hepatocytes.

Other important cell types of the liver are the epithelial cells of the bile ducts (cholangiocytes), the Kupffer cells, which are phagocytotic cells, and Ito cells (hepatic stellate cells, HSC), which store fat and vitamin A. The hepatic stellate cell is the major cell type responsible for synthesis of extracellular collagen and is a critical component of the fibrogenetic response to liver injury. Activation of hepatic stellate cells to a myofibroblast-like state is associated with collagen gene expression, reduction of the intracellular vitamin A content, and profound morphologic changes (Stolz, 1998, Reichen, 2000).

The liver's unique sinusoidal structure is well suited for the bidirectional transfer of a variety of solutes across the liver sinusoidal membrane. Fenestrae within the sinusoidal endothelium and the absence of a basal membrane permit direct contact of the portal blood with the hepatic sinusoidal surface in the subsinusoidal vascular space, the space of Disse (Stolz, 1998).

### 1.3 Pathophysiology of the liver

#### 1.3.1 Cirrhosis

Cirrhosis is among the top ten causes of death in the Western world. Alcohol abuse, chronic hepatitis, biliary disease and iron overload are the main factors leading to cirrhosis. The etiology varies both geographically and socially. Table 1 shows the approximate frequency of etiologic categories in the Western world:
<table>
<thead>
<tr>
<th>ETIOLOGIC FACTOR</th>
<th>FREQUENCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholic liver disease</td>
<td>60-70%</td>
</tr>
<tr>
<td>Viral hepatitis</td>
<td>10%</td>
</tr>
<tr>
<td>Biliary disease</td>
<td>5-10%</td>
</tr>
<tr>
<td>Primary hemochromatosis</td>
<td>5%</td>
</tr>
<tr>
<td>Wilson's disease</td>
<td>rare</td>
</tr>
<tr>
<td>Alpha1-antitrypsin deficiency</td>
<td>rare</td>
</tr>
<tr>
<td>Cryptogenic cirrhosis</td>
<td>10-15%</td>
</tr>
</tbody>
</table>

Tab. 1 Frequency of etiologic factors leading to liver cirrhosis.

Liver cirrhosis is defined by three characteristics: (1) fibrosis is present in the form of bands surrounding cells or broad scars replacing multiple adjacent lobules; (2) the parenchymal architecture of the entire liver is disrupted by interconnecting fibrous scars; (3) parenchymal nodules are created by regeneration of hepatocytes.

Fibrosis is a central feature of cirrhosis and it is therefore important to understand its development. In the normal liver, interstitial collagens (types I and III) are concentrated in portal tracts, with occasional bundles in the space of Disse and around central veins. The collagen framework between hepatocytes is composed of delicate strands of type IV collagen in the space of Disse. In cirrhosis, types I and III collagen are deposited in all parts of the lobule, resulting in severe disruption of blood flow and impaired diffusion of solutes between hepatocytes and plasma. The collagenization of the space of Disse leads to a loss of fenestrations in the sinusoidal endothelial cells and this impairs the movement of proteins between hepatocytes and plasma (Crawford et al., 1994). The major source of excess collagen in cirrhosis appears to be the Ito cell (Maher et al., 1990). The stimuli for deposition of fibrous tissue are not yet clear, but there are several factors proposed which promote fibrosis, for example inflammatory mediators, such as tumor necrosis factors alpha and beta (Crawford et al., 1994).

The clinical features and complications of cirrhosis are common to all forms of the disease regardless of its cause. Cirrhosis is usually latent for many years,
therefore the period of time between the first clinical manifestation and death is usually short. During the long latency period, liver function gradually gets worse. Early symptoms are non-specific and include fatigue, anorexia, dyspepsia, flatulence, a change in bowel habits, slight weight loss, and sometimes nausea and vomiting. Liver failure, portal hypertension and hepatic encephalopathy are major clinical manifestations which occur late during the disease (Wilson et al., 1997). Portal hypertension, a clinical manifestation of cirrhosis, results from increased resistance to portal flow at the level of the sinusoids and compression of the central veins by fibrosis and parenchymal nodules. The clinical consequences of portal hypertension are ascites, the formation of portosystemic venous shunts, and congestive splenomegaly (Crawford et al., 1994).

1.3.1.1 Alcoholic cirrhosis
Chronic alcohol consumption has a variety of adverse effects and leads to mainly three forms of liver disease: (1) hepatic steatosis, (2) alcoholic hepatitis and (3) alcoholic cirrhosis, collectively referred to as alcoholic liver disease (Crawford et al., 1994). Alcoholic cirrhosis, also called Laennec's cirrhosis, accounts for about 50% or more of the cases of cirrhosis in Western countries. The first alteration in the liver caused by alcohol is the gradual accumulation of fat within the liver cells, called fatty infiltration or steatosis. The consequences of this fatty infiltration are various metabolic disturbances, such as excess formation of triglycerides, decreased export of triglycerides from the liver and decreased oxidation of fatty acids due to mitochondrial dysfunction. Patients ingesting excessive amounts of alcohol may also not eat healthy (Wilson et al., 1997). Substantial use of alcohol has considerable effects on the nutritional status. Primary malnutrition is caused by avoiding other nutrients in the diet because of the high energy content of alcoholic beverages. Secondary malnutrition may result from either maldigestion or malabsorption of nutrients caused by gastrointestinal complications associated with alcoholism, involving the pancreas and the small intestine. Such nutritional deficiencies concern thiamine, folic acid, pyridoxine, niacin, ascorbic acid, vitamin A and K as well as calcium, iron and zinc (Lieber, 2000).
In an early state of alcoholism, the fatty degeneration of the liver is reversible, if the person stops drinking excessive amounts of alcohol. It is still not clear why not all patients with excessive alcohol consumption develop cirrhosis. Although a alcoholic hepatitis is considered to be a critical lesion in the development of cirrhosis, not all patients with this lesion progress to cirrhosis. In advanced cases of Laennec’s cirrhosis, thick fibrous bands partition the parenchyma into fine nodules, a condition called “fine nodular cirrhosis”. In the final stage, the liver is shrunken, hard and has almost no parenchyma. Patients with Laennec’s cirrhosis have an increased risk of developing primary liver cell carcinoma (Crawford et al., 1994).

1.3.1.2 Biliary cirrhosis
Liver cell destruction that begins around the bile ducts belongs to a pattern of cirrhosis known as biliary cirrhosis (Wilson et al., 1997). The most common cause of obstruction is an impacted gallstone in the common bile duct; other conditions include biliary atresia, malignancies of the biliary tree and head of the pancreas, and strictures resulting from previous surgical procedures (Crawford et al., 1994). The liver is enlarged, firm, and finely granular and has a green hue. Jaundice, pruritus, malabsorption and steatorrhea are early symptoms of biliary cirrhosis (Wilson et al., 1997).

1.3.1.3 Postnecrotic cirrhosis
Postnecrotic cirrhosis accounts for about 10% of the cases of cirrhosis. About 25% to 75% of the patients with postnecrotic cirrhosis have a prior history of viral hepatitis. Only a small percentage of these cases are the result of intoxication with industrial chemicals, poisons or drugs, such as yellow phosphorus, oral contraceptives, methyldopa, arsenicals, or carbontetrachloride. The main morphologic features of postnecrotic cirrhosis reveal hepatocytes surrounded and partitioned by scar tissue, and an excessive loss of hepatocytes.
As well as patients with alcoholic liver cirrhosis, patients with postnecrotic cirrhosis appear to be predisposed to develop a hepatocellular carcinoma (Wilson et al., 1997).
1.4 Liver tests

There are different tests available to detect and classify liver function. No single test is capable of measuring the total liver function, since the liver is involved in almost every metabolic process in the body and has a large functional reserve; usually a combination of different tests is used (Wilson et al., 1997). The transaminases AST and ALT, the alkaline phosphatase and γ-glutamyl-transpeptidase do not represent a liver function. They are synthesized in liver and to different extents, also in extrahepatic tissue (Reichen, 2000). The transaminases are intracellular enzymes in the heart, liver and skeletal muscle. They are released from damaged tissue (necrosis or altered plasma membrane permeability) and are thus increased in liver cell damage and in other conditions, especially myocardial infarction (Wilson et al., 1997). In most cases, both transaminases are measured, because AST is more sensitive but less specific than ALT and the AST/ALT ratio can give an initial idea of the etiology of the liver disease (Reichen, 2000). Alkaline phosphatase is found in bone, liver, kidney and intestine and is excreted into bile. Therefore increased levels of alkaline phosphatase can reflect biliary obstruction, liver metastasis or bone disease (Wilson et al., 1997). This increase of alkaline phosphatase is due to its re-synthesis, and elevated levels can be measured only hours or days after the acute process. (Reichen, 2000). Bilirubin is the end product of heme degradation. Unconjugated bilirubin, bound to albumin, is transported via the blood to the liver cells. After its uptake, conjugation with one or two glucuronides is catalysed by the enzyme glucuronyltransferase. Conjugated bilirubin is water soluble, non-toxic and can be excreted in both the bile and the urine (Wilson et al., 1997). An increase in unconjugated bilirubin is mainly due to extrahepatic disorders, such as haemolytic anemias, whereas an increase in conjugated bilirubin reflects hepatic dysfunction. Increased serum bile acids also indicate impaired hepatic function (Reichen, 2000).

1.5 Liver metabolism

A major task of the liver is to provide a continuous energy supply for the entire body. Hormonal and neuronal regulation is important for the hepatic metabolic
function. On the one hand, the liver regulates nutrient flux during periods of nutrient absorption (meals). Absorbed nutrients are either metabolised or modified for storage in the liver and/or fat tissue or are made available to the remaining organs as sources of energy metabolism. On the other hand, during nonabsorptive periods such as fasting, the liver is responsible for the metabolic requirements (Bollen et al., 1998).

1.6 Carbohydrate metabolism

1.6.1 Regulation of intrahepatic glucose concentration

Glucose is a central component of the metabolic pathway: it can be converted to either amino acids, fatty acids, or glycogen. Glucose enters or leaves the liver by GLUT 2, a member of a family (GLUT 1-8) of membrane glucose transporters characterized by 12 membrane-spanning domains. GLUT 2 is mainly expressed in the liver, but also in the intestine, kidney and pancreas. It is a low-affinity, high-capacity glucose transport protein, that is different from GLUT 1,2,4, which have a much greater affinity for glucose (Nordlie et al., 1999). It promotes either glucose uptake from or efflux to the sinusoidal space, which is of critical importance during fasting conditions. Its high Km (60 mmol) is well above the peak glucose levels detected during meals or renal transport capacity, thereby ensuring continuous uptake of glucose by the liver.

Glucose homeostasis is maintained in the hepatocyte by interdependent pathways that serve as branch points for the metabolic pathways of glucose metabolism. These interlinking pathways are regulated and integrated by multiple signals that prevent competing pathways from operating at the same time (Stolz, 1998, Fig.2). Figure 2 shows the hepatic carbohydrate and lipid metabolism:
Fig. 2 Hepatic carbohydrate and lipid metabolism.

Gluconeogenic pathways are identified by dashed lines. GK, glucokinase; Glu-6-Pase, glucose-6-phosphatase; 6-Fru Kinase/Pase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Fru 6-P, fructose-6-phosphate; 6PF-1-K, 6-phosphofructo-1-kinase; Fruc-1,6-P2ase, fructose-1,6-bisphosphatase; PK, pyruvate kinase; PEPCK, phosphoenol pyruvate carboxykinase; CPT, carnitine palmitoyltransferase; Glut-2, glucose transporter 2; T, carnitine:acylcarnitine transferase; PEP, phosphoenol pyruvate; PYR, pyruvate; OAA, oxaloacetate; UDPG, uridine diphosphate glucose.
1.6.2 Glycogen metabolism

1.6.2.1 Glycogen

Glycogen is a very large, branched polymer of glucose residues and it is the storage form of glucose (Stryer, 1995). Most mammalian cells store glycogen as a reserve for the production of glucose 6-phosphate which is the metabolic fuel for glycolysis, whereas the liver glycogen serves mainly as a glucose reservoir for other tissues. As a consequence, the level of hepatic glycogen changes with the feeding conditions. The hepatic contribution of glycogenolysis to total glucose production during the first day of starvation varies from 40 to 80%. During longer starvation periods, the hepatic glycogen stores become depleted and the contribution of gluconeogenesis becomes predominant (Bollen et al., 1998). In the gluconeogenetic pathway, pyruvate is converted into glucose. Noncarbohydrates, such as lactate, amino acids and glycerol, can also serve as glucose precursors. Lactate is formed by active skeletal muscle when the rate of glycolysis exceeds the metabolic capacity of the citric acid cycle and the respiratory chain. Amino acids are derived mainly from skeletal muscle (e.g. during starvation). The hydrolysis of triacylglycerides in fat cells yields glycerol and fatty acids (Stryer, 1995, Stolz, 1998).

The general mechanism of glycogen synthesis and degradation is the same in all tissues. The biogenesis of a glycogen molecule starts with the formation of a short oligosaccharide chain (up to 10 residues long), covalently attached to a tyrosine residue in a specialized initiator protein, called glycogenin. This primed glycogenin then serves as a substrate for elongation by glycogen synthase, which, together with the branching enzyme, synthesize the polysaccharide glycogen. A mature glycogen particle has a bush-like structure with branches that form a left-handed helix with 6.5 glucose residues per turn (Bollen et al., 1998).

The degradation of glycogen requires the active form of glycogen phosphorylase and the bifunctional debranching enzyme. The debranching enzyme is able to act as a transferase as well as a glucosidase. In the presence of phosphate, phosphorylase releases the terminal glucose residue of an external chain as glucose-1-phosphate. This process is repeated until the
external chain has been shortened to four glucose units. Subsequently, the transferase activity of the debranching enzyme removes a maltotriose unit from the α-1,6 linked part and attaches it through an α-1,4-glucosidic bond to the free C-4 of the main chain. The remaining single α-1,6 linked glucose unit is then liberated as glucose by the debranching enzyme, acting as α-glucosidase. Additional α-1,4 linked glucose residues are removed by the active form of glycogen phosphorylase (Bollen et al., 1998).

Glycogen is present in the cytosol as granules, which contain regulatory proteins as well as the two enzymes for the synthesis glycogen synthase and for the degradation glycogen phosphorylase (Stryer, 1995).

1.6.2.2 Glycogenin
Glycogenin is a small (37 kDa), self-glucosylating protein involved in the initiation phase of glycogen biosynthesis. In 1975, Krisman and Barengo reported that a liver pellet could synthesize from UDP-glucose, a glycogen-like product precipitable by diluted trichloroacetic acid (Krisman et al., 1975). They proposed that a protein, and not a carbohydrate, was the original primer of glycogen synthesis. Further studies about this primer (called “glycogenin”) were performed, mainly of mammalian muscle glycogenin. The muscle glycogenin was characterized to be autocatalytic, stimulated by Mn^{2+}, and, after it had glucosylated itself, primed glycogen synthesis. The stoichiometry between glycogen and glycogenin molecules in muscle was proposed to be 1:1 (Alonso et al., 1995). In contrast, in liver a 1:1 stoichiometry relationship between glycogen and glycogenin is not present, reflecting the much higher molecular mass of liver glycogen compared to muscle glycogen. It is suggested that liver glycogenin molecules are able to prime more than one glycogen particle (Alonso et al., 1995).

The first glycogenin characterized at the molecular level was from mammalian muscle (glycogenin-1). In 1997, Mu et al. described the existence of glycogenin-2, encoded by a second gene that is expressed in the liver, the heart and, to a lesser extent, in the pancreas. The glycogenin-2 sequence is to 72% identical to glycogenin-1 over the NH₂-terminal 200 residue segment which is considered to contain the catalytic domain. Glycogenin-2 contains required properties: it is
self-glucosylating, it can act as a substrate for glycogen synthase, and is released from glycogen by α-amylase treatment (Mu et al., 1997). These results suggested that glycogenin-2 may play an important role in the metabolism of liver glycogen. Mu et al. showed, that overexpression of glycogenin-2 in Rat-1 fibroblasts resulted in a significant increase in glycogen accumulation in the low speed pellet fraction (Mu et al., 1998). Further characterization of the glycogenin-2 protein revealed that it is also present in human liver (0.15 mg/g of tissue) and covalently linked to carbohydrate. The glycogenin-1 protein was also detected in human liver, but its amount was not measurable (Mu et al., 1998).

The discovery of glycogenin as a self-glucosylating protein that primes glycogen synthesis has much contributed to the understanding of the structure and metabolism of glycogen. However, its role in glycogen metabolism on the molecular level needs to be evaluated in further studies.

1.6.2.3 Glycogen synthase

Glycogen synthase, a homodimer of 81 kDa protomers, is controlled by reversible phosphorylation of multiple serine residues, which induces an inactivation of the enzyme (glycogen synthase b, glycogen synthase a = active form, see Fig. 3).

*In vitro*, liver glycogen synthase is phosphorylated by different protein kinases (protein kinases A and C, phosphorylase kinase, GSK-3, Ca^{2+}- and calmodulin-dependent protein kinase II and AMP-stimulated protein kinase), but it still needs to be evaluated which kinases exactly phosphorylate glycogen synthase. *In vivo* (Bollen et al., 1998). These different protein kinases are activated by hormones such as glucagon, adrenalin or vasopressin (Bollen et al., 1998, Roden et al., 1996, Ercan et al., 1995). The liver glycogen synthase is activated by glucose (Lang et al., 1986), AMP (Carabaza et al., 1990) and glucose 6-phosphate, which is an allosteric activator of glycogen synthase b and also promotes its dephosphorylation (Bollen et al., 1998). *In vitro*, physiological concentrations of glucose stimulate the synthase phosphatase activity, but only in the presence of a second effector such as caffeine, certain methylxanthines and ADP (Gilboe, 1990).
Based on in vitro studies, the pseudo activation as well as the existence of a still-unidentified stimulator of glycogen synthase need to be explored in more detail (Wera et al., 1996, Nuttal et al., 1993, Gilboe, 1990).

1.6.2.4 Glycogen phosphorylase
There are three isoenzymes of glycogen phosphorylase, the "liver", "muscle" and "brain" isoenzymes, depending on where they are preferentially expressed. The liver glycogen phosphorylase is a homodimer of about 100 kDa and can be converted from its inactive \( b \)-form into the active \( a \)-form through phosphorylation of Ser\(^{14} \) by phosphorylase kinase (Fig. 3). The corresponding protein kinase is activated by cAMP-dependent hormones like glucagon and \( \beta \)-adrenergic agonists, or calcium-dependent agents such as vasopressin, parathyroid hormone, angiotensin II, and \( \alpha 1 \)-adrenergic and P\(_2\)-purinergic agonists. (Bollen et al., 1998, Keppens et al., 1993).

The muscle and brain isoenzymes are in addition allosterically activated by AMP and inhibited by glucose-6-phosphate, which enables these enzymes to react to intracellular signals. In contrast, phosphorylation is more important than allosteric regulation for the liver isoenzyme. From a functional viewpoint, the poor allosteric control of liver glycogen phosphorylase is not unexpected, since this isoenzyme is mainly designed to respond to extracellular signals that are involved in the maintenance of the blood glucose level. These signals control glycogenolysis primarily by modulating the phosphorylation state of the phosphorylase (Bollen et al., 1993).

Since the glucose concentration in the blood and in the hepatocytes is the same, phosphorylase acts as a sensor for the blood glucose level. The binding of glucose to the phosphorylase \( a \) does not only lead to a competitive inhibition of phosphorylase \( a \), but makes it more susceptible to inactivation by dephosphorylation (Bollen et al., 1998).

Endotoxins or acute phase proteins, such as IL-1\( \beta \) can affect the glycogen metabolism by stimulation of glycogenolysis. They induce the secretion of prostaglandin \( D_2 \) by Kupffer cells or activate glycogen phosphorylase directly (Casteleijn et al., 1988, Goto et al., 1993, Lee et al., 1993).
1.6.2.5 Insulin and glucagon

It is well established, that the balance between insulin and glucagon is essential for the blood glucose homeostasis. Insulin is involved in the regulation of glycogen synthase and phosphorylase. It decreases glycogenolysis and promotes glycogen synthesis in the liver through different biochemical pathways, where phosphatidylinositol-3-kinase (PI3) plays a key role. PI3 is able to inactivate glycogen synthase kinase 3 after several reactions, which results in a smaller phosphorylation state of glycogen synthase. This leads to more glycogen synthase a, which finally promotes glycogenesis. In addition, PI3-kinase activates a cAMP phosphodiesterase, leading to a lower concentration of the glycogenolytic agent cAMP, which results in increased glycogenesis.

In contrast, glucagon promotes glycogenolysis and reduces glycogen synthesis in the liver by different biochemical reactions. Activation of the glucagon receptor increases the amount of cAMP, which initiates a signalling cascade, leading to the activation of glycogen phosphorylase. Phosphorylase a not only...
increases the rate of glycogenolysis, but also antagonizes glycogen synthesis by inhibiting its dephosphorylation (Bollen et al., 1998).

1.6.3 Glycogen metabolism in rats and humans with liver cirrhosis and chronic cholestasis

Patients and rats with different types of liver cirrhosis have reduced hepatic glycogen stores. Rats with CCl₄-induced liver cirrhosis, as well as rats with secondary biliary cirrhosis induced by bile duct ligation (BLD) for 4 weeks, provide useful animal models to study changes in energy metabolism induced by chronic liver disease. Krähenbühl S. et al. showed in 1991, that hepatic glycogen content in fed animals with CCl₄-induced liver cirrhosis is decreased per hepatocyte and per gram liver (Krähenbühl, S. et al., 1991a). Moreover, rats with secondary biliary cirrhosis induced by bile duct ligation for 2 weeks or for 4 weeks show a progressive decrease in the glycogen stores per gram of liver, per milliliter hepatocyte or per liver (Krähenbühl, L. et al., 1996). This distinction is important, since the volume fraction of hepatocytes decreases in cirrhotic livers and a reduced glycogen content per gram of liver does automatically imply a reduced glycogen content per hepatocyte (Krähenbühl, L. et al., 1996). Humans with alcohol-induced liver cirrhosis have also been described to have reduced hepatic glycogen stores. Owen et al. studied 8 patients with alcohol-induced liver cirrhosis and compared their results with values obtained in 2 control patients (Owen et al., 1981). The results are only given as glycogen per gram of liver wet weight but not per hepatocyte. Therefore it is not known, whether this reduction can be explained by a reduced glycogen content per hepatocyte or only by a decrease in the volume fraction of hepatocytes. Moreover, these results count only for patients with alcoholic liver cirrhosis and not for subjects with viral or biliary cirrhosis.

The metabolic consequences of the hepatic glycogen depletion have been studied in rats with CCl₄-induced liver cirrhosis. When cirrhotic rats are starved, the hepatic glycogen content is more rapidly exhausted, and the increases in the plasma fatty acid, β-hydroxybutyrate and acylcarnitine concentrations appear earlier than in pair-fed control animals (Krähenbühl, S. et al., 1991a). In agreement with these results, cirrhotic patients who fasted overnight have a
metabolic profile that resembles normal humans who fasted for 36-72h, in which fat is the main oxidized fuel (fat: 69%, carbohydrate: 13%, protein: 17%, Owen et al., 1983). Furthermore, hepatic glucose production is reduced in fed rats with CCl₄-induced liver cirrhosis, both under basal conditions and after stimulation with glucagon/L-alanine (Krähenbühl, S. et al., 1993). The reduction in hepatic glucose production in the fed state may be a result of decreased glycogenolysis due to hepatic glycogen depletion. Finally, the results obtained in this study may explain decreased glucose production in patients with cirrhosis following glucagon administration.

The mechanisms leading to this reduction in the hepatic glycogen content are not completely clear. The decrease of glycogen per gram of tissue in cirrhotic livers can partially be explained by the known reduction in the volume fraction of hepatocytes (Krähenbühl, L. et al., 1996). However, since the glycogen content is also reduced per hepatocyte, additional mechanisms must play a role. In rats with secondary biliary cirrhosis, the activities of both glycogen synthase and glycogen phosphorylase were decreased suggesting reduced synthesis of glycogen may be essential. In contrast, these enzymes were not affected in rats with CCl₄-induced liver cirrhosis, suggesting that other mechanisms are responsible for this reduction of the hepatic glycogen content. In 1999 the reversibility of the observed changes in the hepatic glycogen metabolism in rats with long-term bile duct ligation was studied. The rats were observed after bile duct ligation for 4 weeks, or 5 or 14 days after relief of biliary obstruction by Roux-en-Y anastomosis. The liver glycogen content was decreased in BDL rats and normalized within 5 days after RY. Glycogen synthase and glycogen phosphorylase had become completely normal fourteen days after RY (Krähenbühl, L. et al., 1999).

In contrast to the hepatic glycogen content, the skeletal muscle glycogen content has been found to be maintained, but varies in patients with different types of liver cirrhosis (Kruszynska et al., 1988, Selberg et al., 1994). In experimental animals, the studies mentioned above show maintained skeletal muscle glycogen stores in rats with CCl₄-induced liver cirrhosis (Krähenbühl, S. et al., 1991a) but decreased stores in rats with secondary biliary cirrhosis (Krähenbühl, L. et al., 1996).
1.7 Hepatic fatty acid metabolism

1.7.1 The role of fatty acids in the liver

Fatty acids play an important role as an energy source for the liver as well as a storage form of fuel both within and outside the liver. In contrast to the glucose pathway, the oxidation of fatty acids to CO$_2$ and water has the highest ATP production and thus is the most efficient long-term storage form of energy. Synthesis and metabolism of fatty acids are regulated by various factors (Fig. 2), and the liver plays a central role in the regulation of the body's total fatty acid needs (Stolz, 1998).

1.7.2 Mitochondrial metabolism

1.7.2.1 Oxidative metabolism: electron transport chain

The electron transport chain is located in the inner mitochondrial membrane and is composed of four electron transport complexes and of the electron shuttles ubiquinone (Q) and cytochrome c. The electron transport complexes are (I): NADH:ubiquinone oxidoreductase, (II): succinate : ubiquinone reductase, (III): ubiquinol : ferricytochrome c oxidoreductase and (IV): ferrocytochrome c : oxygen oxidoreductase (Fig. 4). Substrates which produce NADH (e.g. L-glutamate and fatty acids) feed the electron transport chain at complex I. Succinate enters the chain at complex II, duroquinone at complex III and ascorbate at complex IV. Molecular oxygen finally accepts the electrons flowing through the chain at complex IV (Krähenbühl, S., 1994). The electron transport chain transports electrons from NADH or FADH$_2$ to molecular oxygen and produces a transmembranous proton gradient (Δp, Fig. 4) used for the generation of ATP and for other reactions. Therefore, a failure in the production of the transmembranous proton gradient impairs not only ATP synthesis, but also leads to a reduced uptake of substrates for mitochondrial metabolism. Most importantly, it may also inhibit the uptake and processing of newly synthesized proteins. An adequate mitochondrial function is very essential for the survival of a given cell and also for provision of functionally intact mitochondria to new cells. The function of isolated mitochondria can be assessed by studying...
oxidative metabolism using the oxygen electrode. By choosing different substrates, a specific complex of the electron transport chain can be analysed. (Krähenbühl, S. et al., 1992).

- ATP synthesis
- Transport of metabolites, electrolytes and nucleotides
- Uptake and processing of proteins for mitochondrial biogenesis

\[ \text{NADH} + \text{H}^+ \rightarrow \text{FMN} \rightarrow \text{Q} \rightarrow \text{b, c, } \rightarrow \text{c} \rightarrow \frac{1}{2} \text{O}_2 \]

\[ \text{succinate} \leftarrow \text{fumarate} \]

Fig. 4 The electron transport chain.

The electron transport chain is located in the inner mitochondrial membrane and consists of 4 enzyme complexes (complexes I, II, III and IV) and of the electron shuttles ubiquinone (Q) and cytochrome c. The cytochromes are indicated with small letters (a, a₃, b, c, cᵢ). NADH is the substrate for complex I, succinate for complex II and ascorbate for complex IV. The energy gained from electron transport from the substrates to oxygen is stored as an electrochemical potential (\(\Delta p\)) which is used for ATP synthesis, transport processes, importing and processing of exogenous proteins and mitochondrial protein synthesis for mitochondrial biogenesis.

1.7.2.2 Mitochondrial β-oxidation

Beta-oxidation of fatty acids provides an essential fuel source for multiple organs, including the liver. In contrast to fatty acid synthesis, which occurs in the cytosol of the cell, the β-oxidation of fatty acids takes place in the mitochondrion. The mitochondrion contains a leaky outer and inner membrane which is impermeable to most molecules. Selective mitochondrial transport
systems allow for shuttling of substrates required for mitochondrial metabolism such as β-oxidation (Stolz, 1998). Long chain fatty acids cross the plasma membrane of target cells and are activated to acyl-CoAs in the cytoplasm by an ATP-utilizing acyl-CoA synthase. The acyl-CoAs, which can not traverse the mitochondrial membranes without modification, are esterified to acyl-carnitines by CPT I and transported across the inner mitochondrial membrane, in exchange for carnitine, by the carnitine/acylcarnitine translocase (Indiveri et al., 1997). Within the mitochondrion, CPT II catalyses the reverse reaction, converting acyl-carnitines back to acyl-CoAs, which are now substrates for β-oxidation (Fig. 5). Medium and short-chain fatty acids are able to cross the mitochondrial membranes independently of CPT I, the carnitine/acylcarnitine translocase and CPT II, and are activated to the CoA-esters by ATP-utilizing reactions in situ before undergoing β-oxidation. The β-oxidation cycle comprises a series of four enzyme-catalysed reactions which cleave a two carbon acetyl-CoA part at each complete cycle (Fig. 5). The residual part re-enters the cycle for further oxidation until two acetyl-CoA molecules are produced. Two oxidation steps release the reducing equivalents FADH₂ and NADH, which transfer their electrons to the electron transport chain. Acetyl-CoA, the product of β-oxidation, can be transported out of the mitochondria by the citrate carrier and can then be used as a substrate for lipogenesis. It can be further oxidized to CO₂ by the reactions of the Krebs cycle in the mitochondrial matrix or can be converted into the ketone bodies acetoacetate or β-hydroxybutyrate by the enzymes of the HMG-CoA cycle (Quant, 1994).
Fig. 5  Hepatic fatty acid metabolism.
CPT I/II, carnitine palmitoyltransferase I/II; HMG-CoA synthase, 3-hydroxy-3-methylglutaryl coenzyme A synthase; o.m., outer mitochondrial membrane; i.m., inner mitochondrial membrane.
1.7.2.3 Ketogenesis

Ketogenesis is a metabolic process that occurs exclusively in the mitochondrial compartment of certain cells in most species, and produces ketone bodies, i.e. acetoacetate and β-hydroxybutyrate, from fatty acids (Fig. 5). Acetone is formed by the non-enzymic breakdown of acetoacetate and is unlikely to be important in the metabolism of the intact animal (Hegardt, 1999). Liver mitochondria are the main producers of ketone bodies, but kidney, small intestine and white adipose tissue mitochondria also synthesize ketone bodies in some metabolic states or during certain stages of development. Ketone bodies are small soluble “fat” molecules. They serve as oxidizable chemical fuels for the brain and peripheral tissues in some metabolic (prolonged starvation), nutritional (high-fat diet) and developmental (during suckling) states. (Quant, 1994). In the transition from the fed to the fasted condition, carbohydrate utilization and fatty acid synthesis in the liver cease and are replaced by the oxidation of fatty acids and the induction of ketogenesis. The influence of fatty acids on ketogenesis depends on the metabolic state of the organism (fed, fasted or diabetic states) (Hegardt, 1999). Due to their ability to cross the blood-brain barrier, ketones can supply up to 75% of the brain's energy needs form oxidation during glycogen depletion. Apart from being important metabolic substrates, ketone bodies are also substrates for myelination of the neonatal brain and, to a lesser extent, precursors for lipogenesis in the lactating mammary gland (Quant, 1994).

1.7.2.3.1 Regulation of ketogenesis

Hepatic ketogenesis is controlled internally by the liver as well as externally by other systems. External controls may include those reactions involved in generating and releasing fatty acids from adipose tissue, regulation of blood flow, secretion of pancreatic hormones, and nervous inhibition. It is also controlled by those reactions producing ketone bodies in the kidney or intestine, and those in the brain and peripheral tissues that oxidize ketone bodies or use them as substrates for lipogenesis and myelin formation. Intrahepatic control reactions include activation of long-chain fatty acids by acyl-CoA synthase, transport of acyl-CoAs into mitochondria involving the CPT I/II-system and the carnitine/acylcarnitine translocase, the enzymes of β-oxidation, the HMG-CoA
pathway and/or the Krebs cycle and efflux of ketone bodies from the mitochondria via pyruvate exchange (Quant et al., 1993)

The physiological rate of ketogenesis varies. However, it is elevated under conditions where the glucagon to insulin ratio is high: during high fat diet, during late pregnancy, during lactation and during prolonged starvation when ketones provide an alternative fuel to spare glucose levels. Under these conditions, the elevated plasma levels are beneficial to the individual and homoeostatic mechanisms ensure that they never exceed safe levels (8 mM, in humans, physiological ketosis). However, with some diseases, e.g. juvenile onset diabetes, where glucagon levels are high and insulin levels are low, the plasma ketone body concentrations can exceed 20 mM (pathological ketosis). The inability of the blood to buffer such a high acid load can result in coma and death. These observations suggest that hormones, nutrition and development are causes of ketogenesis (Quant, 1994).

Moreover, ketogenesis is also controlled within the mitochondria. Such internal controls include mainly two systems: (i) entry of fatty acyl-CoA into mitochondria catalysed by the CPT system (McGarry et al., 1989), and (ii) enzymic activity of mitochondrial HMG-CoA synthase (Quant et al., 1993). CPT I, located in the outer mitochondrial membrane (Fig. 5), seems to be important for the control of entry and β-oxidation of fatty acids in the mitochondria. The activity of CTP I, but not of CPT II, is controlled by malonyl-CoA inhibition (Madsen et al., 1999). Long-term control of CPT I activity by malonyl-CoA seems to play an important role in regulation of ketogenic flux in livers of normal fed adult rats, but the capacity of CPT I and its effect to control ketogenesis has not been measured directly. Moreover, shorter-term controls as well as intramitochondrial sites are discussed. In fact, Quant et al. provide much evidence that HMG-CoA synthase, the second enzyme of ketogenesis (Fig. 5), is also an enzyme which controls the rate of ketogenesis (Quant et al., 1993).

1.7.2.3.2 HMG-CoA synthase

HMG-CoA synthase catalyses the condensation of acetoacetyl-CoA and acetyl-CoA, derived from β-oxidation of fatty acids, to form HMG-CoA plus free CoA. Its activity occurs in two different compartments: the cytosol and the
mitochondria. Cytosolic HMG-CoA is transformed into mevalonate by the action of HMG-CoA reductase. This starts the isoprenoid pathway which produces mainly cholesterol, but also produces other important products such as ubiquinone, isopentenyl adenosine, and farnesyl groups. In the mitochondria, HMG-CoA is transformed to acetoacetate by the action of HMG-CoA lyase (Fig. 5), (Hegardt, 1999).

In 1975, the two isoforms were purified and characterized as having two different chemical natures by Lane's group (Clinkenbeard et al., 1975a; Reed et al., 1975; Clinkenbread et al., 1975b). In 1986, the cloning and the sequencing, first of cDNA(Gil et al., 1986a) and then of the gene (Gil et al., 1986b) of hamster cytosolic HMG-CoA synthase was reported by Goldstein and Brown. The existence of two genes was finally established in 1990 by cloning first the cDNA (Ayté et al., 1990) and then by cloning the gene (Gil-Gomez et al., 1993) for the rat mitochondrial HMG-CoA synthase by Hegardt. Although the percentage of identical amino acid residues between these two synthases was high (65%), it became clear that they were the products of two different genes. Williamson et al. were the first to propose that mitochondrial HMG-CoA synthase is the rate limiting enzyme of ketogenesis in his studies of acetoacetate production in sonicated liver particles (Williamson et al., 1968). This hypothesis was later confirmed by other studies using mitochondrial subfractions (Clinkenbread et al., 1975a).

HMG-CoA synthase is a homodimer of 53-57 kDa monomers (Reed et al., 1975). Its synthesis occurs in three steps. The first, which is rate limiting, is the acetylation of the enzyme in a cysteinyl thiol group. These three reaction are:

1. \[ \text{EnzSH} + \text{acetyl-CoA} \rightarrow \text{acetyl-SEnz} + \text{CoASH} \]
2. \[ \text{Acetoacetyl-CoA} + \text{acetyl-Enz} \rightarrow \text{EnzS-HMG-CoA} \]
3. \[ \text{EnzS-HMG-CoA} + \text{H}_2\text{O} \rightarrow \text{EnzSH} + \text{HMG-CoA} \]

The formation of HMG-CoA proceeds by a Bi Bi Ping Pong kinetic mechanism, in which the enzyme (EnzSH) reacts first with acetyl-CoA to form a covalent acetyl-enzyme intermediate (acetyl-SEnz), with the release of CoA. This
intermediate then condenses with acetoacetyl-CoA to form HMG-CoA bound to the enzyme (EnzS-HMG-CoA), which is then liberated by hydrolysis (Hegardt, 1999). Lowe and Tubbs purified mitochondrial HMG-CoA synthase from ox liver and characterized the enzyme. They showed that the purified synthase catalyses its own succinylation and inhibition by succinyl-CoA: the enzyme binds succinyl-CoA at the active site, in place of acetyl-CoA, to form a stable, succinyl-intermediate, which is not able to bind the second substrate, acetoacetyl-CoA. This study also showed, that purified HMG-CoA synthase desuccinylates with time and will bind acetyl-CoA preferentially. Based on these results, it was proposed that glucagon might increase ketogenesis by lowering the succinyl-CoA concentration and the succinylation of HMG-CoA synthase in vitro (Lowe et al., 1985). Other studies confirm that glucagon increases HMG-CoA synthase activity and decreases the succinyl-CoA content in the liver in vivo (Quant et al., 1989), and that desuccinylation/resuccinylation is a glucagon-induced, insulin-independent control mechanism of mitochondrial HMG-CoA synthase in the rat (Quant et al., 1990). A recent report showed that hepatic HMG-CoA synthase in fed humans is also partially succinylated (Lascelles et al., 1997).

Ketogenesis is also controlled by transcriptional regulation of HMG-CoA synthase. Fasting, cAMP, and fatty acids increase its transcriptional rate, while refeeding and insulin repress it. Fatty acids increase transcription through peroxisomal proliferator regulatory element (PPRE), which binds to the peroxisome proliferator activated receptor (PPAR). Other transcription factors such as chicken ovalbumin upstream promoter transcription factor (COUP-TF) and hepatocyte nuclear factor 4 (HNF-4) compete for the PPRE site, modulating the response of PPAR (Hegardt, 1998).

1.7.3 Mitochondrial function in rats and humans with liver cirrhosis and chronic cholestasis

Mitochondria play a central role in cellular energy metabolism, and maintenance of their function is essential for survival of cells, organs and whole organisms. In addition to the essential functions in energy metabolism, liver mitochondria are responsible for other important metabolic pathways such as amino acid
metabolism, urea production, gluconeogenesis and many others. Mitochondrial dysfunction can be caused in different ways, including defects of the mitochondrial genome as well as inhibition of the respiratory chain. In addition, mitochondrial dysfunction may also be associated with diseases not primarily affecting mitochondrial metabolism. Mitochondria have different strategies to compensate for the inhibition of the oxidative metabolism: they can either increase their oxidative capacity per mg of mitochondrial protein (increased activity or content of the rate limiting enzymes) or increase the mitochondrial content per cell (mitochondrial proliferation and/or decreased mitochondrial destruction), (Krähenbühl, S. et al., 1992a).

Mitochondrial function can be studied using different techniques. In perfused livers, oxygen consumption reflects mitochondrial oxidative metabolism, since under normal conditions most of the hepatic oxygen consumption is used for oxidative phosphorylation (Brown et al., 1990). Other markers of mitochondrial function in perfused livers are the ratios of lactate/pyruvate and β-hydroxybutyrate/acetoacetate in the effluent of the liver. These ratios reflect the cytosolic and mitochondrial redox state, respectively, and increase when reducing equivalents accumulate (Tanaka et al., 1979, Williamson et al., 1966). The function of isolated mitochondria can be assessed by studying oxidative metabolism using the oxygen electrode (Krähenbühl, S. et al., 1990a).

Mitochondrial function has been studied mainly in two rat models of liver cirrhosis: (1) in rats with CCl₄-induced liver cirrhosis and (2) in rats with secondary biliary cirrhosis induced by bile duct ligation. In 1989, Krähenbühl S. et al. showed, that there are both quantitative and qualitative changes of mitochondrial function in cirrhosis. They showed that in perfused livers from rats with CCl₄-induced liver cirrhosis the mitochondrial enzymes, as well as the mitochondrial oxygen consumption, are reduced in parallel with the expected loss of hepatocytes (Krähenbühl, S. et al., 1989). These results were confirmed by another study in rats with CCl₄-induced liver cirrhosis showing that the oxygen consumption is reduced per gram of liver but not per ml hepatocytes (Krähenbühl, S. et al., 1990b). Furthermore, this study showed that the total mitochondrial content of the liver is reduced by a loss of hepatocytes, but the mitochondrial volume per hepatocyte and the mitochondrial structure are intact.
Moreover, mitochondria in rats with CCl₄-induced liver cirrhosis seem to be functionally intact and show higher activities of enzymes located in the inner mitochondrial membrane, which may represent an attempt to maintain hepatic mitochondrial function in liver cirrhosis (Krähenbühl, S. et al., 1990b). In contrast to rats with CCl₄-induced liver cirrhosis, rats with secondary biliary cirrhosis show reduced oxygen consumption per gram of liver and per ml hepatocytes measured in perfused livers, suggesting a defect in hepatic oxidative metabolism in rats with secondary biliary cirrhosis (Krähenbühl, S. et al., 1992b). Furthermore, impaired mitochondrial functions were confirmed in rats with secondary biliary cirrhosis by reduced gluconeogenesis and increased ratios of lactate/pyruvate and β-hydroxybutyrate/acetoacetate in the perfusate (Krähenbühl, S. et al., 1992b). Rats with secondary biliary cirrhosis seem to use another compensatory mechanism to maintain hepatic energy metabolism: they show increased mitochondrial volume per hepatocyte and a 50% increase in mitochondrial protein content expressed per total liver, but no change in activities of inner mitochondrial membrane or matrix enzymes (Krähenbühl, S. et al., 1992b,c). The increase in hepatic mitochondrial protein content seems to occur early after bile duct ligation and is probably a result of increased synthesis of nuclearly encoded mitochondrial proteins and/or of decreased degradation of mitochondrial proteins (Forestier et al., 1997).

Rats with long-term bile duct ligation starved for 24 hours show decreased plasma β-hydroxybutyrate concentrations (Krähenbühl, S. et al., 1994). In isolated hepatocytes, reduced β-hydroxybutyrate production was measured after administration of different fatty acids (Krähenbühl, S. et al., 1994). These results suggest a defect in the common part of the hepatic fatty acid metabolism, which include the β-oxidation, the electron transport chain, and ketone body production (Fig. 5).

In isolated liver mitochondria from rats with CCl₄-induced liver cirrhosis, state 3 oxygen consumption was reduced for β-hydroxybutyrate and succinate as substrates when expressed per whole liver, but unchanged compared to control animals when expressed per mg of mitochondrial protein (Krähenbühl, S. et al., 1990b). More detailed information about the hepatic fatty acid metabolism, and therefore about the function of the respiratory chain, was provided by studying
liver mitochondria of rats with secondary biliary cirrhosis. First, it was shown that state 3 and state 3u (dinitrophenol-uncoupled) oxidation rates were reduced for β-hydroxybutyrate and succinate as substrates, but not for ascorbate/TMPD in liver mitochondria of rats with secondary biliary cirrhosis (Krähenbühl, S. et al., 1992c). Then, these results were confirmed and expanded by another study in the same animal model where decreased state 3 oxidation rates for L-glutamate, succinate, duroquinone, and fatty acids, but not for ascorbate/TMPD as substrate were shown. In addition, this study revealed reduced activities of the subunits of the electron transport chain, mainly for the complexes I, II and III (Krähenbühl, S. et al., 1994).

Therefore, isolated mitochondria from rats with secondary biliary cirrhosis show more pronounced defects in the function of the respiratory chain than mitochondria from rats with CCl₄-induced liver cirrhosis. A decreased activity of the respiratory chain can be affected by an altered lipid composition of the inner mitochondrial membrane, and it is known that rats with long-term bile duct ligation show changes in the composition of membrane lipids (Bengochea et al., 1987). Inner mitochondrial membranes of liver mitochondria from rats with secondary biliary cirrhosis showed a threefold higher cholesterol content but only minor changes in the phospholipid composition, as compared with control rats. (Krähenbühl, S. et al., 1992c). Thus, this increased cholesterol/phospholipid ratio, which leads to a higher rigidity of biological membranes, could be related to the decreased activity of the electron transport chain. Peroxidation of lipids and proteins could interact with membrane bound enzyme complexes or soluble enzymes and decrease their activities. In rats with secondary biliary cirrhosis, increased peroxidation of lipids was shown (Sokol et al., 1991), which can be associated with reduced mitochondrial ubiquinone concentrations (Mellors et al., 1966). It was shown that critical antioxidative defense mechanisms such as the mitochondrial content of glutathione and ubiquinone are decreased in rats with secondary biliary cirrhosis (Krähenbühl, S. et al., 1995). These findings suggest that mitochondrial dysfunction in rats with secondary biliary cirrhosis may result from oxidative damage of mitochondrial proteins and lipids. Another mechanism that may decrease the function of the electron transport chain in liver mitochondria is the accumulation of toxic
substrates, such as unconjugated bilirubin or bile acids. The toxicity of bile acids on biological membranes and its functions are well established (Schölmerich et al., 1984), and it has been shown that lipophilic bile acids reduce the activity of the respiratory chain in isolated rat liver mitochondria (Lee et al., 1965). The electron transport chain, mainly complexes I and III, is considered to be rate limiting for normal fatty acid metabolism in isolated rat liver mitochondria (Bremer et al., 1972, Latipää et al., 1986). However, it is also well established that the β-oxidation pathway can become rate limiting for hepatic mitochondrial fatty acid metabolism (Hoppel et al., 1979, Latipää et al., 1986). The four enzymes of the β-oxidation were shown to have reduced activities in liver mitochondria from rats with secondary biliary cirrhosis (Krähenbühl, S. et al., 1994). Thus, the decreased fatty acid oxidation shown in isolated hepatocytes and liver mitochondria may result from the reduced activity of the electron transport chain as well as from the reduced activities of enzymes of the β-oxidation. To answer the question whether the impaired function of the electron transport chain or the reduced activities of enzymes of the β-oxidation are responsible for the impaired fatty acid metabolism in mitochondria of rats with secondary biliary cirrhosis, further studies were performed. In 1998, Krähenbühl L. et al. characterized the reversibility of hepatic mitochondrial damage in rats with long-term bile duct ligation. The biliary obstruction was reversed by Roux-en-Y anastomosis (Zimmermann et al., 1992). It was shown that the activities of complex I and III recover within days after reversal of bile duct ligation, whereas hepatic fatty acid metabolism did not normalize during this observation period (Krähenbühl, L. et al., 1998). These results imply that decreased mitochondrial fatty acid metabolism can not be explained by impaired activity of the respiratory chain but is more likely the result of alterations in mitochondrial β-oxidation and/or ketogenesis. In the same animal model, the hepatic carnitine pool was measured to study hepatic fatty acid metabolism and carnitine homeostasis in more detail (Wächter et al., 1999). It was shown that hepatic metabolism of fatty acids is impaired in rats with secondary biliary cirrhosis and does not recover during 14 days after Roux-en-Y anastomosis. The analysis of the hepatic carnitine pool provided increased content of acetylcarnitine and
decreased content of β-hydroxybutyrate, suggesting intact β-oxidation but impaired ketogenesis (Wächter et al., 1999).

Mitochondrial function has been shown to be reduced in humans with liver cirrhosis. Cirrhotic livers show fewer mitochondria and less functioning of the respiratory chain than normal livers (Gil et al., 1977). As far as mitochondrial structure is concerned, it is well known that patients with alcohol-induced liver cirrhosis possess giant mitochondria (Bruguera et al., 1977), compatible with impaired function.
2 AIMS OF THE DISSERTATION

Long-term cholestasis in the rat leads to secondary biliary fibrosis and cirrhosis and is associated with alterations in hepatic energy metabolism. In fact, the study of Krähenbühl L. et al. reveals a progressive decrease in the hepatic and the skeletal muscle glycogen content in rats with long-term cholestasis (Krähenbühl L. et al., 1996). Furthermore, hepatic fatty acid metabolism is impaired in rats with long-term cholestasis. These rats show reduced ketosis during starvation and decreased fatty acid oxidation in isolated hepatocytes and liver mitochondria (Krähenbühl S. et al., 1994).

The aim of the first two projects is to assess hepatic energy metabolism in rats with short-term cholestasis, before the development of secondary biliary cirrhosis. Two studies in rats with short-time bile duct ligation are designed to answer the following questions: 1) are the hepatic and skeletal muscle glycogen contents reduced in rats with short-term cholestasis and which are the responsible mechanisms 2) is short-term cholestasis in the rat associated with alterations in hepatic fatty acid metabolism?

In the third project, the reversibility of the impaired hepatic fatty acid metabolism in rats with long-term cholestasis will be investigated. Krähenbühl L. et al. showed in 1998, that the activities of complex I and III of the respiratory chain recover within days after reversal of bile duct ligation, whereas hepatic fatty acid metabolism did not normalize during this observation period (Krähenbühl L. et al., 1998). To explore these alterations in hepatic fatty acid metabolism in more detail, rats will be studied after long-term bile duct ligation and after reversal of biliary obstruction. The role of β-oxidation as well as of the HMG-CoA cycle will precisely be evaluated in this project.

Parallel to these three projects, liver specimens will be collected from patients with different kinds of liver cirrhosis and hepatic glycogen metabolism will be studied. Owen et al. showed that patients with alcohol-induced liver cirrhosis have reduced hepatic glycogen stores (Owen et al., 1981). To answer the question if all patients with liver cirrhosis have reduced hepatic glycogen stores, patients with alcoholic, biliary and viral cirrhosis will be studied in this project.
3 EXPERIMENTAL CONDITIONS

3.1 Animal model

3.1.1 Bile duct ligation (BDL)
Acute cholestasis or cirrhosis was induced by ligation and transection of the common bile duct in male Sprague-Dawley rats (BRL, CH-4414 Füllinsdorf, Switzerland). Rats were housed individually on a 12-hour dark and light cycle and were fed a standard rat chow with tap water ad libitum. Glycogen metabolism was studied in rats in the fed state, whereas hepatic fatty acid metabolism was assessed in fasted rats. Surgery was performed under ether anesthesia. After a midline incision, the duodenum and the common bile duct were exposed and three sutures were placed around the bile duct. The bile duct was then transected between the two sutures closest to the duodenum. In sham-operated animals, laparotomy was performed and the common bile duct was exposed, but not transected. Laparotomy was closed in two layers by running absorbable sutures (Krähenbühl, L. et al., 1996).

3.1.2 Roux-en-Y anastomosis
The reversal of biliary obstruction was achieved by Roux-en-Y anastomosis. First, the abdomen was opened through a 3-4 cm midline incision under pentobarbital anesthesia. Then, the dilated proximal common bile duct stump was exposed and minimally freed from surrounding tissue in order to avoid bleeding. The jejunum was divided 3 cm distal to the ligament of Treitz and a short 4-cm Roux-en-Y limb created. End-to-side jejunojejunostomy was performed with a 6-0 Prolene® single-layer running suture. In sham-operated animals, laparotomy was performed and the abdominal cavity exposed without any surgical procedure (Krähenbühl, L. et al., 1999, Zimmermann et al., 1992).
3.2 Glycogen metabolism

3.2.1 Determination of the tissue glycogen content

The glycogen content in liver and skeletal muscle was determined enzymatically as glucose using a COBAS analyser (Hoffmann-La Roche Diagnostics, Basel, Switzerland) after alkaline destruction of free glucose and enzymatic hydrolysis of glycogen (Lust et al., 1975). 20 mg of frozen liver or 100 mg of skeletal muscle were homogenized at 0°C with 9.8 and 9 vol of 30 mmol/l HCl, respectively. 100 μl of the homogenate were incubated with 10 μl of 1 mol/l NaOH for 10 minutes at 95°C (pH > 10). 10 μl of 1.5 mol/l acetic acid and 15 μl amyloglucosidase (Boehringer Mannheim AG, Rotkreuz, Switzerland, 14 U/mg lyophilizate, suspension in 3.2 M ammonium sulfate solution) were added to the mixture and then incubated for 60 minutes at 37°C with slow shaking (pH 4.6). The homogenate was centrifuged at 10'000 x g for 5 minutes at 4°C, and the supernatant was directly assayed for glucose.

3.2.2 Glycogen synthase and glycogen phosphorylase: activity measurements in tissue

For the determination of glycogen synthase and glycogen phosphorylase activities, 100 mg frozen liver or skeletal muscle were homogenized at 0°C with 9 vol (wt/vol) of a solution containing 50 mmol/l potassium fluoride and 10 mmol/l EDTA (pH 7.0). The homogenate was centrifuged at 10'000 x g for 10 minutes at 4°C, and the supernatant was directly assayed for the active and the total activity of glycogen synthase (Thomas et al., 1968). To distinguish between the active and the total activity of glycogen synthase, the assay was performed at a low and a high glucose-6-phosphate concentration (Guinovart et al., 1979). The assay for glycogen synthase was started with 30 μl of the supernatant which was preheated for 5 minutes at 37°C. Then 60 μl of a mixture containing 50 mmol/l Tris-HCl buffer (pH 7.8), 8 mmol/l EDTA (pH 7.8), 25 mmol/l potassium fluoride, glycogen (35 mg/ml), 0.25 mmol/l (active form) or 10 mmol/l (total activity) glucose-6-phosphate, 0.3 mmol/l UDPG and U-14C-UDPG (25 μCi/ml) was added to these 30 μl preheated supernatant and incubated for 10 minutes at 37°C. 70 μl of this incubation mixture were then
transferred on a labelled filter paper (Whatman no. 3, cat. Number 1003 150). The filter papers were immediately put into 600 ml stirred 66% ethanol and washed twice with 600 ml 66% ethanol for 25 and 15 minutes. Finally the filter papers were washed in 600 ml acetone for 5 minutes. The air dried filter papers were transferred into scintillation vials containing 1 ml H₂O and 10 ml scintillation fluid. The vials were shaken and then counted in a β-counter.

For the measurement of glycogen phosphorylase, an aliquot of the supernatant was diluted 1:2 (vol/vol) with a solution containing 50 mmol/l 2-(N-morpholino)-ethanesulfonic acid (MES), 50 mmol/l potassium fluoride and 5 mmol/l DTT (pH 6.1), (Gilboe et al., 1972). The active form of glycogen phosphorylase was measured in the presence of 0.75 mmol/l caffeine (Theen et al., 1982), whereas the total activity was assessed in the presence of 7.5 mmol/l AMP. The assay was started with 30 µl of the diluted supernatant which was preheated for 5 minutes at 37°C. Then 60 µl of a mixture containing 150 mmol/l potassium fluoride, 100 mmol/l glucose-1-phosphate (pH 6.1), glycogen (35 mg/ml), 0.75 mmol/l (active form) caffeine, 7.5 mmol/l AMP (total activity) and U⁻¹⁴C-glucose-1-phosphate (25 µCi/ml) was added to these 30 µl preheated supernatant and incubated for 20 minutes at 37°C. 70 µl of this incubation mixture was then transferred on a labelled filter paper (Whatman no. 3, cat. Number 1003 150). The filter papers were immediately put into 600 ml stirred 66% ethanol and washed twice with 600 ml 66% ethanol for 25 and 15 minutes. Finally the filter papers were washed in 600 ml acetone for 5 minutes. The air dried filter papers were transferred into scintillation vials containing 1 ml H₂O and 10 ml scintillation fluid. The vials were shaken and then counted in a β-counter.

3.2.3 Isolation of total RNA from rat liver and skeletal muscle

Total RNA was isolated by a single-step guanidinium thiocyanate/phenol/chloroform extraction procedure (Chomczynski et al., 1987, Sambrook et al., 1989). One gram of rat liver and one gram of skeletal muscle of male Sprague-Dawley rats were dissected and homogenized on ice with a polytron at 30'000 rpm for 1 minute in 10 ml solution D consisting of 4 mol/l guanidinium isothiocyanate, 25 mmol/l sodium citrate, pH 7.0, 0.5% (wt/vol) SLS and 0.1 mol/l 2-mercaptoethanol in a 50 ml sterile polypropylene tube. 1 ml of 2 mol/l
sodium acetate, pH 4.0, and 10 ml of water saturated phenol were sequentially added to the homogenate, with gentle mixing by inversion after the addition of each reagent. After addition of 2 ml of chloroform-isoamylalcohol (49:1), the suspension was shaken vigorously for 10 seconds, incubated on ice for 15 minutes, and centrifuged at 2500 x g for 10 minutes at 4°C. After centrifugation, RNA was present in the aqueous phase, whereas DNA and proteins were present in the interphase and the phenol phase. The aqueous phase was transferred to a new sterile 15 ml polypropylene tube, mixed with an equal volume of isopropanol and then placed for at least 1 hour at -20°C. The precipitated RNA was centrifuged at 9200 x g for 10 minutes at 4°C in a SS-34 rotor™ (Kontron Instruments AG, Basel, Switzerland). The RNA pellet was then dissolved in 3 ml solution D and precipitated again with 3 ml isopropanol for 90 minutes at -20°C. After centrifugation at 2300 x g for 10 minutes at 4°C, the RNA pellet was resuspended in 100 µl 75% ethanol, recentrifuged at 2300 x g, dried at room temperature for 5 minutes, dissolved in 500 µl Rnase free water, quantified by measuring OD 260 nm as described (Sambrook et al., 1989) and stored at -70°C.

3.2.4 Isolation of mRNA from rat liver
mRNA was isolated from total RNA using the PolyATRACT mRNA isolation system (Promega Corporation, Madison, WI, USA) as described by the manufacturer.

3.2.5 RNA gel electrophoresis
RNA gel electrophoresis was performed according to Sambrook et al., 1989 with some modifications. One gram agarose was melted in 64 ml of water and cooled to 50°C. After addition of 20 ml of 10x MOPS (0.2 mol/l MOPS, 5 mmol/l sodium acetate, 10 mmol/l EDTA, pH 7.0) and 16 ml formaldehyde solution, the gel was cast in the vapour-hood and left to solidify. Sample buffer consisting of 420 µl formamide, 147 µl formaldehyde, 84 µl 10x MOPS and RNA loading buffer (25 ml glycerol, 2.5 mg bromphenolblue, 25 ml of 10x MOPS) were prepared. The samples containing up to 2 µg mRNA or 10 µg tot RNA were diluted with water to 10 µl, and 20 µl of sample buffer were added. They were
heated for 5 minutes at 65°C and cooled on ice for 10 minutes. Then, 0.75 µl RNA loading buffer were added to the samples before they were applied on the gel, which was run at a constant voltage of 100 V in 1x MOPS for about 2 hours. 1 µg of a 0.24-9.5 kb RNA ladder was used as RNA size marker.

### 3.2.6 Northern blot analysis

The gel was washed 3 times for 10 minutes with 10x SSC (1.5 mol/l NaCl, 0.15 mol/l sodium citrate). About 50 ml of 20x SSC were put into the middle of a blotting machine followed by a filter paper with a size as big as the blotting machine. After putting the gel on this filter paper, a nylon membrane (Hybond N, Amersham, Little Chalfont, Buckinghamshire, England) was put on the gel. To achieve a complete transfer of mRNA to the nylon membrane, a lot of grey towels and Kleenex™ were added on the nylon membrane and at the end the top of the blotting machine was charged with some weight. On each side of the blotting machine 200 ml of 20x SSC were added. The mRNA was transferred over night. The next day, the mRNA was fixed on the nylon membrane by UV-crosslinking. Finally, the membrane was washed twice for 30 minutes with 2x SSC.

The cDNA probes obtained by RT-PCR and confirmed by sequencing corresponded to bp 192-621 of rat glycogen synthase (J05446) and bp 1307-1768 of rat glycogen phosphorylase (X63515). A 1.3 kb GAPDH cDNA was used as control.

For the pre-hybridisation, the membrane was put into the hybridisation tube and 10 ml of the hybridisation solution (50% formamide, 5x SSPE, 0.2% SDS, 5x Denhardt’s reagent, single strand salmon sperm DNA (100 µg/ml) and distilled water) was added. The pre-hybridisation was performed at 42°C for at least 2 hours. The preparation of the radioactive (hot) probe was assessed similarly for glycogen synthase, glycogen phosphorylase and GAPDH: 25 ng of the cDNA used was brought to a volume of 11 µl with water. The probe was boiled for 10 minutes, put on ice-salt for 1 minute, centrifuged at full speed for 1 minute and put on ice again for 2 minutes, then, 4 µl of HIGH PRIME were added. For the incorporation of the radioactivity, 5 µl of 50 µCi α[^32P] were added to this probe and incubated for 20 minutes at 37°C. The reaction was stopped by adding 2 µl
0.2 mol/l EDTA pH 8.0. The probe was then precipitated with 10 µl tRNA, 8 µl NH₄-acetate and 120 µl 100% ethanol. After storing for at least 1 hour at -70°C, the probe was centrifuged at full speed for 15 minutes. The formed pellet was dissolved in 100 µl water. 1 µl of this solution was measured for radioactivity. The exact amount of the hot probe (1’000’000 cpm/ml hybridisation solution) was boiled for 5 minutes at 95°C and afterwards put on ice to cool down. After discarding the pre-hybridisation solution, 10 ml of a new hybridisation solution and the hot probe were added to the hybridisation tube containing the nylon membrane. The hybridisation was performed over night at 42°C in a rotating oven. After the hybridisation procedure, the nylon membrane was washed as follows: twice for 15 minutes with 2x SSC + 0.1% SDS and once for 15 minutes with 0.1x SSC + 0.1% SDS, at 65°C. The nylon membrane was then wrapped in a plastic sheet and exposed on a X-ray film at -70°C. The whole procedure was performed according to Sambrook et al., 1989 with some modifications.

3.3 Hepatic fatty acid metabolism

3.3.1 Isolation of liver mitochondria

The preparation of mitochondria was performed according to Hoppel et al., 1979 with slight modifications. Eight grams of rat liver were put into ice-cold buffer (approx. 50 ml) containing 220 mmol/l mannitol, 70 mmol/l sucrose and 5 mmol/l MOPS, pH 7.4 (MSM buffer). The liver was minced with scissors, and washed with cold MSM buffer to remove blood particles. A 10% suspension (wt:vol) of the minced liver containing 2 mmol/l EDTA was prepared using a Potter-Elvehjem homogenizer with a loose fitting pestle. All the experiments were performed at 0°C. Nuclei, unbroken cells, and cell debris were removed by centrifugation at 700 x g for 10 minutes at 4°C. Mitochondria were then isolated from the supernatant by centrifugation at 7000 x g for 10 minutes at 4°C. The resulting mitochondrial pellet was washed twice with MSM buffer, diluted to contain approximately 100 mg mitochondrial protein per ml. Protein concentrations in mitochondrial preparations were measured using the biuret method with bovine serum albumin as a standard (Gornall et al., 1949).
3.3.2 Mitochondrial oxidative metabolism

Oxygen metabolism by intact mitochondria was measured in a chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH; USA) at 30°C according to Krähenbühl, S et al., 1991b with some modifications. The incubations contained 1 mg/ml mitochondrial protein in 100 mmol/l KCl, 5 mmol/l MOPS, 1 mmol/l EGTA, and 5 mmol/l KH$_2$PO$_4$. The concentrations of the substrates used were 20 mmol/l for L-glutamate and succinate, 7.2 mmol/l for ascorbate, 40 µmol/l for palmitoyl-L-carnitine, 20 µmol/l for palmitoyl-CoA and 80 µmol/l for palmitate. All incubations with fatty acids contained 5 mmol/l L-malate and defatted bovine serum albumin (1mg/ml, w/v). Incubations with palmitoyl-CoA or palmitate contained additionally 2 mmol/l L-carnitine and incubations with palmitate additionally 250 µmol/l ATP and 250 µmol/l CoASH. L-glutamate, palmitoyl-L-carnitine, and palmitoyl-CoA were measured in the 1000 µl chamber, whereas succinate, ascorbate and palmitate were assayed in the 500 µl chamber. After depletion of endogenous substrates by the addition of ADP, the substrate was added to the incubation, and state 3 respiration was initiated by the addition of ADP (final concentration: 100 nmol/ml).

State 3 and state 4 respiration were defined as ADP-stimulated and ADP-limited respiration, respectively. State 3 and 4 respiration and respiratory control ratios (RCR) were calculated as shown in Table 2.

**Calculations:** at 30°C, 1 ml of the incubation buffer contained 445 natom O$_2$. The distance measured in this experiment was 200 mm.

\[
\begin{align*}
\text{state 3} &= \frac{\text{distance (mm) /min}}{200 \text{ mm}} \times 445 \times \frac{1}{\text{mg protein}} \\
\text{state 4} &= \frac{\text{distance (mm) /min}}{200 \text{ mm}} \times 445 \times \frac{1}{\text{mg protein}} \\
\text{RCR} &= \frac{\text{state 3}}{\text{state 4}}
\end{align*}
\]

**Tab. 2 Calculations for state 3, state 4 respiration and for RCR.**
3.3.3 β-oxidation and Krebs cycle

The β-oxidation of [1-14C] palmitic acid (Amersham Pharmacia Biotech, Dübendorf, Switzerland) by liver mitochondria was assessed as described by Fréneaux et al., 1988 with slight modifications. The preincubation medium (1.8 ml of 70 mmol/l sucrose, 43 mmol/l KCl, 3.6 mmol/l MgCl₂, 7.2 mmol/l potassium phosphate, 36 mmol/l Tris-HCl buffer, pH 7.4) contained 0.2 μmol/l ATP, 50 μmol/l L-carnitine, 15 μmol/l CoASH and 1 mg mitochondrial protein. After 5 minutes of preincubation at 30°C, the incubation mixture was brought to 2 ml by adding 200 μl of the preincubation medium containing [1-14C] palmitic acid (final concentration 40 μmol/L, 0.1 μCi per 2 ml) with bovine serum albumin (final concentration in the assay solution 0.5 mg per 2 ml). The tubes were closed with covers containing a filter paper soaked in 100 mmol/l NaOH for trapping of [14C]CO₂ and incubated at 30°C with slow shaking. After 15 minutes, the reaction was stopped by adding 400 μl of 5% perchloric acid to the incubation mixture. Trapping of CO₂ was continued for 60 minutes. The filter papers were then transferred into scintillation vials containing 10 ml scintillation fluid and counted for [14C]CO₂ activity. The incubation mixture was subsequently centrifuged at 4000 x g for 10 minutes. An aliquot (400 μl) of the supernatant was counted for [1-14C] activity. By this technique acid soluble products of mitochondrial palmitate metabolism are measured, which represent the formation of ketone bodies.

3.3.4 Ketogenesis

3.3.4.1 Determination of ketone body formation by disrupted, isolated liver mitochondria

Ketone body formation by disrupted liver mitochondria was measured directly according to Chapman et al., 1973 with some modifications. Frozen mitochondria were thawed, put quickly into liquid nitrogen and thawed again. Two milligram mitochondrial protein were incubated for 15 minutes at 37°C with an acetyl-CoA-regenerating system in a final volume of 900 μl. This system contained 25 U of phosphotransacetylase (Boehringer Mannheim AG, Rotkreuz, Switzerland, >1500 U/mg lyophilizate, suspension in 3.2 mM ammonium sulfate
solution) and the following components (final concentrations): 10 mmol/l sodium phosphate, 4 mmol/l ATP, 30 mmol/l lithium acetyl phosphate, 1 mmol/l CoASH, 35 mmol/l KCl, 3 mmol/l MgCl₂ and 0.5 mmol/l DTT, pH 7.4. The reaction was stopped by adding 100 μl of 30% perchloric acid. The suspension was centrifuged twice at full speed for 3 minutes and the resulting supernatant was analysed for acetoacetate (Olson et al., 1971). 100 μl of this supernatant were incubated with 2 ml 1 mol/l potassium phosphate buffer pH 7.0, containing 4.6 μg NADH and 10 μl 3-hydroxybutyrate dehydrogenase (Boehringer Mannheim AG, Rotkreuz, Switzerland) for 30 minutes at room temperature. Finally, the disappearance of NADH was measured fluorimetrically at an extinction of 365 nm and an emission of 470 nm.

3.3.4.2 Determination of ketone bodies in liver homogenate
100 mg of frozen liver was homogenized at 0°C with 500 μl 3% PCA and then transferred into a graduated 2 ml Eppendorf tube. The homogeniser was washed twice with 500 μl 3% PCA. The homogenate (1500 μl) was centrifuged for 3 minutes at full speed. The supernatant was transferred into a new graduated 2 ml Eppendorf tube, the volume was adjusted to 2.0 ml by adding 3% PCA. The supernatant was analysed for β-hydroxybutyrate (Olson et al., 1971). 100 μl were incubated with 2 ml 1 mol/l bicarbonate buffer pH 9.5, containing 640 μg NAD and 40 μl 3-hydroxybutyrate dehydrogenase for 90 minutes at room temperature. Finally, the appearance of NADH was measured fluorimetrically at an extinction of 365 nm and an emission of 470 nm.

3.3.4.3 Determination of ketone bodies in plasma
100 μl plasma and 100 μl 6% PCA were put together, mixed vigorously and centrifuged for 3 minutes at full speed. The supernatant was again centrifuged for 3 minutes at full speed and then analysed for β-hydroxybutyrate (Olson et al., 1971). 50 μl were incubated with 2 ml 1 mol/l bicarbonate buffer pH 9.5, containing 640 μg NAD and 40 μl 3-hydroxybutyrate dehydrogenase for 90 minutes at room temperature. Finally, the appearance of NADH was measured fluorimetrically at an extinction of 365 nm and an emission of 470 nm.
3.3.4.4 Total activity of HMG-CoA synthase

The total activity of HMG-CoA synthase was assessed as described by Quant et al., 1989 with slight modifications. Fifteen μl of liver mitochondria were treated with 7.5 μl of Triton x-100 to expose HMG-CoA synthase. The standard 1 ml assay system contained (final concentrations) 50 mmol/l Tris-HCl, 10 mmol/l MgCl₂ and 2 mmol/l DTT, pH 8.0. 300 μg protein of lysed mitochondria, 100 mmol/l acetyl-CoA, 10 U phosphotransacetylase, 5 mmol/l acetyl phosphate and 10 mmol/l acetoacetyl-CoA were added simultaneously. Total HMG-CoA synthase activity was measured at 30°C as the initial velocity of the decrease in absorbance at 303 nm. The absorption coefficient of acetoacetyl-CoA under these conditions is 12.2 x 10³ M⁻¹cm⁻¹. By definition, 1 unit of enzyme activity causes 1 μmol of acetoacetyl-CoA to be transformed/minute.

3.3.4.5 Western blot analysis

Immunoblotting of mitochondrial HMG-CoA synthase was performed as described by Serra et al., 1993 with some modifications. 500 milligram of rat liver of male Sprague-Dawley rats were dissected and homogenized on ice with a polytron at 30'000 rpm for 1 minute in 4 ml of a buffer composed of 20 mmol/l HKPO₄⁻, 0.1 mmol/l EDTA, 10 mmol/l 2-mercaptoethanol, and 0.5% (vol/vol) Triton-X-100, pH 7.1. Protein concentrations in these preparations were measured using the Lowry method with bovine serum albumin as a standard (Lowry et al., 1951). A 10% acrylamide-SDS gel (10 ml) was prepared and consisted of the following solutions: 375 mmol/l Tris-HCl buffer (pH 8.8), 0.1% SDS, 10% acrylamide/N'N'-bis-methylene-acrylamide, 0.05% APS and 0.05% N,N,N',N'-tetramethyl-p-phenylenediamine. The stacking gel contained 125 mmol/l Tris-HCl buffer (pH 6.8), 0.1% SDS, 4% acrylamide/N'N'-bis-methylene-acrylamide, 0.05% APS and 0.1% N,N,N',N'-tetramethyl-p-phenylenediamine. A sample buffer consisting of 1.56 mmol/l Tris-HCl buffer (pH 6.8), 2.125% glycerol, 0.5% SDS, 178 mmol/l 2-mercaptoethanol, and 0.05% bromphenolblue was prepared. The samples containing up to 20 μg protein were diluted with water to 10 μl, and 20 μl of sample buffer were added. They were heated for 5 minutes at 95°C and cooled down on ice. Then, the samples (30 μl) were applied on the gel, which was run at a constant voltage of 150 V in
a running buffer consisting of 25 mmol/l Tris-base, 190 mmol/l glycine, and 1 %o SDS for about 1 hour. A special transfer sandwich was built as follows:

- grey plate at the positive pool
- green sponge
- filter paper
- nitrocellulose transfer membrane (*Protran®, Schleicher & Schuell GmbH, Dassel, Germany)
- gel
- filter paper
- green sponge
- grey plate at the negative pool

The proteins were transferred to the nitrocellulose transfer membrane at a constant voltage of 30 V in a running buffer consisting of 30 mmol/l Tris-base, 200 mmol/l glycine, 0.2 %o SDS, and 20% methanol for about 1 hour. Finally, the nitrocellulose membrane was transferred into a box containing 20 ml of a solution of 10 mmol/l Tris-HCl buffer, pH 7.6, 100 mmol/l NaCl, 0.1% tween-20®, and 5% milk powder. Immunoblotting of mitochondrial HMG-CoA synthase itself was performed in Barcelona by Dr. D. Serra, as described (Serra et al., 1993). Antibodies against mitochondrial HMG-CoA synthase were prepared by injecting rabbits with a peptide corresponding to the amino acid sequence 37-49 of the mitochondrial HMG-CoA synthase protein as described previously (Serra et al., 1993). The autoradiograms were quantified using a Luminescent Image Analyser LAS-1000 with Image Reader LAS-1000 for Windows® software (Raytest, Urdorf, Switzerland).

3.3.4.6 DNase digestion and reverse transcription

Total RNA was extracted from rat liver as described in chapter 3.2.3. DNA digestion was performed with the RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA). The incubation mixture (10.0 µl) contained four µg of total RNA, 2.0 µl 5x first strand buffer, RQ1 RNase-free DNase (1U/µg RNA) and RNase free H₂O. The mixture was incubated for 30 minutes at 37°C. After adding 1.0 µl RQ1 DNase Stop Solution, the mixture was again incubated for 10 minutes at 65°C and afterwards put on ice. The reverse
transcription was assessed by using the MMLV reverse transcriptase (Molony Murine Leukemia Virus reverse transcriptase; Gibco BRL, Life Technologies AG, Basel, Switzerland). 11.0 µl RQ1 DNase treated RNA, 2.0 µl RNase free water and 1.0 µl Oligo d\(T\)\(_{15}\) (250 mg/ml) were mixed, incubated for 10 minutes at 70°C and afterwards put on ice. 6.0 µl of a RT-Mix were added to this incubation mixture containing 2.0 µl 5x first strand buffer, 2.0 µl 100 mmol/l DTT, 1.0 µl 10 mmol/l dNTP, 1.0 µl MMLV reverse transcriptase. The mixture was first incubated for 60 minutes at 37°C and then for 15 minutes at 70°C and afterwards put on ice.

3.3.4.7 Real-time quantitative PCR
Real-time quantitative PCR analysis was performed with a PE Applied Biosystems 7700 Sequence Detector (PE Biosystems), which is a combined thermocycler and fluorescent detector. Sets of primers were chosen for HMG-CoA synthase to receive a PCR product of less than 100 base pairs. A dual-labelled fluorogenic probe complementary to a sequence within the PCR product was added to the PCR reaction. The primers and the dual-labelled fluorogenic probe for GAPDH, which served as internal standard, were chosen accordingly. One fluorescent dye (6-carboxyfluorescein) serves as a reporter, and its emission is quenched by a second fluorescent dye (6-carboxytetramethylrhodamine). During elongation, the 5' to 3' exonuclease activity of the Taq DNA polymerase hydrolyses the probe, thus releasing the reporter from the quencher, resulting in increased fluorescence which is detected. For HMG-CoA synthase, the forward and reverse primers were TGAACGGTGAATAGACACAGCAG and GTGGTGCTACTGCTTACCAGG, respectively, with the probe CTGCCGTCCCGGCGTGGAGG. For GAPDH, the corresponding forward and reverse primers were CTGCCCAAGTATGATGACAATGAGAA and AGCCCAAGTGCTTATGT, respectively, with the probe TCGGCCGTTCCTACCAG (Dufour et al., 1999). Primers and probes were custom-synthesized by PE Biosystems. Complementary DNA was amplified in a 50 µl volume containing 25 µl of the 2x TaqMan Universal PCR Master Mix (PE Biosystems), 100 nmol/l probe and 300 nmol/l of each primer. After a denaturating step of 10 minutes at 95°C, 40 cycles were performed: 95°C for 15
seconds and 60°C for 1 minute. The mathematical analysis of the results was performed as recommended by the manufacturer after having performed all the necessary validation experiments (PE Biosystems, DOD Doc# 4303859, user bulletin # 2).
4 RESULTS

4.1 Hepatic and skeletal muscle glycogen metabolism in rats with short-term cholestasis

4.1.1 Abstract

Background/ Aims: Rats with long-term cholestasis have reduced hepatic glycogen stores due to impaired glycogen synthesis. Studies on hepatic and skeletal muscle glycogen metabolism in rats with short-term cholestasis are so far lacking.

Methods: Rats were bile duct ligated (BDL rats) for four or eight days and compared to sham-operated control rats pair-fed to BDL rats (pair-fed CON rats) or fed ad libitum (ad libitum-fed CON rats). All animals were investigated in the fed state.

Results: Four days after surgery, the hepatic glycogen content was 21.6±7.6 versus 21.2±8.5 mg/g liver in BDL and pair-fed CON rats, respectively, while ad libitum-fed CON rats had a glycogen content of 72.9±7.7 mg/g liver. Eight days after surgery, the hepatic glycogen content was significantly reduced in BDL compared to pair-fed (31.2±8.9 vs. 59.1±5.4 mg/g liver) or ad libitum-fed CON rats (58.3±4.7 mg/g liver). Similar patterns were observed with the hepatic glycogen content expressed per ml hepatocytes or per liver on both occasions examined. Histological analysis of livers from BDL rats confirmed the decrease in the hepatic glycogen stores and showed that almost all hepatocytes were affected. At both time points, the total activities of glycogen synthase and phosphorylase were significantly reduced in BDL as compared to CON rats, while the active fractions of these enzymes were not affected. The hepatic mRNA levels of both glycogen synthase and phosphorylase were significantly reduced in BDL as compared to pair-fed CON rats at both time points. The plasma glucagon and endotoxin (portal and systemic) levels were significantly increased in BDL versus pair-fed CON rats at both time points, while the corresponding insulin concentrations were decreased in BDL rats at four, but not at eight days after surgery. The skeletal muscle glycogen content and
activities of glycogen synthase and phosphorylase remained unaffected at both
time points.

Conclusions: Acute cholestasis leads to a rapid decrease in the hepatic
glycogen content, as well as activities and expression of glycogen synthase and
phosphorylase. While reduced intake of food is sufficient to explain the
decrease in the hepatic glycogen stores in BDL and pair-fed CON rats four days
after surgery, reduced glycogen synthesis, possibly related to endotoxinemia, is
the most probable cause of the decrease in hepatic glycogen content in BDL
rats eight days after surgery.

Keywords
Acute cholestasis, bile duct ligation, endotoxin, glycogen, glycogen synthase,
glycogen phosphorylase

4.1.2 Introduction
Hepatic and muscle glycogen metabolism are essential for glucose
homeostasis and they represent important energy sources during early
starvation and physical exercise (1-3). Glycogen synthesis and breakdown are
regulated by two key enzymes, glycogen synthase and glycogen phosphorylase
(1,4,5). The activity of these enzymes is controlled by phosphorylation and
dephosphorylation, respectively (1,6). Glycogen synthase, the rate limiting
enzyme for glycogen synthesis, is activated either by metabolites such as
glucose (3,7,8), AMP (9) or glucose-6-phosphate (10) or by insulin via
stimulation of protein phosphatase 1-G (1,11-15). Glycogen synthase is
inactivated by various protein kinases that are activated by hormones such as
glucagon, adrenalin or vasopressin (1,13,16-18). The key enzyme for glycogen
breakdown is glycogen phosphorylase, which is activated by phosphorylation
(4,19,20). The corresponding protein kinase is activated by hormones like
glucagon, vasopressin or adrenalin (1,17,21-24). The liver enzyme is mainly
controlled by phosphorylation, whereas the muscle and brain isoenzymes are
also activated by AMP and glucose-6-phosphate, which enables these enzymes
to react quickly to intracellular energy needs (1). Endotoxins or acute phase
proteins such as IL-1β stimulate glycogenolysis either by inducing secretion of
prostaglandin D2 by Kupffer cells (25,26) or by affecting glycogen phosphorylase directly (27). Glycogenolysis is inhibited by insulin either via a cAMP- or a calcium-dependent pathway.

Bile duct ligation (BDL) for two to four weeks leads to progressive fibrosis and eventually cirrhosis in rats, similar to secondary biliary cirrhosis in humans (28-30). We were recently able to demonstrate a progressive reduction in the hepatic glycogen content per g liver and per ml of hepatocytes (31), and a decrease in activity and expression of both glycogen synthase and phosphorylase (32) in rats with bile duct ligation for two to four weeks. These findings indicate that reduced glycogen synthesis is the major mechanism for reduced hepatic glycogen stores in rats with long-term cholestasis. However, whether cirrhosis or cholestasis is responsible for reduced glycogen stores in the liver is so far not clear investigated.

We therefore aimed to assess the effect of acute (short-term) cholestasis on hepatic and skeletal muscle glycogen metabolism in liver and skeletal muscle. We designed a study to answer the following questions: 1) are the hepatic and skeletal glycogen contents reduced in rats with bile duct ligation for four or eight days without cirrhosis? 2) if so, what are the potential mechanisms leading to alterations in glycogen metabolism?

4.1.3 Materials and methods

Animals
Male Sprague-Dawley rats (BRL, CH-4414 Füllinsdorf, Switzerland) were used throughout the experiments. Rats were housed individually on a 12-hour dark and light cycle and were fed a standard rat chow with tap water ad libitum. All the animal experiments were approved by the State Animal Ethics Board and were performed according to these guidelines.

Surgical procedures
All surgical procedures were performed as described previously (31). There was no mortality in the BDL rats studied four days after surgery or in either control group, whereas the mortality in the BDL group eight days after surgery was 25%. One animal died during laparotomy due to anaesthesia problems,
whereas the remaining two died on postoperative days five or six of septic complications.

**Study design**

Six different groups of animals were investigated with all animals in the fed state. Animals were either bile duct ligated (BDL rats) for four (n = 12) or eight (n = 9) days, or sham-operated on and pair-fed to BDL rats (pair-fed CON rats) for four (n = 8) or eight (n = 8) days. Two other control groups were sham-operated on and fed *ad libitum* for four (n = 5) or eight (n = 5) days (ad libitum-fed CON rats).

**Characterization of the animals**

The rats were characterized by their body and spleen weights, activities of alkaline phosphatase and aspartate aminotransferase (AST) in plasma and by the plasma concentrations of glucose, insulin, glucagon, bilirubin and bile acids. Alkaline phosphatase, AST, glucose and bilirubin were analysed on a COBAS analyser (Hoffmann-La Roche Diagnostics, Basel, Switzerland). Bile acids were determined with a radioimmunoassay (Becton and Dickinson, Orangeburg, SC, USA). The plasma concentrations of insulin and glucagon were determined using a radioimmunoassay (LINCO Research, Inc. St. Louis, MO, USA).

For endotoxin measurements, blood was obtained from the portal and inferior cava vein in a 1 ml sterile syringe with pyrogen-free heparin (1,000 U/ml). Blood samples were immediately transferred to pyrogen-free glass tubes (heated at 180°C for 24 h). Plasma was then centrifuged (150g) and all samples quantified the same day. Just before assay, samples were diluted 1:10 and heated to 75°C for 10 minutes to denature endotoxin-binding proteins. Levels of endotoxin were measured using the Limulus amebocyte lysate pyrogen test (Kinetic test, Kinetic-QCL, Santa Clara, CA, USA; Biowhittaker).

**Sample preparation**

The rats were decapitated at the time points indicated above and two mixed venous/arterial blood samples were collected in heparinised tubes, or, for the determination of insulin or glucagon, in tubes containing the protease inhibitor Trasylofil® (Bayer AG, Pharma, Zürich, Switzerland). The abdomen was opened quickly and a freeze-clamped liver sample was obtained with clamps previously
cooled in liquid nitrogen. Tissue samples were also obtained from skeletal muscle (triceps femoris) and rapidly frozen in liquid nitrogen. For the determination of portal and systemic endotoxin levels, additional rats (BDL rats for 4 and 8 days and pair-fed control rats for 8 days, n=6 per group) were studied. Rats were kept in ether anesthesia and a systemic and portal blood sample were obtained under sterile conditions and immediately analysed for endotoxin as described above. All liver and muscle biopsy specimens were kept at -80°C until analysis.

From the remainder of the liver, additional samples were obtained in 4% buffered formaldehyde for stereological analysis and in alcohol for histological analysis of glycogen.

**Tissue glycogen content**

The glycogen content in liver and skeletal muscle was determined enzymatically as glucose using a COBAS analyser (Hoffmann-La Roche Diagnostics, Basel, Switzerland) after alkaline destruction of free glucose and enzymatic hydrolysis of glycogen as described by Lust et al. (33) with the modifications reported previously (31). The glycogen content is expressed as milligram glycogen per gram tissue wet weight.

**Enzyme assays**

Glycogen synthase activity (active form and total activity) was determined as described originally by Thomas et al. (34) and modified by Guinovart et al. (35). Total activity of glycogen phosphorylase was determined according to Gilboe et al. (36), and the active form according to Theen et al. (37).

**Northern blotting**

Total liver RNA was prepared according to Chomczynski and Sacchi (38), and mRNA was isolated using the PolyAtract system (Promega, Madison, WI, USA). Two μg of mRNA were separated by electrophoresis on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham, Little Chalfont, Buckinghamshire, England) and hybridised after UV crosslinking. The cDNA probes obtained by RT-PCR and confirmed by sequencing corresponded to bp 192-621 of rat glycogen synthase (J05446) and bp 1307-1768 of rat glycogen phosphorylase (X63515). A 1.3 kb GAPDH cDNA was
used as a control. The probes were labelled with [α-^32P]-dCTP according to the general protocol of Sambrook et al. (Molecular Cloning, A Laboratory Manual, 2nd Edition). After a 2h prehybridisation period, the blots were hybridised overnight with 2 x 10^6 cpm/ml hybridisation solution at 65°C according to the general protocol of Sambrook et al. (Molecular Cloning, A Laboratory Manual, 2nd Edition). After washing (three times for 30 minutes with 2 x SSC containing 0.5% SDS at 65°C) the blots were exposed to an autoradiographic film (Kodak, X-Omat, Rochester, NY, USA) at -70°C. Relative abundance of mRNA levels was determined by densitometric analysis of the film with a Sharp Scanner JX 325.

Stereological and histological analysis of the livers

The histological sections of the liver samples obtained randomly from all bile duct ligated and pair-fed control rats (39) were analysed by stereological methods using the point counting procedure described by Weibel (40). Each point was classified as overlying either hepatocytes, connective tissue or 'other structures'. In the results section, only the volume densities (V_v) and volumes per liver (V) of hepatocytes (h_c) and connective tissue (ct) are reported. Other liver samples were fixed in alcohol and stained with PAS to visualize tissue glycogen. Stains pretreated with diastase served as a negative control.

Statistical methods

All results are expressed as mean ± standard deviation (SD). Means were compared by ANOVA, followed by Scheffe’s test. A p<0.05 was considered to be statistically significant.

4.1.4 Results

Glycogen metabolism was investigated in rats with bile duct ligation for four or eight days and compared to pair-fed or ad libitum-fed control rats. The rats used in the current study are characterized in Table 3. Four days after surgery, BDL rats had a slightly higher body weight than pair-fed control rats but there was no difference after eight days. The body weights between BDL and ad libitum-fed CON rats were not different. Both BDL and pair-fed CON rats showed an initial drop in body weight which reached its maximum two to three
days after surgery (Figure 6). In comparison, such a drop was not observed in ad libitum-fed CON rats. The spleen weights did not differ between BDL and CON rats four days after surgery, but were increased in BDL rats after eight days. The activities of alkaline phosphatase, AST and the plasma concentrations of bilirubin and bile acids all showed significant increases in BDL rats four and eight days after surgery in comparison to CON rats. Endotoxin levels were elevated in BDL rats at both time points in plasma from portal and peripheral blood.

<table>
<thead>
<tr>
<th></th>
<th>BDL 4 days (n=12)</th>
<th>BDL 8 days (n=9)</th>
<th>Pair-fed CON 4 days (n=8)</th>
<th>Pair-fed CON 8 days (n=8)</th>
<th>Ad libitum-fed CON 4 days (n=5)</th>
<th>Ad libitum-fed CON 8 days (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (bw, g)</td>
<td>293±11a</td>
<td>304±24</td>
<td>268±11c</td>
<td>283±22c</td>
<td>292±8</td>
<td>314±17</td>
</tr>
<tr>
<td>Liver weight (g/100 g bw)</td>
<td>5.06±0.36ab</td>
<td>5.23±0.47ab</td>
<td>3.33±0.30c</td>
<td>3.63±0.36</td>
<td>4.49±0.23</td>
<td>4.28±0.23</td>
</tr>
<tr>
<td>Spleen weight (g/100 g bw)</td>
<td>0.28±0.04</td>
<td>0.30±0.05ab</td>
<td>0.27±0.02</td>
<td>0.24±0.002</td>
<td>0.23±0.05</td>
<td>0.24±0.02</td>
</tr>
<tr>
<td>Plasma AST (U/L)</td>
<td>556±178ab</td>
<td>509±173ab</td>
<td>154±40</td>
<td>175±33</td>
<td>174±41</td>
<td>261±170</td>
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<tr>
<td>Plasma ALP (U/L)</td>
<td>486±84ab</td>
<td>418±100ab</td>
<td>172±12</td>
<td>223±28</td>
<td>208±59</td>
<td>287±124</td>
</tr>
<tr>
<td>Plasma bile acids (μmol/L)</td>
<td>89±37ab</td>
<td>74±54ab</td>
<td>0.9±0.5c</td>
<td>1.0±0.3c</td>
<td>1.8±0.7</td>
<td>2.5±0.7</td>
</tr>
<tr>
<td>Plasma bilirubin (μmol/L)</td>
<td>148±25ab</td>
<td>145±53ab</td>
<td>0.3±0.1</td>
<td>0.3±0.2</td>
<td>0.2±0.1</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>Plasma endotoxin (systemic, pg/ml)</td>
<td>41.9±20.9a</td>
<td>78.5±18.9a</td>
<td>n.d.</td>
<td>10.6±4.6</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Plasma endotoxin (portal, pg/ml)</td>
<td>57.8±20.6a</td>
<td>95.2±20.4a</td>
<td>n.d.</td>
<td>9.7±3.9</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

**Tab. 3 Characterization of the animals**

Rats studied were either bile duct ligated (BDL), sham-operated control rats pair-fed to BDL rats (pair-fed CON) or control rats fed ad libitum (ad libitum-fed CON). Data are given as mean±SD. For endotoxin determinations, n=6 animals for each group were studied. *p<0.05 BDL vs. pair-fed CON, ^p<0.05 BDL vs. ad libitum-fed CON, "p<0.05 pair-fed CON vs. ad libitum-fed CON
Fig. 6 Body weights and food intake

Body weights are expressed as the percentage of the initial body weights. All rats studied were included in the analysis. The initial body weights were 312±6 g for BDL rats, 293±11 g for pair-fed and 277±8 g for ad libitum-fed CON rats. Data are given as mean±SD.

<table>
<thead>
<tr>
<th></th>
<th>BDL</th>
<th>Pair-fed CON</th>
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<td>4 days (n=12)</td>
<td>8 days (n=9)</td>
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<tr>
<td></td>
<td>4 days (n=8)</td>
<td>8 days (n=8)</td>
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</table>

**Hepatocytes (hc)**

<table>
<thead>
<tr>
<th></th>
<th>4 days</th>
<th>8 days</th>
<th>4 days</th>
<th>8 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_v ) (hc,liver) (ml hc per ml liver)</td>
<td>0.87±0.04*</td>
<td>0.85±0.04*</td>
<td>0.94±0.03</td>
<td>0.95±0.02</td>
</tr>
<tr>
<td>( V ) (hc) (ml hc per liver)</td>
<td>12.9±0.9*</td>
<td>13.9±1.4*</td>
<td>8.3±0.8</td>
<td>10.7±1.2</td>
</tr>
</tbody>
</table>

**Connective tissue (ct)**

<table>
<thead>
<tr>
<th></th>
<th>4 days</th>
<th>8 days</th>
<th>4 days</th>
<th>8 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_v ) (ct,liver) (ml ct per ml liver)</td>
<td>0.09±0.03*</td>
<td>0.06±0.03*</td>
<td>0.04±0.02</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>( V ) (ct) (ml ct per liver)</td>
<td>1.26±0.09*</td>
<td>1.01±0.10*</td>
<td>0.32±0.03</td>
<td>0.23±0.02</td>
</tr>
</tbody>
</table>

Tab. 4 Stereological analysis of the livers

Rats studied were either bile duct ligated (BDL) or sham-operated control rats pair-fed to BDL rats (pair-fed CON). Stereological analysis was performed by the point-counting procedure as described in Methods. \( V_v \) stands for volume fraction, \( V \) for volume, hc for hepatocytes and ct for connective tissue. Data are given as mean±SD. *p<0.05 BDL vs. pair-fed CON

The stereological analysis of the livers revealed a decrease in the volume fraction of hepatocytes and a corresponding increase in the volume fraction of
collagen in BDL as compared to pair-fed CON rats (Table 4). Due to the increase in liver weight mentioned above, both the volume of hepatocytes and the volume of collagen per liver were increased in BDL rats.

The characterization of the carbohydrate metabolism of the rats studied is given in Table 5. Four days after surgery, the hepatic glycogen content expressed per g liver or ml hepatocytes was not different between BDL and pair-fed CON rats, but was decreased in BDL compared to ad libitum-fed CON rats. When expressed per liver, both BDL and pair-fed CON rats had a lower glycogen content than ad libitum-fed CON rats. Eight days after surgery, the glycogen content per g liver or per ml hepatocytes was lower in BDL rats than in both CON groups. Also, when expressed per liver, the glycogen content was lowest in BDL rats, but the difference reached statistical significance only in comparison to ad libitum-fed CON rats.

<table>
<thead>
<tr>
<th></th>
<th>BDL 4 days (n=12)</th>
<th>BDL 8 days (n=9)</th>
<th>Pair-fed CON 4 days (n=8)</th>
<th>Pair-fed CON 8 days (n=8)</th>
<th>Ad libitum-fed CON 4 days (n=5)</th>
<th>Ad libitum-fed CON 8 days (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tissue glycogen content</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/g liver</td>
<td>21.6±7.6</td>
<td>31.2±8.9</td>
<td>21.2±8.5</td>
<td>59.1±5.4</td>
<td>73.0±7.7</td>
<td>58.4±4.7</td>
</tr>
<tr>
<td>mg/ml hepatocytes</td>
<td>24.8±8.7</td>
<td>36.9±10.6</td>
<td>22.7±9.1</td>
<td>62.2±5.8</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>mg/liver</td>
<td>324±124</td>
<td>517±160</td>
<td>191±81</td>
<td>670±122</td>
<td>960±134</td>
<td>783±72</td>
</tr>
<tr>
<td>mg/g skeletal muscle</td>
<td>5.2±1.4</td>
<td>6.0±1.9</td>
<td>5.2±0.8</td>
<td>5.8±1.4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>Plasma metabolites and hormones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>7.4±0.3</td>
<td>7.6±0.7</td>
<td>8.1±0.7</td>
<td>7.7±0.7</td>
<td>8.5±0.9</td>
<td>8.2±0.4</td>
</tr>
<tr>
<td>Insulin (ng/L)</td>
<td>923±371</td>
<td>1613±669</td>
<td>1819±499</td>
<td>1804±466</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Glucagon (ng/L)</td>
<td>142±31</td>
<td>147±66</td>
<td>74±24</td>
<td>62±14</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Tab. 5 Metabolic characterization of the animals

Rats studied were either bile duct ligated (BDL), sham-operated control rats pair-fed to BDL rats (pair-fed CON) or control rats fed ad libitum (ad libitum-fed CON). Tissue glycogen was determined as glucose after enzymatic hydrolysis and hormone concentrations by a RIA as described in Methods. Data are given as mean±SD.

*p<0.05 BDL vs. pair-fed CON, *p<0.05 BDL vs. ad libitum-fed CON, *p<0.05 pair-fed CON vs. ad libitum-fed CON
As shown in Figure 7, the histological analysis also revealed a lower glycogen content in the hepatocytes of livers from BDL rats. The loss of glycogen shows no zonal distribution and affects most hepatocytes evenly.

![A](image1) ![B](image2)

**Fig. 7 A/B Histological analysis of the hepatic distribution of glycogen**

Liver tissue was fixed in alcohol and stained with PAS. Sections pretreated with diastase served as controls and showed no difference between livers from BDL and CON rats. Figure 7 A shows liver tissue from a pair-fed CON animal eight days after surgery. PAS reactivity for glycogen is diffuse (no zonal distribution) and equal for most hepatocytes. Fig. 7 B shows liver tissue from a BDL rat eight days after surgery. PAS staining for glycogen in the parenchymal areas (center of the figure) is reduced in comparison to the control rat in Figure 7 A. The adjacent non-hepatocyte tissue consists of fibrosed portal tracts and septa showing marked ductular proliferation.

In contrast to the liver, the glycogen content in skeletal muscle showed no significant difference between BDL and pair-fed or ad libitum-fed CON rats at both time points investigated. The plasma glucose concentration tended to be slightly lower in BDL or pair-fed CON rats as compared to ad libitum-fed CON rats. The plasma glucagon concentrations were increased in BDL compared to pair-fed CON rats by approximately 50% at both time points. In contrast, the plasma insulin concentration was decreased by 50% in BDL rats four days after surgery, whereas after eight days no difference between BDL and pair-fed CON rats could be detected.

To characterize hepatic and skeletal muscle glycogen metabolism, the activities of both glycogen synthase and glycogen phosphorylase were determined in liver and skeletal muscle (Table 6). In comparison to pair-fed or ad libitum-fed CON rats, the total activity and also the active part (α-form) of both enzymes was reduced in BDL rats. There was no difference in the active fraction (α-form...
divided by total activity) between BDL and CON rats at both time points for both enzymes (data not shown). In contrast to liver, the activity of both enzymes was not different between BDL and pair-fed CON rats in skeletal muscle.

To explain further the mechanism leading to the observed reduction in the activities of glycogen synthase and phosphorylase in BDL rats, Northern blots for both enzymes were prepared. As shown in Table 7, the hepatic mRNA levels were reduced in BDL as compared to pair-fed CON rats for both glycogen synthase and phosphorylase at both time points investigated.

<table>
<thead>
<tr>
<th></th>
<th>BDL</th>
<th>Pair-fed CON</th>
<th>Ad libitum-fed CON</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 days (n=12)</td>
<td>8 days (n=9)</td>
<td>4 days (n=8)</td>
</tr>
<tr>
<td><strong>Glycogen synthase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active (a) form (U/g liver)</td>
<td>0.036±0.01ab</td>
<td>0.028±0.01ab</td>
<td>0.062±0.014</td>
</tr>
<tr>
<td>Total (a+b) activity (U/g liver)</td>
<td>0.11±0.02ab</td>
<td>0.10±0.03ab</td>
<td>0.21±0.03</td>
</tr>
<tr>
<td>Active (a) form (U/g muscle)</td>
<td>0.075±0.042</td>
<td>0.094±0.043</td>
<td>0.089±0.05</td>
</tr>
<tr>
<td>Total (a+b) activity (U/g muscle)</td>
<td>5.2±1.4</td>
<td>6.0±1.9</td>
<td>5.2±0.8</td>
</tr>
<tr>
<td><strong>Glycogen phosphorylase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active (a) form (U/g liver)</td>
<td>18.2±2.5ab</td>
<td>13.3±4.6ab</td>
<td>25.0±3.6</td>
</tr>
<tr>
<td>Total (a+b) activity (U/g liver)</td>
<td>19.7±2.0ab</td>
<td>15.6±4.3ab</td>
<td>28.5±3.2</td>
</tr>
<tr>
<td>Active (a) form (U/g muscle)</td>
<td>49.4±22.6</td>
<td>47.0±21.7</td>
<td>46.4±19.8</td>
</tr>
<tr>
<td>Total (a+b) activity (U/g muscle)</td>
<td>84.4±28.7</td>
<td>86.2±30.4</td>
<td>82.4±25.7</td>
</tr>
</tbody>
</table>

**Tab. 6 Glycogen synthase and phosphorylase activity in liver and skeletal muscle**

Rats studied were either bile duct ligated (BDL), sham-operated control rats pair-fed to BDL rats (pair-fed CON) or control rats fed ad libitum (ad libitum-fed CON). Enzyme activities were determined using radioactive substrates as described in Methods. Data are given as mean±SD. aP<0.05 BDL vs. pair-fed CON, bP<0.05 BDL vs. ad libitum-fed CON
### Tab. 7 Hepatic mRNA content of glycogen synthase and phosphorylase

Rats studied were either bile duct ligated (BDL) or sham-operated control rats pair-fed to BDL rats (pair-fed CON). The values shown are the densities (unitless) of the bands on the Northern blots prepared as described in Methods. Values were normalized to the values obtained for GAPDH. The control groups (4 days and 8 days after surgery) were pooled since the values were not different for the two time points. Data are given as mean±SD. *p<0.05 BDL vs. pair-fed CON

<table>
<thead>
<tr>
<th></th>
<th>BDL (n=3)</th>
<th>BDL (n=3)</th>
<th>Pair-fed CON (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycogen synthase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative density</td>
<td>0.15±0.13a</td>
<td>0.22±0.12a</td>
<td>0.67±0.39</td>
</tr>
<tr>
<td><strong>Glycogen phosphorylase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative density</td>
<td>0.24±0.06a</td>
<td>0.37±0.05a</td>
<td>2.05±0.72</td>
</tr>
</tbody>
</table>

#### 4.1.5 Discussion

Our study shows that the hepatic glycogen content is decreased in rats with acute cholestasis as a consequence of both reduced food intake and cholestasis. In contrast to the liver, skeletal muscle glycogen content is not affected.

Four days after surgery, the glycogen content expressed per ml hepatocytes or g liver was not different between BDL and pair-fed CON rats. However, in comparison to ad libitum-fed CON rats, both BDL and pair-fed CON rats had a significantly decreased hepatic glycogen content. Since the only difference between pair-fed and ad libitum-fed CON rats is the amount of food ingested (the surgical procedure was the same), reduced food intake has to explain the differences in the hepatic glycogen content four days after surgery. The question remains why BDL rats ingest less food than ad libitum-fed control rats. This cannot be a consequence of anaesthesia or laparotomy, since the CON rats had undergone exactly the same procedures as the BDL rats. Reduced food intake in BDL rats is therefore likely to be due to the specific surgical procedure (ligation of the common bile duct) and/or to cholestasis itself. Bile duct ligation is more likely to be the reason than cholestasis since BDL rats normalized their food intake over the observation period of eight days despite cholestasis (Figure 6). As shown in Figure 6, and also in a previous publication
BDL and pair-fed CON rats had almost identical body weights during the post-surgical period, indicating that assimilation of food is not affected by cholestasis in the rat.

Already four days after surgery, the hepatic mRNA expression and activity of glycogen synthase and phosphorylase were decreased in BDL as compared to CON rats. Since, as discussed above, the hepatic glycogen content per g liver was not different between BDL and pair-fed CON rats at this time point, the decreased activity of glycogen synthase was not associated with a significant decrease in the hepatic glycogen stores in BDL rats. It is possible that such a decrease was prevented by the contemporary decrease in the activity of glycogen phosphorylase, or, more likely, that it was overridden by the effect of the reduction in food intake.

The activity of glycogen synthase and phosphorylase is regulated short-term by phosphorylation and long-term by altered expression (1,6). Since the active fractions of both glycogen synthase and phosphorylase were not altered in BDL as compared to CON rats, decreased expression but not acute changes in the phosphorylation state of these enzymes is the likely cause of reduced activity. This explanation is in agreement with the results of the Northern blots, which revealed reduced hepatic mRNA levels of both enzymes at four and eight days after surgery in BDL rats. These findings are in perfect agreement with those from rats with long-term cholestasis (32) and indicate that cholestasis and not secondary biliary cirrhosis is the primary cause of decreased expression of these enzymes. Interestingly, contrary to hormonal short-term regulation, which increases the activity of one and decreases the activity of the other enzyme (1), cholestasis decreases mRNA expression and activity of both glycogen synthase and phosphorylase at the same time, suggesting a common regulatory pathway. We have observed a similar constellation in rats with long-term cholestasis (32), but the precise mechanism for this unusual finding is not clear. Since glycogen phosphorylase and synthase are located on different chromosomes (1), post-transcriptional alterations are the most likely reasons for this finding.

Similar to humans (41,42) and rats (31) with liver cirrhosis, acute cholestasis was associated with increased plasma concentrations of glucagon in BDL rats,
whereas the plasma insulin levels were either decreased or unchanged. Since glucagon accelerates glycogen breakdown in the liver (1,13,16), increased plasma concentrations of this hormone could theoretically explain a decrease in the hepatic glycogen content. If this were the case, however, an increase in the active fraction of glycogen phosphorylase and a corresponding decrease of glycogen synthase would have been expected in BDL rats (1), which was not the case. The observed decrease in the hepatic glycogen stores in BDL rats is therefore most likely not due to the increased plasma glucagon concentrations. If this interpretation is correct, further studies are needed to explain why glycogen phosphorylase is irresponsive to glucagon in livers of cholestatic rats. Similar findings were obtained in perfused livers from rats with long-term cholestasis, where glucagon failed to stimulate glucose production (43).

Eight days after surgery, the glycogen content per ml of hepatocytes or per g liver was clearly decreased in BDL rats if compared to pair-fed or ad libitum fed CON rats, but not different between the two control groups. Since the intake of food (and also body weight) was not different between BDL and pair-fed CON rats at this time point (indicating that BDL rats had recovered from the surgical procedure), this decrease in the hepatic glycogen content is due to cholestasis, which led to decreased expression and activity of glycogen synthase and phosphorylase. In accordance with these findings and interpretation, our studies in rats with bile duct ligation for two to four weeks showed a progressive decrease in the hepatic glycogen content with prolonged duration of cholestasis (31). A possible explanation for reduced hepatic glycogen stores in BDL rats is endotoxinemia, which has been described in BDL rats also in other studies (44), and which is known to impair hepatic glycogen synthesis (25, 45). Since prostaglandins produced from Kupffer cells mediate the hypoglycogenic effect of endotoxins (25), the skeletal muscle glycogen stores in BDL rats are maintained despite elevation of endotoxins in peripheral blood.

Physiologically more important than the glycogen content per g liver or ml of hepatocytes, is the glycogen content per whole liver. Liver glycogen is particularly important during the postabsorptive state and early starvation, when it is the main source of body glucose (1). When the hepatic glycogen stores are exhausted, the body glucose needs must be met by gluconeogenesis, which
depends on amino acids mainly from skeletal muscle (46). Since long-term cholestasis and/or liver cirrhosis are associated with low hepatic glycogen stores (47,48), this may be one of the mechanisms leading to muscle wasting in patients with chronic liver disease (49,50). Four days after surgery, both pair-fed and BDL rats had a lower total liver glycogen content than ad libitum-fed CON rats, whereas eight days after surgery, total liver glycogen was equal in both CON groups but lower in BDL rats. Due to an increase in liver weight (and volume of hepatocytes per liver), the reduction in glycogen per liver was less pronounced than the reduction per g liver tissue or volume of hepatocytes in BDL rats. Proliferation of hepatocytes at least partially compensates for loss of hepatic glycogen stores during the early phase of bile duct ligation in rats, and may therefore be regarded as a way for the liver to compensate for reduced function.

In contrast to the liver, the skeletal muscle glycogen content and the activities of glycogen synthase and phosphorylase did not differ between BDL and CON rats at both time points. These findings are in contrast to patients with alcoholic liver cirrhosis (51) or rats with long-term bile duct ligation (31) where the activity of glycogen synthase or the glycogen content, respectively, has been found to be reduced. The duration of liver disease seems therefore to be important for the development of changes in skeletal muscle glycogen metabolism. These findings also suggest that local rather than systemic factors contribute to alterations in glycogen metabolism in rats with acute cholestasis. As suggested by the histological pattern showing a more diffuse and non zonal reduction in the hepatic glycogen stores in BDL rats, such factors affect the whole liver and may therefore include for instance high hepatic concentrations of bile acids and/or high endotoxin levels. Further studies are therefore needed to investigate the nature of these local factors in more detail.

**Acknowledgements**

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4.1.6 References


4.2 Impaired hepatic fatty acid oxidation in rats with short-term cholestasis: characterization and mechanism

4.2.1 Summary

Rats with long-term cholestasis have reduced ketosis during starvation. Since it is unclear whether this is also the case in short-term cholestasis, we investigated hepatic fatty acid metabolism in rats with bile duct ligation for 5 (BDL5, n=11) or 10 days (BDL10, n=11) and compared the findings to pair-fed control rats (CON5 and CON10, n=11). The plasma β-hydroxybutyrate concentration was reduced in BDL rats (0.54±0.10 vs. 0.83±0.30 mmol/l at 5 and 0.59±0.24 vs. 0.88±0.09 mmol/l at 10 days in BDL and control rats, respectively). In isolated liver mitochondria, state 3 oxidation rates for various substrates were not different between BDL and control rats. Production of ketone bodies from $^{14}$C-palmitate was reduced by 40% in mitochondria from BDL rats at both time points, whereas production of $^{14}$CO$_2$ was maintained. These findings indicated intact function of the respiratory chain, Krebs cycle and β-oxidation and suggested impaired ketogenesis (HMG-CoA pathway). Accordingly, the formation of acetoacetate from acetyl-CoA by disrupted mitochondria was reduced in BDL rats at 5 (2.1±1.0 vs. 4.8±1.9 nmol/min/mg protein) and at 10 days (1.7±1.0 vs. 6.2±1.9 nmol/min/mg protein). The principle defect could be localized at the rate-limiting enzyme of the HMG-CoA pathway, HMG-CoA synthase, which revealed decreased activity, and reduced hepatic mRNA and protein levels. We conclude that short-term cholestasis in rats leads to impaired hepatic fatty acid metabolism due to impaired ketogenesis. Ketogenesis is impaired due to decreased mRNA levels of HMG-CoA synthase, leading to reduced hepatic protein levels and to decreased activity of this key enzyme of ketogenesis.

**Key words:** mitochondria, respiratory chain, β-oxidation, HMG-CoA pathway, ketogenesis
4.2.2 Introduction

Long-term cholestasis in the rat is associated with alterations in hepatic energy metabolism such as decreased glycogen stores (1) and impaired mitochondrial fatty acid metabolism (2,3). As shown in Figure 8, transport of fatty acids into liver mitochondria and mitochondrial metabolism of fatty acids involves a variety of biochemical reactions and metabolic pathways. After activation at the outer mitochondrial membrane (4), the resulting long-chain acyl-CoA is converted to the respective carnitine derivative by carnitine palmitoyltransferase (CPT) I (5). The long-chain acylcarnitine is transported into the mitochondrial matrix, reconverted to the acyl-CoA derivative and metabolized by the β-oxidation pathway (5,6), resulting in the formation of acetyl-CoA, NADH and FADH. Acetyl-CoA can be converted to ketone bodies or can be degraded further by the action of the Krebs cycle, whereas NADH and FADH are oxidized via the respiratory chain.

Early studies had shown that the production of ketone bodies by perfused livers from rats with long-term bile duct ligation is reduced (7). In agreement with these observations, we later found that rats with long-term bile duct ligation starved for 24 hours had decreased plasma ketone body concentrations (3) and that the production of ketone bodies by hepatocytes isolated from such rats was lower than for control rats (2). Long-term bile duct ligation is associated with reduced activities of complex I and III of the respiratory chain of liver mitochondria (2,8), a finding considered to be responsible for the observed impairment of hepatic fatty acid oxidation in this animal model of biliary cirrhosis. However, in recent studies we could show that the activities of complex I and III recover within days after reversal of bile duct ligation whereas hepatic fatty acid metabolism did not normalize during this observation period (9). In the same animals, analysis of the hepatic carnitine pool revealed an increased content of acetylcarnitine whereas the hepatic β-hydroxybutyrate content was decreased, suggesting intact β-oxidation but impaired ketogenesis (10).
Fig. 8 Hepatic long-chain fatty acid metabolism

Long-chain fatty acids (e.g. palmitate) are activated by a specific acyl-CoA synthase (PCS) which is located in the outer mitochondrial membrane. The acyl-CoA formed is converted to the respective carnitine derivative by carnitine palmitoyltransferase (CPT) I, an enzyme located at the inner side of the outer mitochondrial membrane. The newly formed acylcarnitine is transported across the inner mitochondrial membrane by a carnitine translocase (CTL) and reconverted to the acyl-CoA derivative by CPT II. Within the mitochondrial matrix, the long-chain acyl-CoA undergoes β-oxidation, leading to the formation of acetyl-CoA, NADH and FADH. Acetyl-CoA can be converted to ketone bodies (formation of acetoacetate and β-hydroxybutyrate via the HMG-CoA pathway) or can be degraded further by the action of the Krebs cycle. NADH and FADH are oxidized by the enzyme complexes of the respiratory chain (not shown).
4.2.3 Materials and Methods

Reagents

[1-14C] palmitic acid was obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). Phosphotransacetylase was obtained from Boehringer Mannheim GmbH (Rotkreuz, Switzerland). All other chemicals were of reagent grade.

Animals

All animal experiments had been approved by the State Animal Ethics Board and were performed according to these guidelines. Male Sprague-Dawley rats (BRL, Füllinsdorf, Switzerland) were used throughout the experiments. Rats were housed individually in wire-bottom cages on a 12 hour dark and light cycle. Ligation and transection of the common bile duct were performed as described previously (1). Rats were studied either after 5 days, or after 10 days of bile duct ligation (BDL rats, n = 11 for both groups). Two groups of control rats (n = 11 for both groups) were sham-operated (laparotomy and manipulation of the bile duct) and pair-fed to treated rats throughout the study with normal rat chow (Kliba Futter, Basel, Switzerland). All studies were performed with the rats starved for 24 hours before investigation. There was no mortality in any of the groups studied.

Characterization of the animals

The animals were characterized by their body and spleen weights, activities of alkaline phosphatase and alanine aminotransferase (ALT) in plasma and by their plasma concentrations of glucose, free fatty acids, β-hydroxybutyrate, bilirubin and bile acids. Alkaline phosphatase, ALT, glucose, free fatty acids and bilirubin were analyzed on a COBAS analyzer (Hoffmann-La Roche Diagnostics, Basel, Switzerland) with commercially available kits. Bile acids were determined with a radioimmunoassay (Becton and Dickinson, Orangeburg, SC, USA). Beta-hydroxybutyrate was determined fluorimetrically as described by Olsen (14).
Isolation of liver mitochondria and mitochondrial oxidative metabolism

For the isolation of mitochondria, rats were decapitated and a mixed venous/arterial blood sample was obtained into heparinized tubes. A freeze clamped liver sample was obtained quickly and stored at -70°C. Mitochondria were isolated from the remainder of the liver by differential centrifugation as described by Hoppel et al. (15). As shown previously, this method yields mitochondria of good quality with only minor contamination of peroxisomes or lysosomes also in rats with bile duct ligation (2). An aliquot of freshly isolated mitochondria was used to determine mitochondrial protein by the Biuret method with bovine serum albumin as a standard (16).

Oxygen consumption by intact mitochondria was measured in a chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) at 30°C as described previously (17). The concentrations of the substrates used was 20 mmol/l for L-glutamate and succinate, 7.2 mmol/l for ascorbate, 40 μmol/l for palmitoyl-L-carnitine, 20 μmol/l for palmitoyl-CoA and 80 μmol/l for palmitate. All incubations with fatty acids contained 5 mmol/l L-malate, incubations with palmitoyl-CoA or palmitate additionally 2 mmol/l L-carnitine and incubations with palmitate additionally 250 μmol/l ATP and 250 μmol/l CoASH.

In vitro mitochondrial β-oxidation and Krebs cycle

The β-oxidation of [1-14C] palmitic acid by liver mitochondria was assessed as described by Fréneaux et al. (18), with some modifications. The preincubation medium (1.8 ml of 70 mmol/l sucrose, 43 mmol/l KCl, 3.6 mmol/l MgCl₂, 7.2 mmol/l potassium phosphate, 36 mmol/l Tris-HCl buffer, pH 7.4) contained 0.2 mmol/l ATP, 50 μmol/l L-carnitine, 15 μmol/l CoASH and 1 mg mitochondrial protein. After 5 minutes of preincubation at 30°C, the incubation mixture was brought to 2 ml by adding 200 μl of the preincubation medium containing [1-14C] palmitic acid (final concentration 40 μmol/L; 0.1 μCi per 2 ml) with bovine serum albumin (final concentration in the assay solution 0.5 mg per 2 ml). The tubes were closed with covers containing a filter paper soaked in 100 mmol/l NaOH for trapping of [14C]CO₂ and incubated at 30°C with slow shaking. After 15 minutes, the reaction was stopped by adding 400 μl of 5% perchloric acid to the incubation mixture. Trapping of [14C]CO₂ was
continued for 60 minutes. The filter papers were then transferred into scintillation vials and counted for \([^{14}C]CO_2\) activity. The incubation mixture was subsequently centrifuged at 4000 \( \times \) g for 10 minutes. An aliquot (400 \( \mu \)L) of the supernatant was counted for \([1-^{14}C]\) activity. This activity measures acid soluble products of mitochondrial palmitate metabolism which equals the formation of ketone bodies (18).

**Direct determination of ketone body formation by disrupted, isolated liver mitochondria**

Ketone body formation by liver mitochondria was determined directly according to Chapman et al. (19), with some modifications. Frozen mitochondria were thawed, put quickly into liquid nitrogen and thawed again. Two mg mitochondrial protein were incubated at 37°C for 15 minutes with an acetyl-CoA generating system in a final volume of 900 \( \mu \)L. This system contained 25 U of phosphotransacetylase and the following components (final concentrations): 10 mmol/l sodium phosphate, 4 mmol/l ATP, 30 mmol/l lithium acetyl phosphate, 1 mmol/l CoASH, 35 mmol/l KCl, 3 mmol/l MgCl₂ and 0.5 mmol/l dithiothreitol, pH 7.4. The reaction was stopped by adding 100 \( \mu \)L of 30% perchloric acid. After removing the precipitate by centrifugation the supernatants were analyzed for acetoacetate according to Olsen (14).

**Assay of acetoacetyl-CoA thiolase activity**

The activity of acetoacetyl-CoA thiolase was assayed with disrupted mitochondria using a spectrophotometric method with acetoacetyl-CoA as a substrate (15).

**Assay of HMG-CoA synthase activity**

The activity of HMG-CoA synthase was assessed according to Quant et al. (20), with some modifications. Fifteen \( \mu \)L of liver mitochondria (300 \( \mu \)g protein) were treated with 7.5 \( \mu \)L Triton X-100 to expose HMG-CoA synthase and were assayed immediately. The standard 1 ml assay system contained 50 mmol/l Tris/HCl, 10 mmol/l MgCl₂ and 2 mmol/l dithiothreitol, pH 8.0. Lysed mitochondria (300 \( \mu \)g protein), 100 \( \mu \)mol/l acetyl-CoA, 10 U phosphoacetyltransferase, 5 mmol/l acetyl phosphate and 10 mmol/l acetoacetyl-CoA were added simultaneously. HMG-CoA synthase activity was
measured at 30 °C as the initial velocity of the decrease in absorbance at 303
nm. Under these conditions HMG-CoA synthase is desuccinylated and the total
activity is measured.

RNA isolation and reverse transcription
Total RNA was extracted from rat liver according to the general protocol of
Sambrook et al. (21). The RNA concentration was determined by the
absorbance at 260 nm, and the quality of the RNA was controlled by running an
aliquot on a 1% agarose formaldehyde gel. Four μg of total RNA from rat liver
were used as a template for first-strand cDNA synthesis with reverse
transcriptase (Molony Murine Leukemia Virus reverse transcriptase; Gibco BRL,
Life Technologies AG, Basel, Switzerland) and oligo (dT) primer.

Real-time quantitative PCR analysis of HMG-CoA synthase
Real-time quantitative PCR analysis was performed with a PE Applied
Biosystems 7700 Sequence Detector (PE Biosystems), which is a combined
thermocycler and fluorescent detector. Sets of primers were chosen for HMG-
CoA synthase to receive a PCR product of less than 100 base pairs. A dual-
labelled fluorogenic probe complementary to a sequence within the PCR
product was added to the PCR reaction. The primers and the dual-labelled
fluorogenic probe for GAPDH, which served as internal standard, were chosen
accordingly. One fluorescent dye (6-carboxyfluorescein) serves as a reporter,
and its emission is quenched by a second fluorescent dye (6-carboxy-
tetramethylrhodamine). During elongation, the 5' to 3' exonuclease activity of
the Taq DNA polymerase hydrolyzes the probe, thus releasing the reporter from
the quencher, resulting in increased fluorescence which is detected. For HMG-
CoA synthase, the forward and reverse primers were TGAACG GTGAATAGA
CAC AGC G and GTG GTG CTC ACT GCT TCA GG, respectively, with the
probe CTG CTC CGC GGT GAA GGGCC. For GAPDH, the corresponding
forward and reverse primers were CTG CCAAGTATG ATG ACA TCA AGAA
and AGCCCA GGATGC CCTTTA GT, respectively, with the probe TCG GCC
GCC TGC TTC ACC A (22). Primers and probes were custom-synthesized by
PE Biosystems. Complementary DNA was amplified in a 50 μl volume
containing 25 μl of the 2 x TaqMan Universal PCR Master Mix (PE Biosystems),
100 nmol/l probe and 300 nmol/l of each primer. After a denaturating step of 10 min at 95°C, 40 cycles were performed: 95°C for 15 seconds and 60°C for 1 minute. The mathematical analysis of the results were performed as recommended by the manufacturer after having performed all the validation experiments necessary (PE Biosystems, DOD Doc# 4303859, user bulletin # 2).

Preparation of antibodies and Western blot analysis of HMG-CoA synthase
Antibodies against mitochondrial HMG-CoA synthase were prepared by injecting rabbits with a peptide corresponding to the amino acid sequence 37-49 of the mitochondrial HMG-CoA synthase protein as described previously (23). Immunoblotting of mitochondrial HMG-CoA synthase was carried out as described by Serra et al. (23). The autoradiograms were quantified using a Luminescent Image Analyser LAS-1000 with Image Reader LAS-1000 for Windows® software (Raytest, Urdorf, Switzerland).

Data presentation and statistical analysis
Data are presented as mean±sd unless specified otherwise. Data were analyzed by analysis of variance (ANOVA) followed by a t-test with Bonferroni correction to localize the differences in case of a significant ANOVA. A p<0.05 was considered to be statistically significant.

4.2.4 Results
The studies were carried out to investigate the effect of short-term cholestasis on hepatic mitochondrial fatty acid metabolism and to find out the mechanisms, if hepatic metabolism of fatty acids were found to be impaired. Rats were bile duct ligated for 5 or 10 days (BDL rats) and control rats were pair-fed to BDL rats throughout the study. The final studies were carried out with all rats starved for 24 hours. As shown in Table 8, body weights were not different between BDL and control rats 5 days after surgery, but slightly increased in BDL rats after 10 days. Liver and spleen weights were significantly increased in BDL rats at both time points. A so far unpublished histological analysis of livers from rats 5 or 10 days after bile duct ligation showed no massive hepatic steatosis, fibrosis or proliferation of bile ducts, suggesting that fluid retention is the most likely cause for liver weight gain in rats with short-term bile duct ligation (C.
Lang, unpublished results). As expected, activities of alanine aminotransferase and alkaline phosphatase as well as the plasma concentrations of bilirubin and bile acids were all increased in BDL rats. Similar to previous studies after a longer period of bile duct ligation, BDL rats had reduced plasma glucose levels at both time points (9). While the plasma free fatty acid concentration was not different between BDL and control rats, the plasma β-hydroxybutyrate concentration was decreased in BDL rats at both 5 and at 10 days after surgery, compatible with impaired hepatic fatty acid metabolism.

<table>
<thead>
<tr>
<th></th>
<th>CON5</th>
<th>BDL5</th>
<th>CON10</th>
<th>BDL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (end of study) (g)</td>
<td>266±33</td>
<td>279±30</td>
<td>276±20</td>
<td>299±25*</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>7.2±0.6</td>
<td>12.0±1.0*</td>
<td>8.2±0.8</td>
<td>14.8±1.6*</td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>0.59±0.10</td>
<td>0.80±0.16*</td>
<td>0.64±0.10</td>
<td>1.01±0.19*</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>47±13</td>
<td>213±109*</td>
<td>44±16</td>
<td>123±41*</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>187±35</td>
<td>379±105*</td>
<td>185±25</td>
<td>491±68*</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>0.3±0.4</td>
<td>131±42*</td>
<td>0.2±0.3</td>
<td>181±35*</td>
</tr>
<tr>
<td>Bile acids (µmol/L)</td>
<td>1±1</td>
<td>138±52*</td>
<td>1±1</td>
<td>103±34*</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>6.9±1.1</td>
<td>5.7±0.8*</td>
<td>6.8±1.0</td>
<td>5.7±0.3*</td>
</tr>
<tr>
<td>Free fatty acids (mmol/L)</td>
<td>1.04±0.36</td>
<td>1.07±0.14</td>
<td>0.93±0.23</td>
<td>0.98±0.15</td>
</tr>
<tr>
<td>β-Hydroxybutyrate (mmol/L)</td>
<td>0.83±0.30</td>
<td>0.54±0.10*</td>
<td>0.88±0.09</td>
<td>0.59±0.24*</td>
</tr>
</tbody>
</table>

Tab. 8 Characterization of the rats

Rats were bile duct ligated for 5 (BDL5, n=11) or 10 days (BDL10, n=11). Control rats (CON5 and CON10, n=11 for both groups) were sham-operated and pair-fed to BDL rats throughout the study. Enzyme activities and metabolite concentrations were determined in heparinized plasma as described in Methods. Results are given as mean ±sd. *p<0.05 vs. the respective control group.

As illustrated in Figure 8, hepatic fatty acid metabolism can be impaired due to reduced activation or transport of fatty acids, impaired β-oxidation or activity of the respiratory chain, or due to reduced activity of the HMG-CoA pathway. As a first step, as shown in Table 9, we therefore investigated oxidative metabolism of different substrates by isolated liver mitochondria. State 3 and state 4 (results not shown) oxidation rates were not different for L-glutamate, succinate or ascorbate/TMPD as substrates, excluding a defect in the respiratory chain (17). State 3 and state 4 (results not shown) oxidation rates for palmitoyl-CoA and palmitoylcarnitine were also not different between BDL and control rats,
excluding impaired transport into mitochondria and impaired \(\beta\)-oxidation of fatty acids as an explanation for reduced hepatic fatty acid metabolism in BDL rats. Using palmitate as a substrate, there was no difference in state 3 oxidation at 5, but a slight reduction in BDL rats at 10 days after bile duct ligation. Theoretically, reduced activation of palmitate could therefore have contributed to impaired hepatic fatty acid metabolism in BDL rats, at least 10 after surgery.

<table>
<thead>
<tr>
<th>Activity</th>
<th>CON5</th>
<th>BDL5</th>
<th>CON10</th>
<th>BDL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamate (20 mmol/L)</td>
<td>64±12</td>
<td>68±9</td>
<td>70±13</td>
<td>72±12</td>
</tr>
<tr>
<td>Succinate (20 mmol/L)</td>
<td>85±21</td>
<td>94±34</td>
<td>102±28</td>
<td>84±24</td>
</tr>
<tr>
<td>Ascorbate (7.2 mmol/L)</td>
<td>275±74</td>
<td>253±84</td>
<td>245±58</td>
<td>256±69</td>
</tr>
<tr>
<td>Palmitate (80 (\mu)mol/L)</td>
<td>28±13</td>
<td>29±4</td>
<td>46±8</td>
<td>34±4*</td>
</tr>
<tr>
<td>Palmitoyl-CoA (20 (\mu)mol/L)</td>
<td>53±17</td>
<td>52±15</td>
<td>58±20</td>
<td>58±16</td>
</tr>
<tr>
<td>Palmitoyl-L-carnitine (40 (\mu)mol/L)</td>
<td>53±19</td>
<td>50±14</td>
<td>63±21</td>
<td>55±16</td>
</tr>
</tbody>
</table>

Tab. 9 State 3 oxidation rates by isolated rat liver mitochondria

Rats were bile duct ligated for 5 (BDL5, n=11) or 10 days (BDL10, n=11). Control rats (CON5 and CON10, n=11 for both groups) were sham-operated and pair-fed to BDL rats throughout the study. Mitochondria were isolated by differential centrifugation and state 3 oxidation rates were determined using a Clark-type oxygen electrode as described in Methods. Units are natoms/min/mg mitochondrial protein. Results are presented as mean±SD. *p<0.05 BDL vs. control rats.

As a next step, the formation of ketone bodies and CO\(_2\) from palmitate was determined using \(^{14}\)C-palmitate as a substrate. As shown in Table 10, the formation of acid soluble products (representing ketone bodies, reference 18), was decreased in BDL rats at both time points, compatible with impaired ketogenesis, possibly due to reduced activity of the HMG-CoA pathway (Figure 8). In favor of this hypothesis, production of CO\(_2\) from palmitate, determined in the same incubations as the formation of ketone bodies and reflecting the activity of the Krebs cycle, was increased in mitochondria from BDL by 17% (5 days) or 43% (10 days) but without reaching statistical significance due to high variations. The ratio of CO\(_2\)-production and formation of ketone bodies was increased significantly in BDL rats at both time points.
<table>
<thead>
<tr>
<th>Activity</th>
<th>CON5</th>
<th>BDL5</th>
<th>CON10</th>
<th>BDL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid soluble β-oxidation products</td>
<td>1.29±0.32</td>
<td>0.70±0.34*</td>
<td>1.17±0.43</td>
<td>0.69±0.37*</td>
</tr>
<tr>
<td>CO₂ production</td>
<td>0.030±0.007</td>
<td>0.035±0.020</td>
<td>0.037±0.021</td>
<td>0.053±0.035</td>
</tr>
<tr>
<td>CO₂ production/(acid soluble products + CO₂ production)</td>
<td>2.3±0.04</td>
<td>4.9±1.3*</td>
<td>3.2±1.0</td>
<td>5.1±2.1*</td>
</tr>
</tbody>
</table>

Tab. 10 In vitro β-oxidation and Krebs cycle activity by isolated liver mitochondria

Rats were bile duct ligated for 5 (BDL5, n=11) or 10 days (BDL10, n=11). Control rats (CON5 and CON10, n=11 for both groups) were sham-operated and pair-fed to BDL rats throughout the study. Mitochondria were isolated by differential centrifugation. β-Oxidation and Krebs cycle activity were determined using 1-14C-palmitate as a substrate as described in Methods. Units for acid soluble β-oxidation products and CO₂ production are nmoles/min/mg mitochondrial protein. Results are presented as mean±SD. *p<0.05 BDL vs. control rats.

The next step was therefore to assess the activity of the HMG-CoA pathway in more detail (Table 11). With acetyl-CoA as a substrate and using disrupted mitochondria, the formation of acetoacetate can be assessed, whereas β-hydroxybutyrate is not formed under these conditions (19). In agreement with the hypothesis of a specific defect in the HMG-CoA pathway, acetoacetate production from acetyl-CoA was reduced in mitochondria from BDL rats at both time points. As shown in Table 11, this reduction could be explained primarily by impaired activity of HMG-CoA synthase, the rate-limiting enzyme of the HMG-CoA pathway, whose activity was reduced in BDL rats at both time points studied. In contrast, the activity of the first enzyme of the HMG-CoA pathway, acetoacetyl-CoA thiolase, was not reduced at 5 days and showed an only slight reduction at 10 days after surgery.
<table>
<thead>
<tr>
<th>Activity</th>
<th>CON5</th>
<th>BDL5</th>
<th>CON10</th>
<th>BDL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation of acetoacetate from acetyl-CoA</td>
<td>4.8±1.9</td>
<td>2.1±1.0*</td>
<td>6.2±1.9</td>
<td>1.7±1.0*</td>
</tr>
<tr>
<td>Acetoacetyl-CoA thiolase</td>
<td>220±43</td>
<td>194±41</td>
<td>233±47</td>
<td>183±35*</td>
</tr>
<tr>
<td>HMG-CoA synthase</td>
<td>1.46±0.47</td>
<td>0.94±0.50*</td>
<td>1.82±0.39</td>
<td>0.89±0.69*</td>
</tr>
</tbody>
</table>

**Tab.11 Activity of the HMG-CoA pathway in disrupted liver mitochondria**

Rats were bile duct ligated for 5 (BDL5, n=11) or 10 days (BDL10, n=11). Control rats (CON5 and CON10, n=11 for both groups) were sham-operated and pair-fed to BDL rats throughout the study. Mitochondria were isolated by differential centrifugation and disrupted by freeze-thawing. Production of acetoacetate from acetyl-CoA was determined fluorimetrically and enzyme activities spectrophotometrically as described in Methods. Units are nmoles/min/mg mitochondrial protein. Results are presented as mean±SD. *p<0.05 BDL vs. control rats.

**Fig.9 Analysis of hepatic HMG-CoA synthase mRNA expression by real-time quantitative PCR**

Rats studied were either bile duct ligated (BDL) or sham-operated (CON). Control rats were pair-fed to BDL rats, and all animals were starved for 24 h before the final experiments. Figure A represents a representative amplification plot of one BDL and one CON animal. The x-axis denotes the number of cycles and the y-axis the fluorescence intensity over background. The horizontal line is the fluorescence at the 17th cycle of GAPDH which was taken as a threshold. The two curves for GAPDH (blue = CON, green = BDL) are superimposed and cannot be separated from each other. The fluorescence curve for HMG-CoA synthase with liver RNA of CON animals reaches the threshold after 17 cycles (yellow line) and the curve with liver RNA of BDL rats after 19 cycles (red line). The relative quantification is shown in the Figure B (control rats are set at 100%). Data are given as mean ± S.D. *p < 0.05 BDL vs. CON rats.
Fig. 10 Effect of bile duct ligation on mitochondrial HMG-CoA synthase protein

Rats studied were either bile duct ligated (BDL) or sham-operated (CON). Control rats were pair-fed to BDL rats, and all animals were starved for 24 h before the final experiments. Rats were decapitated and their livers quickly removed and frozen in liquid nitrogen, processed for electrophoresis and Western transfer which was followed by incubation with a specific antibody for mitochondrial HMG-CoA synthase. Blots were quantitatively analyzed using a Luminescent Image Analyzer. The autoradiographs for both time points studied are shown in Figure A and the relative quantification is shown in Figure B (control rats are set at 100%). Data are given as mean ± S.D. *p < 0.05 BDL vs. CON rats.

In order to confirm the biochemical determinations and to define the mechanism of reduced ketogenesis by acute cholestasis more precisely, we determined the hepatic steady state levels of mRNA and protein of HMG-CoA synthase. As shown in Figure 9, liver mRNA levels of HMG-CoA synthase were reduced by approximately 70% at day 5 and by 65% at day 10 after surgery. Accordingly, as shown in Figure 10, a Western blot of HMG-CoA synthase showed reduced hepatic protein levels of this enzyme at both time points studied.

4.2.5 Discussion

The current studies demonstrate that, similar to rats with long-term bile duct ligation, hepatic fatty acid metabolism is impaired also in rats with short-term cholestasis. Impaired hepatic fatty acid metabolism in BDL rats is explained by decreased ketogenesis due to reduced activity of HMG-CoA synthase, the rate limiting enzyme of the HMG-CoA pathway.
Our studies show a good agreement between the biochemical and the molecular characterization of the principle enzyme affected, HMG-CoA synthase. The biochemical characterization of mitochondrial fatty acid metabolism revealed reduced ketogenesis with $^{14}$C-palmitate or acetyl-CoA as a substrate, indicating that at least one of the four ketogenetic enzymes had to be impaired by acute cholestasis (see Figure 8). A reduced activity of enzyme complexes of the respiratory chain could be excluded, because state 3 and 4 respiration in the presence of L-glutamate, succinate or ascorbate/TMPD had been found to be unchanged in mitochondria from BDL rats. Concerning the function of the respiratory chain, acute is clearly different from long-term cholestasis, since long-term cholestasis is associated with decreased activities of enzyme complexes I, II, III and V (2,8,9). The development of a decrease in the function of the respiratory chain of liver mitochondria requires therefore either cholestasis over several weeks and/or the development of liver cirrhosis. Since the respiratory chain and/or fatty acid metabolism of liver mitochondria appear not to be grossly affected in rats with CCl$_4$- (24) or thioacetamide-induced liver cirrhosis (25,26), long-term cholestasis appears to be more important for the mitochondrial defect in BDL rats than cirrhosis itself.

Since the production of $^{14}$CO$_2$ from $^{14}$C-palmitate was not impaired (or even increased) in mitochondria from BDL rats and oxidative metabolism of palmitoyl-L-carnitine and palmitoyl-CoA was also not affected, mitochondrial β-oxidation and Krebs cycle activity had to be normal (or increased) in mitochondria from BDL rats. These findings also differ from the situation in rats with long-term cholestasis, where various enzymes of mitochondrial β-oxidation have been found to have a reduced activity (2). The only finding compatible with impaired fatty acid metabolism proximal to the HMG-CoA pathway in mitochondria from rats with acute cholestasis was a reduced state 3 oxidation rate with palmitate as a substrate. Taking into account normal mitochondrial metabolism of palmitoyl-CoA and palmitoylcarnitine, this finding is compatible with impaired activation of long-chain fatty acids on the outer mitochondrial membrane (Figure 8). However, reduced activation of fatty acids in BDL rats was considered to be of only minor importance and to offer no satisfactory explanation for impaired hepatic fatty acid metabolism in BDL rats for different reasons. First, activation
of fatty acids is normally not considered to be rate-limiting for hepatic fatty acid metabolism (5,27). Second, $^{14}\text{CO}_2$-production from $^{14}\text{C}$-palmitate was not different between mitochondria from BDL and control rats, suggesting that the mitochondrial pool of acetyl-CoA, which is at the same time the end-product of β-oxidation (distal to activation of fatty acids, see Figure 8) and the substrate for the HMG-CoA pathway and the Krebs cycle, was not lower in BDL as compared to control rats. This assumption is in agreement with a previous study showing that the mitochondrial short-chain acyl-CoA pool is not different between rats with bile duct ligation for 4 weeks and control rats (28).

The formation of ketone bodies from acetyl-CoA is performed by four mitochondrial enzymes, namely acetoacetyl-CoA thiolase, HMG-CoA synthase, HMG-CoA lyase and β-hydroxybutyrate dehydrogenase (27). Since disrupted mitochondria form only acetoacetate but not β-hydroxybutyrate from acetyl-CoA (19), a deficiency of β-hydroxybutyrate dehydrogenase could not be the reason of our findings and was therefore not assessed further. The activity of the first enzyme of the HMG-CoA pathway, acetoacetyl-CoA thiolase, showed a slight reduction in mitochondria from BDL rats 10 days after surgery. Due to the high activity of this enzyme (see Table 11), this finding provided no satisfactory explanation for reduced activity of the HMG-CoA pathway in mitochondria from BDL rats. In this context, it is important to emphasize that, in contrast to long-term cholestasis, acute cholestasis is not associated with a broad, unspecific mitochondrial damage. This is well illustrated by maintained activity of acetoacetyl-CoA thiolase at the early time point studied, and maintained functions of the respiratory chain, β-oxidation and Krebs cycle in liver mitochondria from rats with acute cholestasis.

The rate-limiting enzyme of the HMG-CoA pathway is HMG-CoA synthase (29,30). In hepatocytes, two different HMG-CoA synthases can be detected, one in the cytosol and the other one in mitochondria. Both enzymes have been cloned and they are encoded by different genes (31). While the cytosolic enzyme is important for cholesterol biosynthesis, the mitochondrial one catalyzes the rate-limiting step of the HMG-CoA pathway. Short-term regulation of mitochondrial HMG-CoA synthase is achieved by succinylation and desuccinylation (23,32,33). The enzyme is more active in the desuccinylated
state, which can be achieved by treatment with glucagon (23,32). In our studies, we measured total activity of HMG-CoA synthase and can therefore not exclude the possibility that impaired desuccinylation may also contribute to reduced ketogenesis in acute cholestasis in vivo. On the other hand, the reduction in the activity of HMG-CoA synthase observed in our studies is in the same order of magnitude as that for hepatic HMG-CoA synthase protein or mRNA levels, suggesting that the major effect of acute cholestasis is on long- and not short-term regulation of HMG-CoA synthase.

Long-term control of HMG-CoA synthase activity is achieved by regulation of gene transcription (27). Physiological factors which upregulate transcription include starvation, long-term exercise, high-fat diet and also the fetal-suckling transition (23,27). In addition, HMG-CoA synthase transcription is also upregulated in experimental animals with diabetes (23) or treated with glucocorticoids (34). A number of transcription factors have been identified, among them hormones such as glucocorticoids and glucagon, but also cellular proteins such as the peroxisome proliferator activating receptor (PPAR) (27). Our results clearly show that hepatic HMG-CoA synthase mRNA and protein levels are decreased in livers from BDL rats, compatible with impaired transcription of the HMG-CoA synthase gene. However, our results do not allow to draw firm conclusions about the mechanisms responsible for the suspected decrease in HMG-CoA synthase transcription. Since BDL rats have generally increased glucagon but unchanged insulin concentrations in comparison to control rats (1), a hormonal cause for this finding is unlikely. Similarly, nutritional factors are also an unlikely cause for impaired transcription of HMG-CoA synthase in acute cholestasis, since control rats were pair-fed to BDL rats and the free fatty acid concentrations were not different between BDL and control rats. Further studies, which were beyond the scope of the current project, will therefore be necessary to find out the reasons for reduced hepatic HMG-CoA synthase mRNA levels in rats with acute cholestasis.

A question not asked in the current investigations concerns the faith of the fatty acids entering the liver when ketogenesis is impaired. The results of the current studies suggest that probably a larger portion is oxidized by the Krebs cycle. Another possibility is esterification to triglycerides and storage in the liver and/or
export into the blood. Histological analysis of livers from rats after 14 or 28 days of bile duct ligation showed no steatosis (1), excluding massive hepatic accumulation of triglycerides. Previous studies have shown increased plasma triglyceride levels in rats with bile duct ligation for 4 weeks (3), suggesting that more fatty acids are esterified and exported as VLDL particles into the blood.

Acknowledgements

We thank Monika Ledermann for technical support and animal care, and Dr. R. Kretschmer for the determination of the plasma free fatty acid concentration. The studies were supported by the grants 3200-051127.97-1 (L.K.) and 31-46792.96 (S.K.) of the Swiss National Science foundation.
4.2.6 References


4.3 Impaired ketogenesis is a major mechanism for disturbed hepatic fatty acid metabolism in rats with long-term cholestasis

4.3.1 Abstract

Rats with long-term cholestasis have reduced ketosis which persists after restoring bile flow. Since the precise mechanisms for this metabolic abnormality are unclear, we investigated fatty acid metabolism in starved rats with biliary obstruction for 4 weeks (BDL rats), and 3, 7, 14, 28 and 84 days after reversal of biliary obstruction by Roux-en-Y anastomosis (RY rats), and in sham-operated control rats. In comparison to controls, BDL rats had reduced β-hydroxybutyrate concentrations in plasma (0.25±0.10 vs. 0.75±0.20 mmol/L) and liver (2.57±0.20 vs. 4.63±0.61 μmol/g) which increased significantly after restoring bile flow. Hepatic expression and activity of carnitine palmitoyltransferase I (CPT I) was unaffected in BDL rats and increased after restoring bile flow. CPT II expression and activity was reduced in BDL rats, and also increased after restoring bile flow. Oxidative metabolism of L-glutamate, succinate, palmitate, palmitoyl-CoA and palmitoylcarnitine by isolated mitochondria was reduced in BDL rats and recovered 7 to 14 days after reversal of biliary obstruction. Mitochondrial β-oxidation, as assessed by the formation of acid soluble products and 14CO2 from 1-14C-palmitate by intact mitochondria, was decreased in BDL rats and recovered 7 days after restoring bile flow. Ketogenesis, determined by the formation of acetoacetate from acetyl-CoA by disrupted mitochondria, was decreased in BDL rats and recovered 3 months after restoring bile flow. Both mRNA and protein expression of HMG-CoA synthase, the rate-limiting enzyme of ketogenesis, was reduced in livers of BDL rats and increased significantly after reversal of biliary obstruction. While impairment of hepatic fatty acid metabolism is multifactorial in BDL rats, impaired expression and activity of HMG-CoA synthase is the major factor after reversal of biliary obstruction.

Key words: mitochondria, respiratory chain, β-oxidation, HMG-CoA pathway (ketogenesis), HMG-CoA synthase
4.3.2 Introduction

Long-term cholestasis in the rat is associated with alterations in hepatic energy metabolism such as impaired glycogen storage (Krähenbühl et al., 1996) and mitochondrial fatty acid metabolism (Krähenbühl and Brass, 1991; Krähenbühl et al., 1994). Rats with long-term bile duct ligation have decreased ketosis during starvation, compatible with reduced production of ketone bodies (Krähenbühl and Brass, 1991). Similar findings were obtained in perfused livers from BDL rats, which showed reduced production of ketone bodies when long- or short-chain fatty acids were used as substrates (Koyama et al., 1975; Koyama et al., 1981). We have conducted several studies on the mechanisms causing impaired ketogenesis in BDL rats. In initial studies, we could show that liver mitochondria from BDL rats have a reduced activity of enzyme complexes of the respiratory chain and of enzymes of the mitochondrial ß-oxidation which could both explain these findings (Koyama et al., 1975). Further studies revealed, however, that after reversing bile duct ligation, ketogenesis remained impaired, whereas the function of complex I and III of the respiratory chain, which are both essential for hepatic fatty acid metabolism, normalized quickly (Krähenbühl et al., 1998).

As shown and explained in Figure 11, beside complexes I and III of the respiratory chain, formation of ketone bodies can be impaired at many other sites, among them activation and transport of long-chain fatty acids, ß-oxidation and the hydroxymethylglutaryl-CoA (HMG-CoA) cycle (ketogenesis). Regarding activation and transport of fatty acids, the activity of carnitine palmitoyltransferase I (CPT I) is considered to be rate-limiting (Spurway et al.,

Abbreviations:

- AST: Aspartate aminotransferase
- BDL rats: bile duct ligated rats
- CoASH: coenzyme A, reduced form
- CPT: carnitine palmitoyltransferase
- HMG-CoA cycle: Hydroxymethylglutaryl-CoA cycle
- TMPD: N,N,N'N'-tetramethyl-p-phenylenediamine
Fig. 11 Hepatic fatty acid metabolism.

Long-chain fatty acids are first activated by palmitoyl-CoA synthase (PCS) on the outer mitochondrial membrane (o.m.). They are then converted to the carnitine derivative by carnitine palmitoyltransferase I (CPT I) and transported across the inner mitochondrial membrane (i.m.) by carnitine translocase (CTL). In the mitochondrial matrix, they are reconverted to the CoA derivative by CPT II and undergo β-oxidation. NADH and FADH produced by β-oxidation are metabolized by the electron transport chain consisting of the enzyme complexes I, II, III and IV. Ubiquinol (UQ) and cytochrome c (cyt c) transport electrons between complexes I or II and III, and between complex III and IV, respectively. Complexes I, III and IV can shift protons from the mitochondrial matrix into the intermembranaceous space, building up a proton gradient. This gradient is necessary to produce ATP from ADP by complex V or F0F1-ATPase. Beside NADH and FADH, β-oxidation produces also acetyl-CoA, which can be used for the formation of ketone bodies or is degraded to CO₂ and H₂O by the Krebs cycle. See text for further explanations.

1997; Drynan et al., 1996). The activity of the HMG-CoA cycle, which produces ketone bodies from acetyl-CoA, the end product of β-oxidation, is controlled by HMG-CoA synthase, an enzyme located in the mitochondrial matrix (Hegardt, 1999). Short-term control of the activity of HMG-CoA synthase is achieved by
succinylation (Lowe and Tubbs, 1985), whereas gene transcription controls long-term activity (Hegardt, 1999).

In recent studies, we were able to show that rats with short-term cholestasis have also impaired ketogenesis and that this finding can be explained entirely by a reduced activity of HMG-CoA synthase (Lang et al., 2001). This mechanism may therefore contribute to impaired ketogenesis also in rats with long-term cholestasis and may explain the observation that ketogenesis remains decreased after reversing biliary obstruction. In order to find an answer to this question, we decided to study hepatic fatty acid metabolism in rats with bile duct ligation for 4 weeks and up to 3 months after reversal of bile duct ligation by biliodigestive anastomosis. The specific questions asked were i) which mechanisms contribute to impaired hepatic fatty acid metabolism in rats with long-term cholestasis, ii) are these changes reversible after biliodigestive anastomosis for 3 months, and iii) which are the mechanisms, if hepatic fatty acid metabolism does not recover.

4.3.3 Materials and Methods

Reagents

[1-14C] palmitic acid was obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). Phosphotransacetylase was obtained from Boehringer Mannheim GmbH (Rotkreuz, Switzerland). All other chemicals were of the highest purity available obtained from different providers.

Animals

Male Sprague-Dawley rats (BRL, Füllinsdorf, Switzerland) were used throughout the experiments. Rats were housed in wire-bottom cages on a 12 hour dark and light cycle. All animals were fed a standard rat chow and tap water ad libitum. The studies were performed with the rats starved for 24 hours before investigation. All animal experiments had been approved by the Animal Ethics Board of the State of Berne and were performed according to these guidelines.
Surgical procedure
The rats were fasted overnight before surgery but had free access to water until immediately before and after surgery. Bile duct ligation (BDL) and sham operation were performed as reported previously (Krähenbühl et al., 1998). A 3-4 cm midline incision was made and the common bile duct was exposed, ligated with three sutures and transected between the two sutures closest to the duodenum. In sham-operated animals (control rats), laparotomy was performed and the common bile duct was exposed but not transected. The reversal of biliary obstruction was achieved by Roux-en-Y anastomosis (Krähenbühl et al., 1998). First, the abdomen was opened through a 3-4 cm midline incision under pentobarbital anesthesia. Then, the dilated proximal common bile duct stump was exposed and minimally freed from surrounding tissue in order to avoid bleeding. The jejunum was divided 3 cm distal to the ligament of Treitz and a short 4-cm Roux-en-Y limb created. End-to-side jejunoojejunostomy was performed with a 6-0 Prolene® (Ethicon, Spreitenbach, Switzerland) single-layer running suture. In sham-operated animals, laparotomy was performed and the abdominal cavity exposed without any surgical procedure.

Study design
Seven different groups were investigated with all animals starved for 24 hours. Rats of group 1 were bile duct ligated for 4 weeks (BDL, n=9). In rats of groups 2 to 6, bile duct ligation was reversed by Roux-en-Y anastomosis for 3 (group 2, n=4), 7 (group 3, n=6), 14 (group 4, n=6), 28 (group 5, n=6) or 84 days (group 6, n=6). Group 7 represented sham-operated control rats (n=8). Control rats were studied at the same time point as BDL rats.

Characterization of the animals
The animals were characterized by their body, liver and spleen weights, activities of alkaline phosphatase and aspartate aminotransferase (AST) in plasma and by their plasma concentrations of glucose, free fatty acids, β-hydroxybutyrate, bilirubin and bile acids. Alkaline phosphatase, AST, glucose, free fatty acids and bilirubin were analyzed on a COBAS analyzer (Hoffmann-La Roche Diagnostics, Basel, Switzerland) with commercially available kits. Bile
acids were determined with a radioimmunoassay (Becton and Dickinson, Orangeburg, SC, USA). Beta-hydroxybutyrate was determined fluorimetrically as described by Olsen (Olsen, 1971).

*Isolation of liver mitochondria and mitochondrial oxidative metabolism*

For the isolation of mitochondria, rats were decapitated and a mixed venous/arterial blood sample was obtained into heparinized tubes. A freeze clamped liver sample was obtained quickly and stored at −70°C. Mitochondria were isolated from the remainder of the liver by differential centrifugation as described by Hoppel et al. (Hoppel et al., 1979). As shown previously, this method yields mitochondria of good quality with only minor contamination of peroxisomes or lysosomes also in rats with bile duct ligation (Krähenbühl et al., 1994). An aliquot of freshly isolated mitochondria was used to determine mitochondrial protein by the Biuret method with bovine serum albumin as a standard (Gornall et al., 1949). Oxygen consumption by intact mitochondria was measured in a chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) at 30°C as described previously (Krahnenbühl et al., 1991). The concentrations of the substrates used was 20 mmol/l for L-glutamate and succinate, 7.2 and 0.24 mmol/l for ascorbate and N,N,N′N′-tetramethyl-p-phenylenediamine (TMPD), respectively, 40 μmol/l for palmitoyl-L-carnitine, 20 μmol/l for palmitoyl-CoA and 80 μmol/l for palmitate. All incubations with fatty acids contained 5 mmol/l L-malate, incubations with palmitoyl-CoA or palmitate additionally 2 mmol/l L-carnitine and incubations with palmitate additionally 250 μmol/l ATP and 250 μmol/l CoASH.

*In vitro mitochondrial β-oxidation and Krebs cycle*

The β-oxidation of [1-14C] palmitic acid by liver mitochondria was assessed as described by Fréneaux et al. (Fréneaux et al., 1988), with some modifications. The preincubation medium (1.8 ml of 70 mmol/l sucrose, 43 mmol/l KCl, 3.6 mmol/l MgCl₂, 7.2 mmol/l potassium phosphate, 36 mmol/l Tris-HCl buffer, pH 7.4) contained 0.2 mmol/l adenosine triphosphate, 50 μmol/l L-carnitine, 15 μmol/l CoASH and 1 mg mitochondrial protein. After 5 minutes of preincubation at 30°C, the incubation mixture was brought to 2 ml by adding 200 μl of the preincubation medium containing 1-14C-palmitic acid (final concentration 40
µmol/L; 0.1 µCi per 2 ml) with bovine serum albumin (final concentration in the assay solution 0.5 mg per 2 ml). The tubes were closed with covers containing a filter paper soaked in 100 mmol/l NaOH for trapping of 14CO2 and incubated at 30°C with slow shaking. After 15 minutes, the reaction was stopped by adding 400 µl of 5% perchloric acid to the incubation mixture. Trapping of 14CO2 was continued for 60 minutes. The filter papers were then transferred into scintillation vials and counted for 14C radioactivity. The incubation mixture was subsequently centrifuged at 4000 x g for 10 minutes. An aliquot (400 µL) of the supernatant was counted for 14C radioactivity. This activity measures acid soluble products of mitochondrial 1-14C-palmitate metabolism which equals the formation of ketone bodies and ketone body precursors (Fromenty et al., 1990).

Formation of ketone bodies by disrupted, isolated liver mitochondria

Ketone body formation by liver mitochondria was determined directly according to Chapman et al. (18), with some modifications. Frozen mitochondria were thawed, put quickly into liquid nitrogen and thawed again. Two mg mitochondrial protein were incubated at 37°C for 15 minutes with an acetyl-CoA generating system in a final volume of 900 µl. This system contained 25 U of phosphotransacetylase and the following components (final concentrations): 10 mmol/l sodium phosphate, 4 mmol/l ATP, 30 mmol/l lithium acetyl phosphate, 1 mmol/l CoASH, 35 mmol/l KCl, 3 mmol/l MgCl2 and 0.5 mmol/l dithiothreitol, pH 7.4. The reaction was stopped by adding 100 µl of 30% perchloric acid. After removing the precipitate by centrifugation the supernatants were analyzed for acetoacetate according to Olsen (12).

Ketone bodies in liver homogenate

100 mg of frozen liver was homogenized at 0°C with 500 µl 3% PCA and then transferred into a graduated 2 ml Eppendorf tube. The homogenizer was washed twice with 500 µl 3% perchloric acid. The homogenate (1500 µL) was centrifuged for 3 minutes at full speed. The supernatant was transferred into a new graduated 2 ml Eppendorf tube, the volume was adjusted to 2.0 ml by adding 3% PCA. The supernatant was analyzed for β-hydroxybutyrate (Lang et al., 2001). 100 µl were incubated with 2 ml 1 mol/l bicarbonate buffer pH 9.5, containing 640 µg NAD and 40 µl β-hydroxybutyrate-rate dehydrogenase for 90
minutes at room temperature. Finally, the appearance of NADH was measured fluorimetrically at an extinction of 365 nm and an emission of 470 nm.

**RNA isolation, Dnase digestion and reverse transcription and real-time quantitative PCR analysis of HMG-CoA synthase**

Total RNA was extracted from rat liver according to the general protocol of Sambrook et al. (Sambrook et al., 1989). The RNA concentration was determined by the absorbance at 260 nm, and the quality of the RNA was controlled by running an aliquot on a 1% agarose formaldehyde gel. DNA digestion was performed with the RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA). The incubation mixture (10 μL) contained 4 μg of total RNA, 2.0 μl 5x first strand buffer, RQ1 RNase-free DNase (1U/μg RNA) and RNase free H2O. The mixture was incubated for 30 minutes at 37°C. After adding 1.0 μl RQ1 DNase stop solution, the mixture was again incubated for 10 minutes at 65°C and afterwards put on ice. Reverse transcription was performed using the MMLV reverse transcriptase (Molony Murine Leukemia Virus reverse transcriptase; Gibco BRL, Life Technologies AG, Basel, Switzerland). 11.0 μl RQ1 DNase treated RNA, 2.0 μl RNase free water and 1.0 μl Oligo dT(15) (250 mg/ml) were mixed, incubated for 10 minutes at 70°C and afterwards put on ice. 6.0 μl of a RT-Mix were added to this incubation mixture containing 2.0 μl 5x first strand buffer, 2.0 μl 100 mmol/l DTT, 1.0 μl 10 mmol/l dNTP, 1.0 μl MMLV reverse transcriptase. The mixture was first incubated for 60 minutes at 37°C, then for 15 minutes at 70°C and afterwards put on ice and stored at −20°C. Real-time quantitative PCR analysis of HMG-CoA synthase was performed with a PE Applied Biosystems 7700 Sequence Detector (PE Biosystems) as described previously (Lang et al., 2001).

**Preparation of antibodies and Western blot analysis of HMG-CoA synthase**

Antibodies against mitochondrial HMG-CoA synthase were prepared by injecting rabbits with a peptide corresponding to the amino acid sequence 37-49 of the mitochondrial HMG-CoA synthase protein as described previously (Serra et al., 1993). Immunoblotting of mitochondrial HMG-CoA synthase was carried out as described by Serra et al. (Serra et al., 1993). The autoradiograms were quantified using a Luminescent Image Analyser LAS-1000 with Image Reader LAS-1000 for Windows® software (Raytest, Urdorf, Switzerland).
Activity of carnitine palmitoyltransferase (CPT) and protein expression of CPT I and II

Activities were determined in liver homogenate using the CPT assay-1 ("forward direction": palmitoyl-CoA + carnitine → palmitoylcarnitine + CoASH) and the CPT assay-2 ("backward reaction": palmitoylcarnitine + CoASH → palmitoyl-CoA + carnitine) as described by Hoppel and Tomec (Hoppel and Tomec, 1972). Antibodies against rat CPT I and II were a gift from Dr. V.A. Zammit (Hannah Research Institute, Ayr, Scotland) (Fraser and Zammit, 1998). Western blots for CPT I and II were performed as described by Fraser and Zammit (Fraser and Zammit, 1998).

Data presentation and statistical analysis
Data are presented as mean±sd unless specified otherwise. Data were analyzed by analysis of variance (ANOVA) followed by a t-test with Bonferroni correction to localize the differences in case of a significant ANOVA. A p<0.05 was considered to be statistically significant.

4.3.4 Results

Hepatic fatty acid metabolism was investigated in rats with long-term bile duct ligation and after reversal of biliary obstruction by biliodigestive anastomosis. Rats used in the current investigations are characterized in Table 12. Body weights were not different between the different groups of rats investigated with the exception of an increased body weight in RY rats observed for three months, which is explained by the long observation period. Similar as in other studies, liver and spleen weights were increased in BDL rats and had reached control values 1 and 3 months after reversing bile duct ligation, respectively (Krähenbühl et al., 1998; Zimmermann et al., 1992). In comparison to control rats, BDL rats had higher aspartate aminotransferase and alkaline phosphatase plasma activities, as well as higher plasma bilirubin and bile acid concentrations, which had all normalized 3 or 7 days after reversing biliary obstruction.
<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>BDL</th>
<th>RY3</th>
<th>RY7</th>
<th>RY14</th>
<th>RY28</th>
<th>RY84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>358±72</td>
<td>322±27</td>
<td>378±33</td>
<td>321±46</td>
<td>349±65</td>
<td>343±27</td>
<td>482±81ab</td>
</tr>
<tr>
<td>Liver weight (g/100 g BW)</td>
<td>2.63±</td>
<td>5.90±</td>
<td>5.09±</td>
<td>3.86±</td>
<td>4.01±</td>
<td>3.47±</td>
<td>2.80±</td>
</tr>
<tr>
<td></td>
<td>0.56</td>
<td>0.71a</td>
<td>1.33a</td>
<td>0.62ab</td>
<td>0.92ab</td>
<td>0.58b</td>
<td>0.58b</td>
</tr>
<tr>
<td>Spleen weight (g/100 g BW)</td>
<td>0.20±</td>
<td>0.63±</td>
<td>0.62±</td>
<td>0.56±</td>
<td>0.44±</td>
<td>0.44±</td>
<td>0.36±</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.13a</td>
<td>0.23a</td>
<td>0.19a</td>
<td>0.18a</td>
<td>0.11a</td>
<td>0.23b</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>90±20</td>
<td>480±200a</td>
<td>120±20b</td>
<td>130±20b</td>
<td>140±30b</td>
<td>110±20b</td>
<td>150±30b</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>170±60</td>
<td>400±90a</td>
<td>140±70b</td>
<td>160±50b</td>
<td>160±60b</td>
<td>200±80b</td>
<td>120±30b</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>1±1</td>
<td>120±40a</td>
<td>11±4ab</td>
<td>4±3b</td>
<td>3±3b</td>
<td>2±1b</td>
<td>2±1b</td>
</tr>
<tr>
<td>Bile acids (µmol/L)</td>
<td>2±1</td>
<td>130±50a</td>
<td>2±1b</td>
<td>7±5b</td>
<td>5±3b</td>
<td>5±4b</td>
<td>6±4b</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>7.7±0.9</td>
<td>6.7±0.6</td>
<td>7.1±0.2</td>
<td>7.5±0.9</td>
<td>6.7±0.6</td>
<td>6.9±0.9</td>
<td>7.8±1.4</td>
</tr>
</tbody>
</table>

Tab. 12 Characterization of the rats

Rats studied were bile duct ligated for 4 weeks (BDL, n=9), or 3 (RY3, n=4), 7 (RY7, n=6), 14 (RY14, n=6), 28 (RY28, n=6) or 84 days (RY84) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, n=8) were sham-operated and studied at the same time point as the BDL rats. Enzyme activities and metabolite concentrations were determined in heparinized plasma as described in Methods. Results are given as mean±sd. *p<0.05 vs. control, bp<0.05 vs. BDL.

Similar to previous studies, BDL rats had decreased plasma (Krähenbühl and Brass, 1991; Krähenbühl et al., 1994) and liver β-hydroxybutyrate concentrations after starvation for 24 hours in comparison to control rats (Table 13). Over the observation period of 3 months after reversal of bile duct ligation, the plasma β-hydroxybutyrate concentration gradually increased, but did not reach control values, whereas the liver β-hydroxybutyrate concentration had reached control values after 3 months. The plasma free fatty acid concentrations were decreased by 20 to 30% in BDL rats and also up to 4 weeks after restoring bile flow but were still in the range of 1 mmol/l. Since the Km value of long-chain acyl-CoA synthase for long-chain fatty acids is in the
range of 100 µmol/l (Tomoda et al., 1987), this decrease is an unlikely cause of the observed impairment of hepatic fatty acid metabolism in BDL and RY rats.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>BDL</th>
<th>RY3</th>
<th>RY7</th>
<th>RY14</th>
<th>RY28</th>
<th>RY84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma free fatty acids (mmol/L)</td>
<td>1.24±0.30</td>
<td>0.93±0.13</td>
<td>0.71±0.11</td>
<td>0.88±0.16</td>
<td>0.75±0.26</td>
<td>0.79±0.09</td>
<td>1.01±0.19</td>
</tr>
<tr>
<td>β-hydroxybutyrate in plasma (mmol/L)</td>
<td>0.75±0.20</td>
<td>0.25±0.10</td>
<td>0.39±0.10</td>
<td>0.51±0.20</td>
<td>0.42±0.15</td>
<td>0.45±0.17</td>
<td>0.47±0.12</td>
</tr>
<tr>
<td>β-hydroxybutyrate in liver (µmol/g liver)</td>
<td>0.75±0.61</td>
<td>0.25±0.20</td>
<td>0.39±0.26</td>
<td>0.51±0.23</td>
<td>0.42±0.30</td>
<td>0.45±0.68</td>
<td>0.47±0.68</td>
</tr>
</tbody>
</table>

**Tab. 13 Fatty acid metabolism**

Rats studied were bile duct ligated for 4 weeks (BDL, n=9), or 3 (RY3, n=4), 7 (RY7, n=6), 14 (RY14, n=6), 28 (RY28, n=6) or 84 days (RY84) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, n=8) were sham-operated. Beta-hydroxybutyrate in plasma and liver, and plasma fatty acids were determined as described in Methods. Results are given as mean±sd. ^a<p<0.05 vs. control, ^b<p<0.05 vs. BDL

Since the activity of the carnitine palmitoyltransferase (CPT) system can be rate-limiting for hepatic metabolism of fatty acids (Spurway et al., 1997; Drynan et al., 1996), it was studied in more detail (Table 14).

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>BDL</th>
<th>RY5</th>
<th>RY14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;forward reaction&quot; (µmol/min/g liver wet weight)</td>
<td>1.01±0.16</td>
<td>1.10±0.27</td>
<td>1.39±0.20</td>
<td>1.56±0.15^ab</td>
</tr>
<tr>
<td>&quot;backward reaction&quot; (µmol/min/g liver wet weight)</td>
<td>11.2±1.0</td>
<td>5.42±1.44^a</td>
<td>6.05±2.05^a</td>
<td>7.83±0.79^ab</td>
</tr>
<tr>
<td>Protein expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPT I</td>
<td>100±25</td>
<td>105±21</td>
<td>142±16^ab</td>
<td>182±14^ab</td>
</tr>
<tr>
<td>CPT II</td>
<td>100±6</td>
<td>60±1^a</td>
<td>62±7^a</td>
<td>77±4^a</td>
</tr>
</tbody>
</table>

**Tab. 14 Activity and expression of carnitine palmitoyltransferase (CPT) I and II**

Rats studied were bile duct ligated for 4 weeks (BDL, n=9), or 7 (RY7, n=8), or 14 days (RY14, n=6) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, n=8) were sham-operated. Results are presented as mean±SD. Protein expression was studied in n=3 samples per group, and units are arbitrarily (control values were set at 100). ^a<p<0.05 vs. control, ^b<p<0.05 vs. BDL
The activity of CPT was determined in both the forward direction and backward directions, which predominantly reflect the activity of CPT I and CPT II, respectively (Hoppel and Tomec, 1972). The activity in the forward reaction was not different between BDL and control rats and showed a significant increase 14 days after reversal of biliary obstruction. In contrast, the activity in the backward reaction was decreased in BDL as compared to control rats and partially recovered over 14 days after relief of biliary obstruction. Hepatic expression of CPT I paralleled CPT activity in the forward direction with a significant increase in RY rats as compared to BDL or control rats. Similarly, hepatic expression of CPT II paralleled CPT activity in the backward direction with a decrease in BDL as compared to control rats and a partial recovery after relief of biliary obstruction.

Mitochondrial fatty acid metabolism was studied further by assessing oxidative metabolism of fatty acid derivatives in intact, freshly isolated mitochondria (Table 15).

<table>
<thead>
<tr>
<th>Activity</th>
<th>CON</th>
<th>BDL</th>
<th>RY7</th>
<th>RY14</th>
<th>RY28</th>
<th>RY84</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrophilic substrates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glutamate (20 mmol/L)</td>
<td>82±9</td>
<td>66±6a</td>
<td>78±31</td>
<td>73±10</td>
<td>78±20</td>
<td>109±28ab</td>
</tr>
<tr>
<td>Succinate (20 mmol/L)</td>
<td>140±29</td>
<td>90±25a</td>
<td>111±38</td>
<td>102±30a</td>
<td>111±44</td>
<td>152±28b</td>
</tr>
<tr>
<td>Ascorbate (7.2 mmol/L)</td>
<td>257±68</td>
<td>235±33</td>
<td>259±33</td>
<td>220±15</td>
<td>271±26</td>
<td>215±26</td>
</tr>
<tr>
<td><strong>Fatty acids and derivatives</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitate (80 μmol/L)</td>
<td>68±19</td>
<td>33±8a</td>
<td>43±18a</td>
<td>54±9b</td>
<td>53±20b</td>
<td>59±9b</td>
</tr>
<tr>
<td>Palmitoyl-CoA (20 μmol/L)</td>
<td>88±14</td>
<td>67±12a</td>
<td>75±13</td>
<td>75±16</td>
<td>72±20</td>
<td>98±8b</td>
</tr>
<tr>
<td>Palmitoyl-L-carnitine (40 μmol/L)</td>
<td>96±12</td>
<td>77±12a</td>
<td>86±21</td>
<td>89±8</td>
<td>92±22</td>
<td>109±17b</td>
</tr>
</tbody>
</table>

*Tab. 15 State 3 oxidation rates by isolated rat liver mitochondria*

Rats studied were bile duct ligated for 4 weeks (BDL, n=9), or 7 (RY7, n=6), 14 (RY14, n=6), 28 (RY28, n=6) or 84 days (RY84) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, n=8) were sham-operated. Mitochondria were isolated by differential centrifugation and state 3 oxidation rates were determined using a Clark-type oxygen electrode as described in Methods. Units are natoms/min/mg mitochondrial protein. Results are presented as mean±SD. a,p<0.05 vs. control, b,p<0.05 vs. BDL.
In mitochondria from BDL rats, oxidation of palmitate, palmitoyl-CoA and palmitoylcarnitine was decreased by 20 to 30% as compared to control mitochondria, compatible with a defect in mitochondrial fatty acid metabolism distal to activation and also distal to CPT I (see Figure 11 for further explanation). After restoring bile flow, the impairment of mitochondrial fatty acid metabolism was rapidly reversible, normal activities were reached after 7 (palmitoylcarnitine and palmitoyl-CoA) to 14 days (palmitate). The slow recovery of palmitate oxidation is compatible with impaired activation of palmitate. As shown previously, oxidative metabolism of L-glutamate and succinate was impaired in mitochondria from BDL rats, but not oxidation of ascorbate, compatible with reduced activities of complex I and II (and possibly also III) of the electron transport chain (Krähenbühl et al., 1994; Krähenbühl et al., 1998). Mitochondrial metabolism of L-glutamate had normalized 7 days and metabolism of succinate 28 days after restoring bile flow. Since oxidative metabolism of L-glutamate, which involves complexes I, III and IV of the electron transport chain, had normalized 7 days after restoring bile flow, impaired activity of the electron transport chain could not explain reduced hepatic fatty acid metabolism 7 to 28 days after reversing biliary obstruction. The formation of acid soluble products by isolated mitochondria, mainly reflecting formation of ketone bodies and ketone body precursors, and involving activation, transport and β-oxidation of fatty acids (see Figure 11), was decreased in BDL rats but normalized quickly after reversing biliary obstruction (Table 16). Similarly, the formation of 14C02 from 1-14C-palmitate was decreased in BDL rats and reached control values 7 days after reversing biliary obstruction. These findings indicate that mitochondrial β-oxidation is decreased in BDL rats but this decrease can not explain impaired ketogenesis in RY rats observed for longer than 7 days. Therefore, we investigated specifically the activity of the HMG-CoA cycle, which is distal to β-oxidation (see Figure 11).

As shown in Table 16, the formation of acetoacetate from acetyl-CoA was indeed decreased in mitochondria from BDL rats in comparison to control rats, and recovered only 3 months after restoring bile flow. Since HMG-CoA synthase is rate-limiting for the activity of the whole cycle (Dashti and Ontko, 1979), we assessed mRNA and protein expression of this enzyme. Hepatic
expression of HMG-CoA synthase was decreased on both the protein and the mRNA level in BDL as compared to control rats (see Figure 12 for protein expression of HMG-CoA synthase).

Recovery of HMG-CoA expression after reversing biliary obstruction was only partial both for protein and mRNA. The hepatic HMG-CoA synthase mRNA content showed a close linear correlation with the hepatic concentration of β-hydroxybutyrate (Figure 13), indicating that impaired expression of this key enzyme is the major factor in explaining reduced hepatic fatty acid metabolism in rats with chronic biliary obstruction.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>BDL</th>
<th>RY3</th>
<th>RY7</th>
<th>RY14</th>
<th>RY28</th>
<th>RY84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid soluble β-oxidation products (nmol/min/mg protein)</td>
<td>2.75±</td>
<td>1.21±</td>
<td>n.d.</td>
<td>2.37±</td>
<td>3.17±</td>
<td>2.46±</td>
<td>2.55±</td>
</tr>
<tr>
<td>CO₂ production (nmol/min/mg protein)</td>
<td>0.34±</td>
<td>0.06±</td>
<td>n.d.</td>
<td>0.22±</td>
<td>0.25±</td>
<td>0.38±</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

**HMG-CoA synthase**

<table>
<thead>
<tr>
<th></th>
<th>CON ±10</th>
<th>RY3 ±3ab</th>
<th>RY7 ±12ab</th>
<th>RY14 ±12ab</th>
<th>RY28 ±12ab</th>
<th>RY84 ±12b</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA expression (unitless)</td>
<td>100 ±10</td>
<td>43 ±12ab</td>
<td>52 ±12ab</td>
<td>59 ±24ab</td>
<td>46 ±15ab</td>
<td>69 ±12ab</td>
</tr>
<tr>
<td>Protein expression (unitless)</td>
<td>100 ±11</td>
<td>44 ±8a</td>
<td>32 ±5ab</td>
<td>76 ±3ab</td>
<td>76 ±18ab</td>
<td>71 ±10ab</td>
</tr>
</tbody>
</table>

**Tab. 16 Activity of β-oxidation and ketogenesis.**

Rats studied were bile duct ligated for 4 weeks (BDL, n=9), or 3 (RY3, n=4), 7 (RY7, n=6), 14 (RY14, n=6), 28 (RY28, n=6) or 84 days (RY84) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, n=8) were sham-operated. Mitochondria were isolated by differential centrifugation and disrupted by freeze-thawing. Beta-oxidation and 14CO₂ production were determined in freshly isolated mitochondria using 1-14C-palmitate as a substrate as described in Methods. Production of acetoacetate from acetyl-CoA was determined fluorimetrically using disrupted mitochondria. Protein and mRNA expression of HMG-CoA synthase were performed as described in Methods using standard methods. These data are unitless and the control values are arbitrarily set at 100%. Results are presented as mean±SD. *p<0.05 vs. control, **p<0.05 vs. BDL n.d. not determined
Fig. 12 Hepatic expression of HMG-CoA synthase protein

The Western blots were performed as described in the Method section and quantified by densitometric analysis of the bands. The numeric results of these blots are given in Table 5 with the control values set at 100%. Note that all blots contain control (CON) and BDL samples for comparison.
Fig. 13 Relationship between hepatic mRNA expression of HMG-CoA synthase and the hepatic content of β-hydroxybutyrate.

The hepatic level of HMG-CoA synthase mRNA was determined by quantitative PCR and the content of β-hydroxybutyrate by a fluorimetric method as described in Methods. The relation is described by the following equation: \( y = 2.1 + 2.4x \) \( (r^2 = 0.69, p < 0.05) \).
4.3.5 Discussion

The current studies show that impaired hepatic fatty acid metabolism in rats with long-term cholestasis can be explained by several mechanisms including reduced activation of palmitate, reduced activity of CPT II, mitochondrial β-oxidation and the electron transport chain, and, most importantly, reduced activity of the HMG-CoA cycle. While most of these alterations are rapidly reversible after restoring bile flow, this is not the case for reduced activity of the HMG-CoA cycle.

We focused on 4 possible locations which are known to be potentially rate limiting for hepatic fatty acid metabolism and which could be impaired in BDL rats: mitochondrial activation/transport of fatty acids, β-oxidation cycle, electron transport chain and HMG-CoA cycle.

Long-chain fatty acids are first activated by palmitoyl-CoA synthase, an enzyme located on the outer mitochondrial membrane (Ramsay and Arduini, 1993), before they are transported into the mitochondrial matrix for β-oxidation (Figure 11). The results obtained with the oxygen electrode show that oxidative metabolism of palmitate is impaired to a slightly higher extent and improves slower than metabolism of palmitoyl-CoA or palmitoylcarnitine, indicating that activation of palmitate is impaired in BDL rats. We have obtained similar findings in rats with short-term cholestasis (Lang et al., 2001), suggesting that cholestasis (and not secondary biliary cirrhosis) affects synthesis of palmitoyl-CoA. However, since oxidative metabolism of palmitate had reached control values 14 days after reversal of biliary obstruction, impaired activation of palmitate can not explain reduced formation of ketone bodies in RY rats at later time points.

Impaired activation of palmitate can obviously not explain reduced oxidation of palmitoyl-CoA and palmitoylcarnitine (see Figure 11). Palmitoyl-CoA is converted by CPT I to palmitoylcarnitine which is then transported into the mitochondrial matrix. CPT I is located at the contact sites of the outer and inner mitochondrial membranes (Fraser and Zammit, 1998), can be rate-limiting for hepatic fatty acid metabolism (Spurway et al., 1997; Drynan et al., 1996) and is regulated by inhibition by malonyl-CoA (short-term) and by protein expression...
Our results show that both activity and protein expression of CPT I were not affected by long-term cholestasis. Reduced oxidative metabolism of palmitoyl-CoA has therefore to be explained by impairment of hepatic fatty acid metabolism distal from CPT I, e.g. CPT II or \( \beta \)-oxidation.

CPT II is located in the inner mitochondrial membrane, catalyzes the formation of palmitoyl-CoA from palmitoylcarnitine and is considered not to be rate-limiting for hepatic fatty acid oxidation (McGarry and Brown, 1997). On the other hand, patients with very low or lacking activity of hepatic CPT II can develop liver steatosis (Bonnefont et al., 1996; Yamamoto et al., 1996), indicating that also CPT II can become rate-limiting for hepatic fatty acid metabolism. Activity and protein expression of CPT II were reduced in BDL rats with only a partial reversibility after restoring bile flow. On the other hand, oxidative metabolism of palmitoyl-CoA and palmitoylcarnitine was decreased in mitochondria from BDL rats, but this decrease was reversible already 7 days after restoring bile flow. This apparent discrepancy between CPT II activity and expression and mitochondrial metabolism of palmitoylcarnitine and palmitoyl-CoA may reflect the fact discussed above, namely that CPT II is not rate-limiting for hepatic fatty acid metabolism. Furthermore, under \textit{in vivo} conditions, fatty acids may not be metabolized at maximal speed and therefore the observed reduction in activity and expression of CPT II may not be relevant.

An impaired function of the electron transport chain is a well recognized reason for reduced fatty acid metabolism in patients with mitochondrial cytopathies (Goncalves et al., 1995; Krähenbühl et al., 1999) and could therefore contribute to impaired hepatic fatty acid metabolism in chronic cholestasis. As shown in the current and in previous studies (Krähenbühl et al., 1994; Krähenbühl et al., 1998), the function of complexes I and III of the electron transport chain of hepatic mitochondria is decreased in rats with chronic cholestasis but recovers within 7 days after reversal of biliary obstruction. In contrast, the function of complex II (assessed by oxidative metabolism of succinate) had normalized only after 4 weeks. However, complex II is not needed for fatty acid metabolism. A decreased function of the electron transport chain may therefore contribute to
impaired hepatic fatty acid metabolism in BDL rats, but is not the reason for this finding after reversal of biliary obstruction.

Similar to the electron transport chain, an impaired function of enzymes of the β-oxidation cycle is also known to result in decreased hepatic fatty acid metabolism (Coates and Stanley, 1992; Fromenty and Pessayre, 1995; Bennett et al., 2000). Beta-oxidation was assessed using intact mitochondria by measuring oxidative metabolism of fatty acids with the oxygen electrode and by the formation of acid soluble products from 1-14C-palmitate. Under the conditions used in the experiments with the oxygen electrode, in the presence of L-malate, citrate is the end product of fatty acid metabolism and no ketone bodies are produced (Hoppel et al., 1979). This is in contrast to the experiments in which the formation of acid soluble products is determined, which consist of ketone bodies and ketone body precursors (Fromenty et al., 1990). Similar to the results obtained with the oxygen electrode, the formation of acid soluble products and of 14CO2 from 1-14C-palmitate were reduced in mitochondria from BDL rats and had normalized 7 days after reversal of biliary obstruction. Since the results are the same, irrespective of the end product determined, they suggest that impaired β-oxidation contributes to reduced hepatic fatty acid metabolism in BDL rats and also in RY rats but not for longer than 7 days after restoring bile flow. Impaired β-oxidation has been identified as a mechanism for reduced hepatic fatty acid metabolism in BDL rats in a previous study (Krähenbühl et al., 1994).

We have shown in a recent study that an impaired function of the HMG-CoA cycle is the reason for reduced formation of ketone bodies in rats with short-term cholestasis (Lang et al., 2001). After exclusion of other potential causes for impaired hepatic fatty acid metabolism in rats with reversed biliary obstruction (see above), we therefore focused on the activity of the HMG-CoA cycle. The activity of the HMG-CoA cycle, determined by measuring the formation of acetoacetate from acetyl-CoA, was decreased in BDL rats and had recovered only 3 months after reversing biliary obstruction, showing a similar pattern as the hepatic content of ketone bodies. This assay measures the activity of three enzymes, namely acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA lyase. The activity of the HMG-CoA cycle is limited by HMG-CoA synthase,
which is regulated short-term by succinylation and long-term by protein expression (Hegardt, 1999; Lowe and Tubbs, 1985). The assay therefore basically reflects the activity of this key enzyme of the HMG-CoA cycle. Similar to its activity, mRNA and protein expression of HMG-CoA synthase were decreased in BDL rats and slowly increased after reversal of biliary obstruction. In contrast to its activity, however, recovery of mRNA and protein expression was not complete during the observation period. Since short-term regulation is achieved by succinylation (which decreases the function of HMG-CoA synthase) and the assay used measures only the active (not succinylated) portion of the enzyme, the discrepancy between activity and expression may be explained by the activation status of the enzyme. While our studies show clearly that, similar to rats with short-term cholestasis (Lang et al., 2001), HMG-CoA mRNA and protein levels are decreased in BDL rats, they do not allow to draw conclusions about the mechanism leading to these findings. Further studies assessing transcription of the HMG-CoA synthase gene are needed to answer this question.

In conclusion, we provide evidence that multiple factors (in particular impaired activation of long-chain fatty acids, and impaired activities of β-oxidation, electron transport chain and HMG-CoA cycle) lead to reduced hepatic metabolism of fatty acids in rats with long-term cholestasis. After reversing biliary obstruction, hepatic fatty acid metabolism recovers only slowly, a finding explained by reduced mRNA and protein expression, and activity of HMG-CoA synthase, the rate-limiting enzyme of the HMG-CoA cycle.

Acknowledgements

We thank Sabine Jakob and Monika Ledermann for technical support and animal care, and Dr. R. Kretschmer for the determination of the plasma free fatty acid concentration. We thank Dr. V.A. Zammit (Hannah Research Institute, Ayr, Scotland) for providing antibodies against rat CPT I and II.
4.3.6 References


4.4 Hepatic glycogen metabolism in patients with liver cirrhosis

4.4.1 Abstract

**Background & Aims:** Patients with liver cirrhosis have reduced hepatic glycogen stores. The data base for this statement is small, however, and the mechanisms unclear.

**Methods:** Patients with different types of liver cirrhosis (n=22) and control patients undergoing liver surgery (n=14) were studied in the post-absorptive state. Liver glycogen content, activities and mRNA expression of glycogen synthase and phosphorylase, and liver morphology were determined by standard methods.

**Results:** Cirrhotic and control patients were similar regarding age, body weight, serum biochemistry. Cirrhotic patients had a reduced glycogen content per g liver (26±19 vs. 45±17 mg/g), per ml hepatocytes (39±27 vs. 50±21 mg/ml) and per liver (33.1±21.8 vs. 61.3±20.7 g). Focusing on subgroups, the hepatic glycogen content was reduced only in patients with alcoholic or biliary cirrhosis, but not in patients with viral cirrhosis. Liver histology confirmed these findings and revealed that the decrease in liver glycogen in alcoholic or viral cirrhosis was not diffuse but with marked local differences. Activities of glycogen synthase and phosphorylase were not different between cirrhotic and control patients, whereas hepatic mRNA expression was decreased in cirrhotics by 30 to 50%.

**Conclusions:** Patients with alcoholic or biliary cirrhosis have decreased hepatic glycogen stores which is not the case for patients with viral cirrhosis. Maintained activity of glycogen synthase and phosphorylase and the focal pattern of glycogen loss suggest that primarily local hepatic factors are responsible for these findings.

4.4.2 Introduction

Glycogen is the storage form of carbohydrates in liver and skeletal muscle and represents an important source of energy, in particular during exercise and
during early starvation (1, 2). In accordance with the importance of glycogen metabolism for fuel homeostasis, glycogen synthesis and breakdown are tightly regulated. The two key enzymes responsible for this regulation are glycogen synthase and glycogen phosphorylase whose activities are controlled by phosphorylation and dephosphorylation. Glycogen synthase, the rate-limiting enzyme of glycogen formation, is activated by metabolites such as AMP (3) or glucose-6-phosphate (4), and by insulin which stimulates protein phosphatases (2, 5, 6). Inactivation occurs by protein kinases stimulated by hormones such as glucagon, adrenalin or vasopressin (2, 5). Glycogen phosphorylase, the rate-limiting enzyme of glycogen breakdown, is activated by phosphorylation. The protein kinases performing the phosphorylation steps are activated by cAMP-dependent hormones such as glucagon and β-adrenergic agonists, or calcium-dependent agents such as vasopressin, parathyroid hormone, angiotensin II, and α1-adrenergic and P2-purinergic agonists (7-11). Glycogenolysis is inhibited by insulin which impairs both the cAMP- and the calcium-dependent pathways (10).

Alterations in hepatic glycogen metabolism have been described in both rats and humans with liver cirrhosis. It is well established that rats with CCl4-induced or secondary biliary cirrhosis have reduced hepatic glycogen stores (12, 13). In rats with secondary biliary cirrhosis, reduced synthesis of glycogen appears to be the principle mechanism leading to reduced hepatic glycogen stores, whereas in rats with CCl4-induced cirrhosis the mechanisms remain unclear. Humans with alcohol-induced liver cirrhosis have also been described to have reduced hepatic glycogen stores (14). Owen et al. studied 8 patients with alcohol-induced liver cirrhosis and compared their results with values obtained in 2 control subjects (14). In many textbooks of hepatology, this study is cited and the statement is generalized in the sense that hepatic glycogen stores are reduced in all patients with liver cirrhosis, independent of its etiology (15, 16).

A reduction in the hepatic glycogen content is associated with important metabolic consequences. The transition from the fed to the fasted state develops earlier, since the hepatic glycogen stores are exhausted rapidly after onset of starvation (12). Therefore, fatty acids become important substrates for energy production in cirrhotics also in the postabsorptive state (14, 17), a period
of time during which subjects without liver disease have still high hepatic glycogen levels and can produce glucose and energy from hepatic glycogen (18,19). When the hepatic glycogen stores are exhausted, the glucose needs for glucose-dependent tissues such as erythrocytes or brain have to be met by gluconeogenesis. Important substrates for gluconeogenesis are amino acids, many of them originating from skeletal muscle. Accelerated gluconeogenesis may therefore represent a mechanism for muscle wasting in patients with liver cirrhosis (20,21).

Due to the importance of hepatic glycogen metabolism in cirrhosis and the small database currently available we decided to study hepatic glycogen metabolism in patients with different types of liver cirrhosis. We intended to answer the following specific questions i) are the hepatic glycogen stores decreased in all types of liver cirrhosis, independently of etiology ii) can the expected reduction be explained by the reduced content of hepatocytes only and iii) what are the principle mechanism for the expected reduction in the hepatic glycogen stores?

4.4.3 Materials and Methods

Patients

The studies have been reviewed and accepted by the Ethic’s Committee of the State of Berne. Patients with liver cirrhosis (n=22) were studied either during liver transplantation (n=17) or hepatic resection for liver cancer (n=5). Cirrhosis was confirmed histologically in all cases. Twelve patients were in stage Child B (4 with alcoholic, 5 with biliary and 3 with viral cirrhosis) and 10 in Child C (3 with alcoholic, 3 with biliary and 4 with viral cirrhosis). The time interval between the last meal and start of the operation was between 8 and 12 hours. Control patients (n=14) underwent hepatic resection of a colorectal metastasis. None of these patients had a history of liver disease or increased alcohol consumption. Clinical signs of chronic liver disease were absent, and viral hepatitis, chronic cholestasis and autoimmune liver disease were excluded by laboratory analyses in all cases. The time interval between the last meal and surgery in control patients was 10 to 14 hours. All patients (cirrhotics and controls) were treated with a diet containing 3.5 Kcal/kg (25-30% as fat, 15% as amino acids and 55-60% as carbohydrates) for the last week prior to surgery. Routine
inhalation anesthesia with enflurane or isoflurane was performed in all cases. From the liver tissue obtained during surgery a piece was rapidly frozen in liquid nitrogen and stored at –80 °C for isolation of RNA, and determination of enzyme activities and glycogen content. The remainder of the biopsy (or the liver in case of the patients undergoing liver transplantation) was used for histological and stereological analysis as described below.

Characterization of the patients

Patients were characterized by their body weights, body mass index, activities of alanine aminotransferase (ALT) and alkaline phosphatase, concentrations of bilirubin, bile acids, glucose and β-hydroxybutyrate in serum. ALT, alkaline phosphatase and bilirubin were analyzed on a COBAS analyzer (Hoffman-La Roche Diagnostics, Basel, Switzerland). Bile acids were determined with a radioimmuno assay (Becton and Dickinson, Orangeburg, SC). The serum glucose concentrations were determined enzymatically (kit obtained from Sigma Chemicals, Buchs, Switzerland) and the serum β-hydroxybutyrate concentrations fluorimetrically using the method of Olsen (22). The liver weight was determined gravimetrically in the 17 patients undergoing liver transplantation, and by estimating (2% of body weight) (15) in the remaining cirrhotic (n=5) and control patients (n=14).

Tissue preparation and histological analysis

For the stereological analysis, five pieces of liver tissue were obtained by random sampling from each liver biopsy or liver, and fixed in 5% buffered formalin. The samples were embedded into paraffin and 5 randomly chosen sections from each block were colored with Elastica van Gieson. On each of these sections, at least 100 points were counted (23) and classified as described before (24).

For the localization of hepatic glycogen, liver tissue was fixed with alcohol and embedded into paraffin. Several sections from each block were colored with PAS with and without previous treatment with diastase to destroy glycogen (negative control).
Liver glycogen content

The glycogen content in liver was determined enzymatically as glucose (using a commercially available reagent kit, Sigma Chemicals, Buchs, Switzerland) after alkaline destruction of free glucose and enzymatic hydrolysis of glycogen as described originally by Lust et al. (25) with the previously reported modifications (12). The glycogen content is expressed as mg per gram liver wet weight or per ml of hepatocytes and as g glycogen per whole liver.

Enzyme assays

For the determination of the activities of the glycogen synthase and glycogen phosphorylase, frozen liver was homogenized at 0°C with 9 volumes of a solution containing 50 mmol/l potassium fluoride and 10 mmol/l EDTA (pH 7.0). The homogenate was centrifuged at 10,000 g for 10 minutes at 4°C, and the resulting supernatant was assayed directly for glycogen synthase activity (active form and total activity) as described originally by Thomas et al. (26) and modified by Guinoivart et al. (27). An aliquot of the supernatant was diluted 1:2 (vol:vol) with a solution containing 50 mmol/l 2-(N-morpholino)-ethanesulfonic acid (MES), 50 mmol/l potassium fluoride and 5 mmol/l dithiothreitol (pH 6.1). The resulting solution was assayed for total glycogen phosphorylase activity according to Gilboe et al. (28), and for the active form of glycogen phosphorylase according to Teen et al. (29).

RNA isolation and reverse transcription

Total RNA was extracted from rat liver according to the general protocol of Sambrook et al. (30). The RNA concentration was determined by the absorbance at 260 nm, and the quality of the RNA was controlled by running an aliquot on a 1% agarose formaldehyde gel. Four µg of total RNA from rat liver were used as a template for first-strand cDNA synthesis with reverse transcriptase (Molony Murine Leukemia Virus reverse transcriptase; Gibco BRL, Life Technologies AG, Basel, Switzerland) and oligo (dT) primer.

Real-time quantitative PCR analysis glycogen synthase synthase and phosphorylase

Real-time quantitative PCR analysis was performed with a PE Applied Biosystems 7700 Sequence Detector (PE Biosystems), which is a combined thermocycler and fluorescent detector. Sets of primers were chosen for
glycogen synthase and glycogen phosphorylase to obtain a PCR product of less than 100 base pairs. A dual-labelled fluorogenic probe complementary to a sequence within the PCR product was added to the PCR reaction. The primers and the dual-labelled fluorogenic probe for GAPDH, which served as internal standard, were chosen accordingly. One fluorescent dye (6-carboxyfluorescein) serves as a reporter, and its emission is quenched by a second fluorescent dye (6-carboxy-tetramethylrhoda-mine). During elongation, the 5’ to 3’ exonuclease activity of the Taq DNA polymerase hydrolyzes the probe, thus releasing the reporter from the quencher, resulting in increased fluorescence which is detected. The forward and reverse primers were: glycogen synthase: TTA AAT TTT CAG CAG TGC ATG AG and TGA CCT CGAACAAAATCTTGGA with the probe TCA AAA TCT ACA TGC CAT GTA CAA GGC CAG A; glycogen phosphorylase: AGA GGA AGG AAG CAAAAG GAT CA and TGG ATT TTA GCC ACG CCA TT with the probe CAT CTC TGC ATT GGT TCC CAT GC. For GAPDH, the internal standard, a pre-developed TaqMan Assay Reagent Control Kit was used. Primers and probes were custom-synthesized by PE Biosystems. Complementary DNA was amplified in a 50 μl volume containing 25 μl of the 2 x TaqMan Universal PCR Master Mix (PE Biosystems), 100 nmol/l probe and 300 nmol/l of each primer. After a denaturating step of 10 min at 95°C, 40 cycles were performed: 95°C for 15 seconds and 60°C for 1 minute. The mathematical analysis of the results was performed according the Ct method of calculation, where Ct stands for the cycle number at which the fluorescence of the sample crosses a given threshold (see PE Biosystems user bulletin # 2). After individual normalization of all values obtained for GAPDH, the mean value for control livers was calculated, arbitrarily set at 1 and used as a normalization factor for the cirrhotic livers.

**Statistical evaluation**

Results are expressed as mean ± SD unless specified otherwise. Means between two groups (control and cirrhotic patients) were compared by Student’s t-test after having tested for normal distribution of the data. Subgroup analysis was performed by ANOVA, followed by Scheffé’s test. Analysis of mRNA
expression was performed by the Mann-Whitney U test since the data showed no normal distribution. A p<0.05 was considered to be statistically significant.

### 4.4.4 Results

Control and cirrhotic patients are characterized in Table 17. The age of the patients studied was comparable among the different groups, with the exception of patients with viral cirrhosis who were younger than control patients. Body weights and body mass index were also similar between the groups of patients investigated, except for patients with alcoholic cirrhosis who had a higher body weight than control patients. Metabolic characterization revealed no differences in glucose and β-hydroxybutyrate serum concentrations, indicating that all subjects were in the postabsorptive state and not starving. Cirrhotic patients had higher serum concentrations of bilirubin and serum bile acids and a higher activity of alkaline phosphatase, while there were no differences between the groups regarding the serum activity of alanine aminotransferase.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Control (n=14)</th>
<th>Cirrhosis (n=22)</th>
<th>Alcohol (n=7)</th>
<th>Biliary (n=8)</th>
<th>Viral (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>62±12</td>
<td>54±13</td>
<td>56±9</td>
<td>58±8</td>
<td>46±15*</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>72.8±9.3</td>
<td>76.1±11.6</td>
<td>81.7±9.3*</td>
<td>73.9±16.4</td>
<td>72.6±5.7</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.3±2.0</td>
<td>25.6±3.1</td>
<td>26.3±3.26</td>
<td>25.8±3.58</td>
<td>24.6±2.8</td>
</tr>
<tr>
<td>Serum glucose (mmol/L)</td>
<td>9.46±2.98</td>
<td>8.69±5.02</td>
<td>7.89±0.60</td>
<td>10.3±7.0</td>
<td>7.42±4.95</td>
</tr>
<tr>
<td>Serum β-hydroxybutyrate (μmol/L)</td>
<td>0.24±0.09</td>
<td>0.29±0.16</td>
<td>0.37±0.22</td>
<td>0.28±0.15</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>Serum bilirubin (μmol/L)</td>
<td>10±7</td>
<td>43±40*</td>
<td>36±28*</td>
<td>59±55*</td>
<td>30±26*</td>
</tr>
<tr>
<td>Serum bile acids (μmol/L)</td>
<td>2±2</td>
<td>38±34*</td>
<td>26±22*</td>
<td>43±28*</td>
<td>45±51*</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>135±65</td>
<td>218±117</td>
<td>145±55</td>
<td>328±82*</td>
<td>165±62</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>78±40</td>
<td>57±65</td>
<td>27±9</td>
<td>99±90</td>
<td>36±27</td>
</tr>
</tbody>
</table>

**Tab. 17 Characterization of the patients**

Patients were studied in the postabsorptive state after a period of 8 to 14 hours after the last intake of food. Data are given as mean±sd, *p<0.05 vs. control.
A morphometric analysis of the livers was performed to ensure that control patients had a normal liver architecture and to determine the volume fraction \( V_v \) of hepatocytes, in order to be able to relate the glycogen content and enzyme activities to the volume of hepatocytes (Table 18). As expected from similar studies in rats with liver cirrhosis (13,24), the volume fraction of hepatocytes was decreased in all types of liver cirrhosis while the volume fraction of connective tissue was increased. When expressed as an absolute value (volume per liver), a similar pattern emerged, since the liver volumes showed no large variation between the different groups.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=14)</th>
<th>Cirrhosis (n=22)</th>
<th>Alcohol (n=7)</th>
<th>Biliary (n=8)</th>
<th>Viral (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver weight</strong></td>
<td>1.46±0.19</td>
<td>1.44±0.33</td>
<td>1.60±0.25</td>
<td>1.53±0.36</td>
<td>1.20±0.24*</td>
</tr>
<tr>
<td><strong>Hepatocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V ) (ml)</td>
<td>1270±200</td>
<td>912±241*</td>
<td>1042±211*</td>
<td>943±263*</td>
<td>752±170*</td>
</tr>
<tr>
<td>( V_v ) (ml/ml)</td>
<td>0.87±0.04</td>
<td>0.64±0.09*</td>
<td>0.66±0.11*</td>
<td>0.62±0.07*</td>
<td>0.63±0.10*</td>
</tr>
<tr>
<td><strong>Connective tissue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V ) (ml)</td>
<td>139±61</td>
<td>455±172*</td>
<td>486±206*</td>
<td>492±150*</td>
<td>388±159*</td>
</tr>
<tr>
<td>( V_v ) (ml/ml)</td>
<td>0.10±0.04</td>
<td>0.31±0.08*</td>
<td>0.30±0.11*</td>
<td>0.31±0.06*</td>
<td>0.32±0.10*</td>
</tr>
<tr>
<td><strong>Rest</strong> 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V ) (ml)</td>
<td>47±14</td>
<td>73±38*</td>
<td>67±38</td>
<td>95±46*</td>
<td>58±0</td>
</tr>
<tr>
<td>( V_v ) (ml/ml)</td>
<td>0.03±0.01</td>
<td>0.05±0.01*</td>
<td>0.04±0.01</td>
<td>0.07±0.02*</td>
<td>0.04±0.01</td>
</tr>
</tbody>
</table>

**Tab. 18 Morphometric analysis**

Morphometric analysis was performed by counting at least 500 points on 5 different sections of each liver biopsy as described in Methods. Data are given as mean±sd, and reflect the volume per liver \( (V) \) or the volume fraction \( (V_v) \) of the respective compartment. The density of the liver was set at 1 g/ml for conversion of the liver weight to volume. *p<0.05 vs. control. 1Rest: e.g. blood vessels such as sinusoids, and portal and central veins, bile ducts.

The hepatic glycogen content and activities of glycogen synthase and phosphorylase are given in Table 19. When expressed per g liver wet weight, cirrhotic patients had a 42% decrease in the hepatic glycogen content. Considering subgroups, this decrease was 60% for both patients with alcoholic or biliary cirrhosis, whereas patients with viral cirrhosis had normal hepatic glycogen stores. When expressed per ml of hepatocytes, the hepatic glycogen content was still decreased by approximately 45% in patients with alcoholic or biliary cirrhosis compared to control patients, but not different from controls in
patients with viral cirrhosis. A similar pattern could be seen when the hepatic glycogen content was expressed per liver. There were no significant correlations between the hepatic glycogen content and body weight or body mass index of the patients.

In contrast to the hepatic glycogen content, total activities and active forms of glycogen synthase and phosphorylase were not different between patients with liver cirrhosis and control patients. The only difference was a 30% decrease in the activity of the active form of glycogen phosphorylase in patients with viral cirrhosis. When the activity of the active form was expressed as a ratio of total activity, there were no differences between cirrhotic and control patients (results not shown). There were no significant correlations between enzyme activities and hepatic glycogen content of the patients.

<table>
<thead>
<tr>
<th>Glycogen</th>
<th>Control (n=14)</th>
<th>Cirrhosis (n=22)</th>
<th>Alcohol (n=7)</th>
<th>Biliary (n=8)</th>
<th>Viral (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/g liver wet weight</td>
<td>45±17</td>
<td>26±19*</td>
<td>18±*</td>
<td>18±16*</td>
<td>43±21</td>
</tr>
<tr>
<td>mg/ml hepatocytes</td>
<td>50±1</td>
<td>39±27</td>
<td>27±6*</td>
<td>28±4*</td>
<td>64±27</td>
</tr>
<tr>
<td>g/liver</td>
<td>61.3±20.7</td>
<td>33.1±21.8*</td>
<td>28.2±8.6*</td>
<td>21.5±17.9*</td>
<td>49.7±26.1</td>
</tr>
</tbody>
</table>

| Glycogen synthase | | | | | |
| total (mU/ml hepatocytes) | 190±65 | 230±77 | 242±69 | 258±40 | 186±104 |
| active (mU/ml hepatocytes) | 34±14 | 37±16 | 33±13 | 41±16 | 37±20 |

| Glycogen phosphorylase | | | | | |
| total (U/ml hepatocytes) | 17.8±7.5 | 19.2±7.3 | 20.5±6.0 | 20.7±7.8 | 16.1±8.0 |
| active (U/ml hepatocytes) | 14.2±6.9 | 13.3±6.0 | 14.7±4.9 | 15.1±7.0 | 9.9±4.9* |

Tab. 19 Glycogen metabolism

Glycogen and enzyme activities were determined by routine methods as described in the Method section. Enzyme activities are expressed as total activity (a+b), and activity of the active part of the enzyme (a). Data are given as mean±sd, *p<0.05 vs. control.

As shown in Figure 14, the hepatic mRNA expression of glycogen synthase and phosphorylase showed a large variation in control livers. In comparison, the variation was clearly smaller in cirrhotic livers. The median expression of both enzymes was decreased by 30 to 50% in cirrhotic as compared to control livers, but the difference reached statistical significance only for all cirrhotic patients tested as one group (both enzymes) and for patients with viral cirrhosis (glycogen phosphorylase). There were no physiologically significant correlations between hepatic mRNA expression and activity of glycogen synthase or phosphorylase.
Fig. 14 Hepatic mRNA expression of glycogen synthase and phosphorylase

Steady state mRNA levels of the two enzymes were determined using a TaqMan as described in Methods. Data were first normalized individually to GAPDH mRNA expression. The mean of the values of control patients were then arbitrarily set at 1 and the values of cirrhotic patients normalized to it. Data analysis was performed by the Mann-Whitney U test, since the values showed no normal distribution. Data are given as median, 25 and 75 percentiles (boxes), 10 and 90 percentiles (bars) and outliers.

The distribution of glycogen within liver tissue is shown in Figure 15. Figure 15 A shows a liver from a control patient with normal liver histology. PAS reactivity is homogeneous and strong. Figure 15 B shows the corresponding picture of a patient with alcoholic liver cirrhosis. In this situation, PAS reactivity is heterogeneous, irrespective of the presence of fat in hepatocytes. A similar picture emerges in patients with biliary cirrhosis (Figure 15 C), showing that hepatocytes in the periphery of a nodule have a clearly higher glycogen content than those in the center. In agreement with the biochemical results, the hepatocytes in the liver of patients with viral cirrhosis have an almost normal glycogen content without a focal pattern (Figure 15 D).
Fig. 15 A-D. Glycogen distribution in livers from control (15 A) and cirrhotic patients (15 B-D)

Fig. 15 A shows control liver tissue with marked, diffuse and homogeneous PAS staining for glycogen. Fig. 15 B shows a part of a cirrhotic nodule with macrovesicular fatty change of a patient with alcoholic liver cirrhosis. In this situation, PAS reactivity for glycogen is heterogeneous, also in hepatocytes not showing steatotic change. Fig. 15 C shows a PAS stain of a patient with primary biliary cirrhosis. Note that marked PAS reactivity (indicating a high glycogen content) is seen in peripheral parts of a nodule, whereas more central parts show reduced glycogen staining. Similar changes were seen in patients with primary sclerosing cholangitis (not shown). Fig. 15 D shows a cirrhotic nodule in the center and parts of an adjacent nodule on the right side. The biopsy originates from a patient with chronic hepatitis C. In this PAS stain, reactivity for glycogen is homogeneous and diffuse. Similar stains were obtained in biopsies of patients with chronic hepatitis B (not shown).

4.4.5 Discussion

Our study demonstrates that the etiology of liver cirrhosis is important for hepatic glycogen metabolism: hepatic glycogen stores are reduced only in patients with alcoholic or biliary cirrhosis whereas patients with viral cirrhosis have a normal hepatic glycogen content.

In order to obtain comparable results between patients with liver cirrhosis and control patients, several precautions were taken. The diet ingested during the
week before surgery was similar regarding calories and composition, and the time interval between the last meal and surgery was also tried to keep constant. This could not be realized completely for patients undergoing liver transplantation, for some of them the time interval was close to 8 hours and not 10-14 hours as for the control patients. If anything, the hepatic glycogen content would be higher in patients with a short interval between the last meal and surgery. This could possibly explain why patients with viral cirrhosis have a high hepatic glycogen content, but it would even more accentuate the difference in the hepatic glycogen between patients with alcoholic or biliary cirrhosis and control patients. Since starvation is associated with an increase in the serum β-hydroxybutyrate concentrations (12, 31, 32), this metabolite was determined in serum of control and cirrhotic patients. Regarding the identically low values obtained in all groups of patients, no patients were starving at the time point of surgery. In this context, it is also important to mention that control patients had an almost identical hepatic glycogen content as healthy control subjects in the postabsorptive phase (14,18,19). The control patients studied by us represent therefore a valuable control group.

In agreement of the studies by Owen et al. (14), we could also demonstrate that the hepatic glycogen content expressed per g liver is reduced in patients with alcoholic liver cirrhosis. Since liver cirrhosis is associated with replacement of hepatocytes by connective tissue (this study and ref. 13 and 24), a reduction in hepatic glycogen could theoretically be explained by reduced liver parenchyma per g liver only. In order to answer this question, it was necessary to perform a stereological analysis of the livers and to relate the glycogen content and enzyme activities to hepatocellular volume. Our results show clearly that loss of hepatocytes explains the reduction in hepatic glycogen in patients with alcoholic or biliary cirrhosis only partially and that additional mechanisms must be considered.

Glycogen synthase and phosphorylase are the two key enzymes for glycogen formation and breakdown, respectively, and are both under the control of kinases and phosphatases whose activity depends on hormonal (systemic) or local factors (1). Our study shows that the reduced hepatic glycogen stores in patients with alcoholic or biliary cirrhosis cannot be explained by mechanisms
acting through glycogen synthase or phosphorylase, since the activities (total and active form expressed per ml of hepatocytes) of both enzymes were maintained in cirrhotic patients. Interestingly, hepatic mRNA expression of both enzymes was decreased in cirrhotic patients, suggesting an increased translational efficiency and/or increased stability of these two proteins in cirrhotic livers. The only exception was a reduced activity of glycogen phosphorylase in patients with viral cirrhosis, which correlated well with low mRNA expression and which may at least partially explain why this group of patients maintains the hepatic glycogen stores.

Similar results (reduced hepatocellular glycogen stores and maintained activities of glycogen synthase and phosphorylase) have been obtained in rats with CCl₄-induced liver cirrhosis (12). In contrast, in rats with secondary biliary cirrhosis due to bile duct ligation for 4 weeks, both hepatic glycogen stores and activities of glycogen synthase and phosphorylase were reduced (13), suggesting that impaired synthesis is the most important mechanism for reduced hepatic glycogen stores in this animal model of secondary biliary cirrhosis. Unchanged activities and activation of glycogen synthase and phosphorylase exclude hormonal factors as cause for reduced glycogen content in patients with alcoholic or biliary cirrhosis. Regarding the pattern of glycogen distribution in liver biopsies, showing both a general decrease per hepatocyte and areas almost completely devoid of glycogen, local factors appear to be important for glycogen loss in patients with alcoholic or biliary cirrhosis. One possibility which could at least contribute to the observed heterogeneous loss of glycogen in livers with alcoholic or biliary cirrhosis is loss of metabolic zonation due to the formation regenerative nodules (33). However, further studies beyond the scope of the current investigations are needed to define in detail the nature of the postulated local factors leading to focal loss of glycogen in livers with alcoholic or biliary cirrhosis.

Acknowledgements

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5  CONCLUSIONS
The results of the first two projects demonstrate that hepatic energy metabolism in rats with short-term cholestasis is impaired. Both hepatic glycogen metabolism as well as hepatic fatty acid metabolism are affected.

In the first study, glycogen metabolism was investigated 4 or 8 days after bile duct ligation. 4 days after surgery, rats with bile duct ligation and pair-fed control animals had a similar glycogen content per gram liver or per ml hepatocytes, whereas the ad libitum-fed control animals revealed higher hepatic glycogen stores. Due to the fact that the only difference between the two control groups was the amount of food ingested, reduced food intake appears to be the main reason for the decrease of the hepatic glycogen content in rats with bile duct ligation for 4 days. 8 days after surgery, the glycogen content was clearly decreased in bile duct ligated animals compared to both control groups, but not different between the two control groups. Because the intake of food did not differ between bile duct ligated and control animals, cholestasis seems to be the main reason for this decreased glycogen content per ml hepatocytes, gram liver and per whole liver in rats with bile duct ligation for 8 days.
The question remains, why rats with bile duct ligation for 4 days ingest less food than ad libitum-fed control rats. Since both control groups had undergone exactly the same procedures as the bile duct ligated animals concerning anaesthesia or laparotomy, only the surgery itself or cholestasis are possible reasons for this finding. Bile duct ligated animals normalized their food intake over the observation period of 8 days, thus the ligation of the common bile duct has to be responsible for this finding.
The activity as well as the mRNA expression of glycogen synthase and glycogen phosphorylase in the liver were decreased in bile duct ligated rats at both time points compared to both control groups. Due to the fact that the hepatic glycogen content did not differ between bile duct ligated animals and the pair-fed control group 4 days after surgery, the reduced activity of glycogen synthase was not associated with a significant decrease in hepatic glycogen stores at this time. The reduction in food intake or probably the contemporary decrease in the activity of glycogen phosphorylase might have overridden this
effect. Glycogen synthase and glycogen phosphorylase are regulated short-term by phosphorylation and long-term by altered expression. Since the active fractions of both enzymes were not affected in bile duct ligated rats, decreased expression instead of changes in the phosphorylation state is likely to be responsible for the reduced activities of glycogen synthase and phosphorylase. Rats with long-term cholestasis also showed reduced activities and mRNA levels of these two key enzymes of glycogen metabolism (Krähenbühl L. et al., 1999). Thus, cholestasis and not secondary biliary cirrhosis seems to be responsible for decreased expression of glycogen synthase and glycogen phosphorylase.

Plasma glucagon levels were increased in bile duct ligated rats compared to the control animals. Why these elevated glucagon levels did not lead to an increased active fraction of glycogen phosphorylase and to a decreased active fraction of glycogen synthase, has to be evaluated in more detail. In contrast to the hormonal short-term control, which increases the activity of one and decreases the activity of the other enzyme, cholestasis decreases mRNA expression and activity of both enzymes at the same time. The exact mechanism for this finding still needs to be studied.

The glycogen content per whole liver is physiologically more important than the glycogen content per gram liver or ml of hepatocytes. Due to an increase in liver weight, the reduction in glycogen per liver was smaller than the reduction per gram liver tissue, or per volume of hepatocytes in bile duct ligated rats. In conclusion, the proliferation of hepatocytes, which leads to this increased liver weight, partially compensates for the loss of hepatic glycogen stores in the rat at this early state of cholestasis. The proliferation of hepatocytes should therefore be regarded as a possible way for the liver to compensate its reduced function.

The skeletal muscle glycogen metabolism was not affected in rats with short-term cholestasis. These findings differ from results obtained from studies of patients with alcoholic liver disease (Kruzynska et al., 1988), or of rats with long-term cholestasis (Krähenbühl L. et al., 1996), where glycogen metabolism in skeletal muscle is found to be impaired. Therefore, the duration of the liver
disease seems to be very important for the development of defects in skeletal muscle glycogen metabolism.

Finally, this project shows that acute cholestasis in the rat leads to an impaired hepatic glycogen metabolism due to both reduced food intake and cholestasis. The histological pattern of the affected liver, which showed a diffuse reduction in the hepatic glycogen stores in bile duct ligated rats, leads to the conclusion that local rather than systemic factors, such as high concentration of bile acids or endotoxins, are responsible for this impaired liver function.

Acute cholestasis in rats not only leads to reduced glycogen stores, but also to impaired hepatic fatty acid metabolism. A decreased function of activation and import of long-chain fatty acids, of β-oxidation, of the Krebs Cycle, of the respiratory chain or of the HMG-CoA cycle has to be considered responsible for this finding. The activity of the enzyme complexes of the respiratory chain was not affected in rats with short-term bile duct ligation, in contrast to rats with long-term bile duct ligation, which showed reduced activities of complexes I, II, III and V (Krähenbühl S. et al., 1994). It therefore takes cirrhosis or long-term cholestasis to develop a decrease of the function of the respiratory chain. Because the hepatic fatty acid metabolism in rats with CCl₄ induced liver cirrhosis (Krähenbühl S. et al., 1989) is not affected, cholestasis rather than cirrhosis seems to be responsible for this mitochondrial defect in rats with bile duct ligation.

The CO₂ production from palmitate and the oxidative metabolism of palmitoyl-L-carnitine and palmitoyl-CoA did not differ between bile duct ligated rats and the control animals, which implies no defects in β-oxidation and in the Krebs Cycle in rats with short-term cholestasis. On the contrary, rats with long-term bile duct ligation showed reduced activities of some enzymes of β-oxidation (Krähenbühl S. et al., 1994).

All these findings suggest that impaired ketogenesis is the main reason for the decreased function of the hepatic fatty acid metabolism in rats with acute cholestasis. HMG-CoA synthase is the rate limiting enzyme of this pathway and is controlled short-time by succinylation/desuccinylation (Serra et al., 1993) and long-time by regulation of gene transcription (Hegardt, 1999). HMG-CoA synthase is more active in the desuccinylated state, which can be achieved by
glucagon treatment. In this study, only the total activity of HMG-CoA synthase was measured, and therefore it can not be excluded that the succinylation state of this enzyme contributed to impaired ketogenesis in rats with acute cholestasis. On the other hand, the degree of the reduced activity was similar to the degree of the reduced protein, or to the decreased mRNA levels of HMG-CoA synthase, suggesting that the main effect of acute cholestasis is on long- and not short-term regulation of HMG-CoA synthase.

There are a lot of physiological factors (e.g. concentrations of glucagon and insulin, nutritional state) which regulate HMG-CoA synthase gene transcription and therefore its activity. Because the rats were pair-fed in this study, and bile duct ligated animals had higher glucagon plasma concentrations, decreased activity of HMG-CoA synthase is not due to nutritional and hormonal alterations. The defect in the gene regulation of HMG-CoA synthase in rats with short-term cholestasis needs to be studied in more detail.

Some defects in hepatic energy metabolism in rats with short-term cholestasis are in good agreement with the defects found in rats with long-term cholestasis or patients with liver cirrhosis. However, there might exist defects in certain mechanisms which are characteristic of cholestasis or secondary biliary cirrhosis. As a consequence, further studies will be necessary to understand all these mechanisms and to evaluate whether these alterations are a result of cholestasis or cirrhosis.

In the third project, the reversibility of hepatic mitochondrial damage in rats with long-term cholestasis was studied. In 1999, Krähenbühl L. et al. concluded that the persistence of decreased mitochondrial fatty acid metabolism in rats after Roux-en-Y anastomosis can not be explained by impaired activity of the respiratory chain, but is probably due to alterations in mitochondrial β-oxidation. The current study shows that ketogenesis rather than β-oxidation is responsible for this impaired hepatic fatty acid metabolism in rats after Roux-en-Y anastomosis.

We focused on 4 possible locations which are known to be potentially rate limiting for hepatic fatty acid metabolism and which could be impaired in BDL rats: activation/transport of fatty acids, the respiratory chain, the β-oxidation and the HMG-CoA cycle.
As far as the activation of fatty acids is concerned, the results obtained with the oxygen electrode show that oxidative metabolism of palmitate is impaired and improves more slowly than metabolism of palmitoyl-CoA or palmitoyl-carnitine. These results suggest that the activation of palmitate is impaired in BDL rats. Because similar findings were obtained in rats with short-term cholestasis, cholestasis rather than cirrhosis seems to be responsible for this defect. Oxidative metabolism of palmitate had reached control value 14 days after reversal of biliary obstruction. Therefore, impaired activation of palmitate cannot explain reduced formation of ketone bodies in RY rats at a later time.

The transport of long chain fatty acids into the mitochondrial matrix is namely catalysed by CPT I, which can be rate limiting for hepatic fatty acid metabolism (Spurway et al., 1997; Drynan et al., 1996). Palmitoyl-CoA is converted by CPT I to palmitoylcarnitine, which is then transported into the mitochondrial matrix. Both activity and protein expression of CPT I were not affected by long-term cholestasis, which leads to the conclusion that reduced oxidative metabolism of palmitoyl-CoA has therefore to be explained by other impaired functions. CPT II catalyses the formation of palmitoyl-CoA from palmitoyl carnitine and is not considered to be rate limiting for hepatic fatty acid oxidation (McGarry and Brown, 1997). Activity as well as protein expression of CPT II were decreased in BDL rats and recovered only partially after restoring bile flow. On the contrary, the oxidative metabolism of palmitoyl-CoA and palmitoyl carnitine was decreased in mitochondria from BDL rats, but did recover 7 days after restoring bile flow. This discrepancy between CPT II activity and expression, and mitochondrial metabolism of palmitoyl-CoA and palmitoyl carnitine, may reflect the fact that CPT II is not rate limiting for hepatic fatty acid metabolism.

An impaired function of the electron transport chain as well as an impaired function of β-oxidation might be responsible for reduced fatty acid metabolism. The function of complex I and III of the electron transport chain of hepatic mitochondria is decreased in rats with chronic cholestasis, but recovers within 7 days after restoring bile flow. In contrast, the function of complex II normalized only 4 weeks after restoring bile flow, but complex II is not needed for fatty acid metabolism. The formation of acid soluble products and of $^{14}$CO$_2$ from 1-$^{14}$C-palmitate were reduced in mitochondria from BDL rats and also normalized 7
days after reversal of biliary obstruction. In conclusion, the impaired functions of the electron transport chain and of the β-oxidation by intact hepatic mitochondria therefore contribute to impaired hepatic fatty acid metabolism in BDL rats, but are not the reason for this finding after reversal of biliary obstruction.

The second project shows that an impaired function of the HMG-CoA cycle is the main reason for reduced formation of ketone bodies in rats with short-term cholestasis. Therefore, the activity, mRNA and protein expression of HMG-CoA synthase, the rate limiting enzyme of this cycle, were studied in more detail. The activity of HMG-CoA synthase was decreased in BDL rats and recovered only 3 months after reversing biliary obstruction. Similar to its activity, mRNA and protein expression of HMG-CoA synthase were decreased in BDL rats, but did not recover completely within the observations period of 3 months. This discrepancy between activity and expression may be due to the fact that only the active (not succinylated) form of the enzyme was measured. However, similar to rats with short-term cholestasis, HMG-CoA synthase activity, mRNA and protein expression are decreased in BDL rats. It is too early to draw conclusions about the mechanism leading to this finding and further studies will be necessary to answer this question.

The results obtained from patients with different kinds of liver cirrhosis provided a lot of evidence that the etiology of liver cirrhosis is important for hepatic glycogen metabolism. This study showed that patients with alcoholic and biliary cirrhosis have reduced glycogen stores, whereas patients with viral cirrhosis have a normal hepatic glycogen content.

In this project, it was possible to confirm the results reported by Owen et al. in 1981, who claimed that patients with alcoholic liver cirrhosis have decreased hepatic glycogen stores. Our study showed that the glycogen content in this patient group was reduced per gram liver. Due to the fact that liver cirrhosis is associated with a replacement of hepatocytes by connective tissue, this decrease in hepatic glycogen could probably be explained by the reduced liver hepatocellular volume per gram liver. Therefore, stereological analysis of the livers was performed and the glycogen content and the enzyme activities were calculated per ml hepatocytes. These results indicate clearly that the reduction
in hepatic glycogen content in patients with alcoholic or biliary liver cirrhosis can only be partially explained by the loss of hepatocytes, and that other mechanisms might contribute to these findings.

The activities of glycogen synthase and glycogen phosphorylase in the liver were unchanged in all patients studied. As a consequence, these enzymes are not responsible for the reduced hepatic glycogen in patients with alcoholic or biliary cirrhosis. Conversely, the hepatic mRNA expression of both enzymes was decreased in cirrhotic patients, which could be an effect of increased translational efficiency and/or increased stability of these two enzymes in cirrhotic livers.

Similar to rats with acute cholestasis, patients with alcoholic or biliary cirrhosis also showed a general decrease of hepatic glycogen. Since there are some areas with almost no glycogen, it can be concluded that also in patients, local rather than systemic factors are responsible for this impaired liver function.

The results obtained by this study are only partially comparable to the results provided by different studies of rats with liver cirrhosis. Both patients with alcoholic liver cirrhosis and rats with CCl₄-induced liver cirrhosis show reduced hepatocellular glycogen stores and maintained activities of glycogen synthase and glycogen phosphorylase. In contrast, rats with biliary cirrhosis have both reduced glycogen stores and decreased activities of glycogen synthase and glycogen phosphorylase. This shows that it is difficult to correlate results obtained from an animal model with human data. Further research should therefore be aimed at improving the existing and developing new animal models, which are as similar as possible to the pathology in humans.
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CURRICULUM VITAE

Name: Corinna Bettina Lang
Date of birth: 6th April, 1968
Place of birth: Lucerne, LU, Switzerland

Education:

1998-2001: PhD candidate
PhD Thesis: "Glycogen metabolism and mitochondrial function in different kind of liver disease". Institut für Klinische Pharmakologie, Inselspital Bern, Prof. Dr. med. S. Krähenbühl und PD Dr. med. L. Krähenbühl

1997-1998: pharmacist
Winkelried Apotheke, Zürich
Hirschengraben Apotheke, Bern

1990-1996: master in pharmacy
Swiss Federal Institute of Technology Zürich, (ETH)

1981-1988: Maturität Typ E
Gymnasium Luzern