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

Corinne Lang,* Markus Schäfer,† Dolors Serra,§ Fausto G. Hegardt,§ Lukas Krähenbühl,† and Stephan Krähenbühl[1,*]

Institute of Clinical Pharmacology* and Department of Visceral and Transplantation Surgery,† University of Berne, CH-3010 Berne, Switzerland and School of Pharmacy,§ University of Barcelona, 08028 Barcelona, Spain

Abstract **Rats with long-term cholestasis have reduced ketosis during starvation. Because 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 days (BDL5, n = 11) or 10 days (BDL10, n = 11) and compared the findings with those made with 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 mM at 5 days and 0.59 ± 0.24 vs. 0.88 ± 0.09 mM 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 days (2.1 ± 1.0 vs. 4.8 ± 1.9 nmol/min per mg protein) and at 10 days (1.7 ± 1.0 vs. 6.2 ± 1.9 nmol/min per mg protein). The principal 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 because of decreased mRNA levels of HMG-CoA synthase, leading to reduced hepatic protein levels and to decreased activity of this key enzyme of ketogenesis.** —Lang, C., M. Schäfer, D. Serra, F. G. Hegardt, L. Krähenbühl, and S. Krähenbühl. **Impaired hepatic fatty acid oxidation in rats with short-term cholestasis: characterization and mechanism.** ***J. Lipid Res.*** **2001.** 42: **22–30.**

Supplementary key words mitochondria • respiratory chain • β-oxidation • HMG-CoA pathway • ketogenesis

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 **Fig. 1**, transport of fatty acids into liver mitochondria and mitochondrial metabolism of fatty acids involve 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 I (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, when starved for 24 h, 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 complexes 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 more recent studies we could show that the activities of complexes 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).

So far, hepatic fatty acid metabolism has not been studied

Abbreviations: ALT, alanine aminotransferase; CoA, coenzyme A; CoASH, reduced form of coenzyme A; CPT, carnitine palmitoyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; PCR, polymerase chain reaction; TMPD, *N,N,N′*,N′-tetramethyl-*p*-phenylene diamine.

[1] To whom correspondence should be addressed: Stephan Krähenbühl, Division of Clinical Pharmacology, Department of Internal Medicine, University Hospital, CH-4031 Basel, Switzerland.

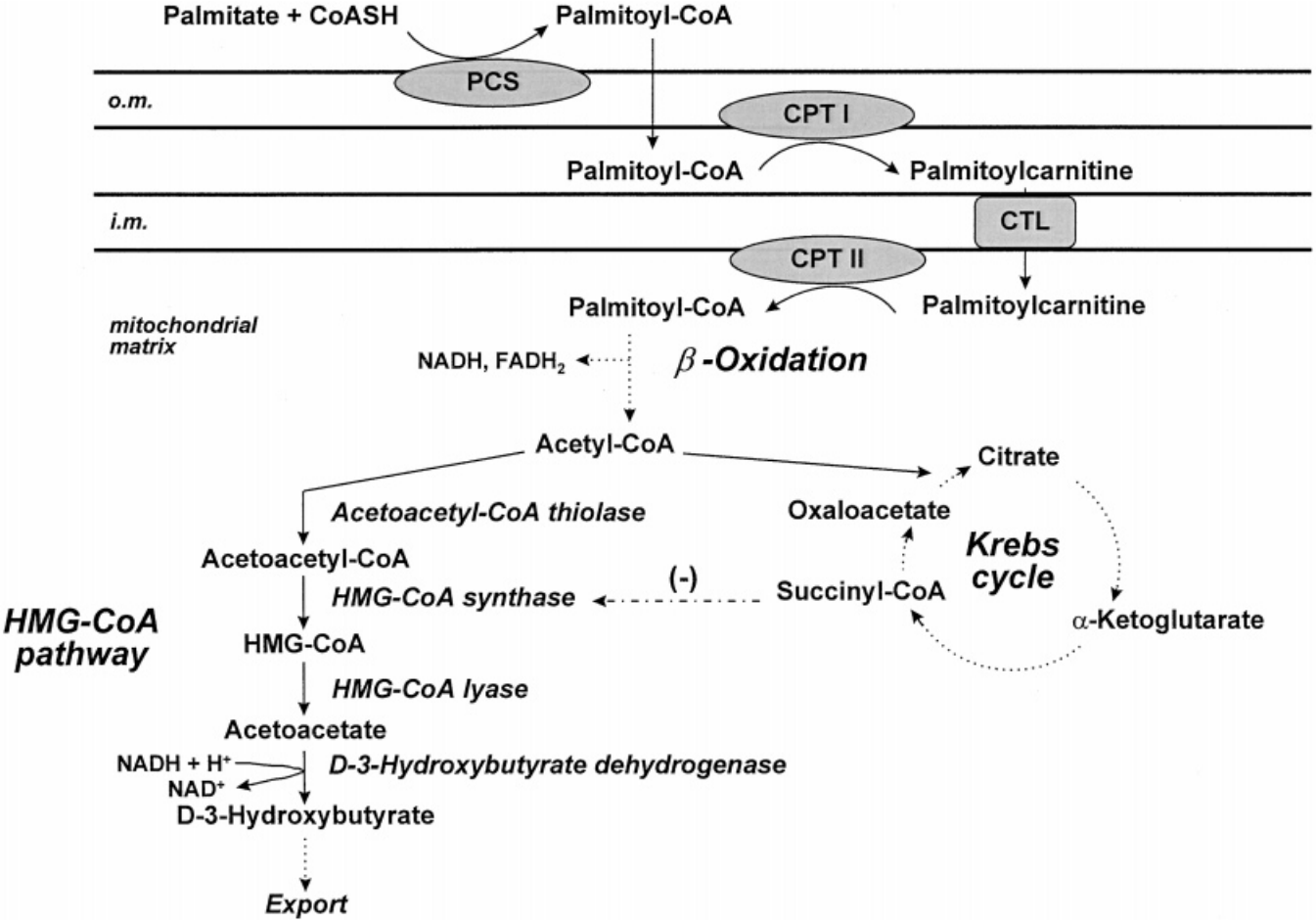

Fig. 1. Hepatic long-chain fatty acid metabolism. Long-chain fatty acids (e.g., palmitate) are activated by a specific acyl-CoA synthase (PCS), which is located in the outer mitochondrial membrane (o.m.). The acyl-CoA formed is converted to the respective carnitine derivative by carnitine palmitoyltransferase I (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 (i.m.) 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).

in detail in rats with short-term bile duct ligation, before the development of secondary biliary cirrhosis. Short-term bile duct ligation in the rat can be regarded as an animal model for acute cholestasis in humans (11), a clinical condition known to be associated with an increased mortality for surgical (12) or other interventions (13). Because increased mortality in acute cholestasis may also be a consequence of deranged hepatic energy metabolism, we decided to study hepatic fatty acid metabolism in rats with bile duct ligation for 5 or 10 days. The specific questions asked by us were *i*) is short-term bile duct ligation associated with alterations in hepatic fatty acid metabolism and *ii*) if yes, which are the mechanisms leading to these alterations.

MATERIALS AND METHODS

Reagents

[1-^{14}C]palmitic acid was obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). Phosphotransacetylase was obtained from Boehringer Mannheim (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-h dark and light cycle. Ligation and transection of the common bile duct were performed as described previously (1). Rats were studied after either 5 or 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 with treated rats throughout the study with normal rat chow (Kliba Futter, Basel, Switzerland). All studies were performed with the rats starved for 24 h before investigation. There was no mortality in any of the groups studied.

Characterization of animals

The animals were characterized by their body and spleen weights and activities of alkaline phosphatase and alanine amino-

transferase (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 Dickinson, Orangeburg, SC). β-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 in 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, DiMarco, and Tandler (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 were 20 mM for L-glutamate and succinate, 7.2 mM for ascorbate, 40 μM for palmitoyl-L-carnitine, 20 μM for palmitoyl-CoA, and 80 μM for palmitate. All incubations with fatty acids contained 5 mM L-malate, incubations with palmitoyl-CoA or palmitate contained in addition 2 mM L-carnitine, and incubations with palmitate contained in addition 250 μM ATP and 250 μM CoASH.

In vitro mitochondrial β-oxidation and Krebs cycle

The β-oxidation of [1-^{14}C]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 mM sucrose, 43 mM KCl, 3.6 mM $MgCl_2$, 7.2 mM potassium phosphate, 36 mM Tris-HCl buffer, pH 7.4) contained 0.2 mM adenosine triphosphate, 50 μM L-carnitine, 15 μM CoASH, and 1 mg of mitochondrial protein. After 5 min of preincubation at 30°C, the incubation mixture was brought to 2 ml by adding 200 μl of the preincubation medium containing [1-^{14}C]palmitic acid (final concentration, 40 μmol/L; 0.1 μCi/2 ml) with bovine serum albumin (final concentration in the assay solution, 0.5 mg/2 ml). The tubes were closed with covers containing a filter paper soaked in 100 mM NaOH for trapping of [^{14}C]CO_2 and incubated at 30°C with slow shaking. After 15 min, the reaction was stopped by adding 400 μl of 5% perchloric acid to the incubation mixture. Trapping of $^{14}CO_2$ was continued for 60 min. The filter papers were then transferred into scintillation vials and counted for $^{14}CO_2$ activity. The incubation mixture was subsequently centrifuged at 4,000 *g* for 10 min. An aliquot (400 μl) 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, Miller, and Ontko (19), with some modifications. Frozen mitochondria were thawed, put quickly into liquid nitrogen, and thawed again. Two milligrams of mitochondrial protein was incubated at 37°C for 15 min 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 mM sodium phosphate, 4 mM ATP, 30 mM lithium acetyl phosphate, 1 mM CoASH, 35 mM KCl, 3 mM $MgCl_2$, and 0.5 mM 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 (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 substrate (15).

Assay of HMG-CoA synthase activity

The activity of 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase was assessed according to Quant, Tubbs, and Brand (20), with some modifications. Fifteen microliters of liver mitochondria (300 μg of protein) was treated with 7.5 μl of Triton X-100 to expose HMG-CoA synthase and was assayed immediately. The standard 1-ml assay system contained 50 mM Tris-HCl, 10 mM $MgCl_2$, and 2 mM dithiothreitol, pH 8.0. Lysed mitochondria (300 μg of protein), 100 μM acetyl-CoA, 10 U of phosphoacetyltransferase, 5 mM acetyl phosphate, and 10 mM 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, Fritsch, and Maniatis (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 micrograms of total RNA from rat liver was used as a template for first-strand cDNA synthesis with reverse transcriptase (Molony murine leukemia virus reverse transcriptase; GIBCO-BRL, Life Technologies, Basel, Switzerland) and oligo(dT) primer.

Real-time quantitative PCR analysis of HMG-CoA synthase

Real-time quantitative polymerase chain reaction (PCR) analysis was performed with a PE Applied Biosystems (Foster City, CA) 7700 sequence detector, which is a combined thermocycler and fluorescence detector. Sets of primers were chosen for HMG-CoA synthase to receive a PCR product of less than 100 base pairs. A dual-labeled fluorogenic probe complementary to a sequence within the PCR product was added to the PCR. The primers and the dual-labeled fluorogenic probe for glyceraldehyde-3-phosphate dehydrogenase (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 that is detected. For HMG-CoA synthase, the forward and reverse primers were TGA ACG GTG AAT AGA CAC AGC G and GTG GTG CTC ACT GCT TCA GG, respectively, with the probe being CTG CTC CGC GGT GAA GGGCC. For GAPDH, the corresponding forward and reverse primers were CTG CCA AGT ATG ATG ACA TCA AGA A and AGC CCA GGA TGC CCT TTA GT, respectively, with the probe being 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× TaqMan Universal PCR Master Mix (PE Biosystems), 100 nM probe, and a 300 nM concentration of each primer. After a denaturating step of 10 min at 95°C, 40 cycles were performed: 95°C for 15 s and 60°C for 1 min. The mathematical analysis of the results was 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 with luminescent image analyzer LAS-1000 with image reader LAS-1000 for Windows® software (Raytest, Urdorf, Switzerland).

Data presentation and statistical analysis

Data are presented as means ± 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. $P < 0.05$ was considered to be significant.

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 was found to be impaired. Rats were bile duct ligated for 5 or 10 days (BDL rats) and control rats were pair fed with BDL rats throughout the study. The final studies were carried out with all rats starved for 24 h. As shown in **Table 1**, 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 of 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 10 days after surgery, compatible with impaired hepatic fatty acid metabolism.

As illustrated in Fig. 1, hepatic fatty acid metabolism can be impaired because of reduced activation or transport of fatty acids, impaired β-oxidation, or activity of the respiratory chain, or because of reduced activity of the HMG-CoA pathway. As a first step, as shown in **Table 2**, 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 β-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 days, 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 days after surgery.

As a next step, the formation of ketone bodies and CO_2 from palmitate was determined with [^{14}C]palmitate as a substrate. As shown in **Table 3**, the formation of acid-soluble products [representing ketone bodies (18)] was decreased in BDL rats at both time points, compatible with impaired ketogenesis, possibly due to reduced activity of the HMG-CoA pathway (Fig. 1). In favor of this hypothesis, produc-

TABLE 1. Characterization of rats

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

Rats were bile duct ligated for 5 days (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 with BDL rats throughout the study. Enzyme activities and metabolite concentrations were determined in heparinized plasma as described in Materials and Methods. Results are given as means ± SD.

[a] $P < 0.05$ versus the respective control group.

TABLE 2. State 3 oxidation rates by isolated rat liver mitochondria

Activity	CON5	BDL5	CON10	BDL10
L-Glutamate (20 mM)	64 ± 12	68 ± 9	70 ± 13	72 ± 12
Succinate (20 mM)	85 ± 21	94 ± 34	102 ± 28	84 ± 24
Ascorbate (7.2 mM)	275 ± 74	253 ± 84	245 ± 58	256 ± 69
Palmitate (80 μM)	28 ± 13	29 ± 4	46 ± 8	34 ± 4[a]
Palmitoyl-CoA (20 μM)	53 ± 17	52 ± 15	58 ± 20	58 ± 16
Palmitoyl-L-carnitine (40 μM)	53 ± 19	50 ± 14	63 ± 21	55 ± 16

Rats were bile duct ligated for 5 days (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 with BDL rats throughout the study. Mitochondria were isolated by differential centrifugation and state 3 oxidation rates were determined with a Clark-type oxygen electrode as described in Materials and Methods. Units are n atoms per minute per milligram of mitochondrial protein. Results are presented as means ± SD.

[a] $P < 0.05$, BDL versus control rats.

tion 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 rats by 17% (5 days) or 43% (10 days) but without reaching statistical significance because of high variations. The ratio of CO_2 production and formation of ketone bodies was increased significantly in BDL rats at both time points.

The next step was therefore to assess the activity of the HMG-CoA pathway in more detail (**Table 4**). 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 4, this reduction could be explained primarily by impaired activity of HMG-CoA synthase, the rate-limiting enzyme of the HMG-CoA pathway, the activity of which 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 only a slight reduction 10 days after surgery.

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 **Fig. 2**, liver mRNA levels of HMG-CoA synthase were reduced by approximately 70% on day 5 and by 65% on day 10 after surgery. Accordingly, as shown in **Fig. 3**, a Western blot of HMG-CoA synthase showed reduced hepatic protein levels of this enzyme at both time points studied.

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 principal 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 Fig. 1). 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 un-

TABLE 3. In vitro β-oxidation and Krebs cycle activity by isolated liver mitochondria

Activity	CON5	BDL5	CON10	BDL10
Acid-soluble β-oxidation products	1.29 ± 0.32	0.70 ± 0.34[a]	1.17 ± 0.43	0.69 ± 0.37[a]
CO_2 production	0.030 ± 0.007	0.035 ± 0.020	0.037 ± 0.021	0.053 ± 0.035
CO_2 production/(acid soluble products + CO_2 production)	2.3 ± 0.04	4.9 ± 1.3[a]	3.2 ± 1.0	5.1 ± 2.1[a]

Rats were bile duct ligated for 5 days (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 with BDL rats throughout the study. Mitochondria were isolated by differential centrifugation. β-Oxidation and Krebs cycle activity were determined with [1-^{14}C]palmitate as a substrate as described in Materials and Methods. Units for acid-soluble β-oxidation products and CO_2 production are nanomoles per minute per miligram of mitochondrial protein. Results are presented as means ± SD.

[a] $P < 0.05$, BDL versus control rats.

TABLE 4. Activity of the HMG-CoA pathway in disrupted liver mitochondria

Activity	CON5	BDL5	CON10	BDL10
Formation of acetoacetate from acetyl-CoA	4.8 ± 1.9	2.1 ± 1.0[a]	6.2 ± 1.9	1.7 ± 1.0[a]
Acetoacetyl-CoA thiolase	220 ± 43	194 ± 41	233 ± 47	183 ± 35[a]
HMG-CoA synthase	1.46 ± 0.47	0.94 ± 0.50[a]	1.82 ± 0.39	0.89 ± 0.69[a]

Rats were bile duct ligated for 5 days (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 with 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 were determined spectrophotometrically as described in Materials and Methods. Units are nanomoles per minute per milligram of mitochondrial protein. Results are presented as means ± SD.

[a] $P < 0.05$, BDL versus control rats.

changed in mitochondria from BDL rats. Concerning the function of the respiratory chain, acute cholestasis is clearly different from long-term cholestasis, because 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. Because the respiratory chain and/or fatty acid metabolism of liver mitochondria appear not to be grossly affected in rats with CCl_4-induced (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.

Because 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

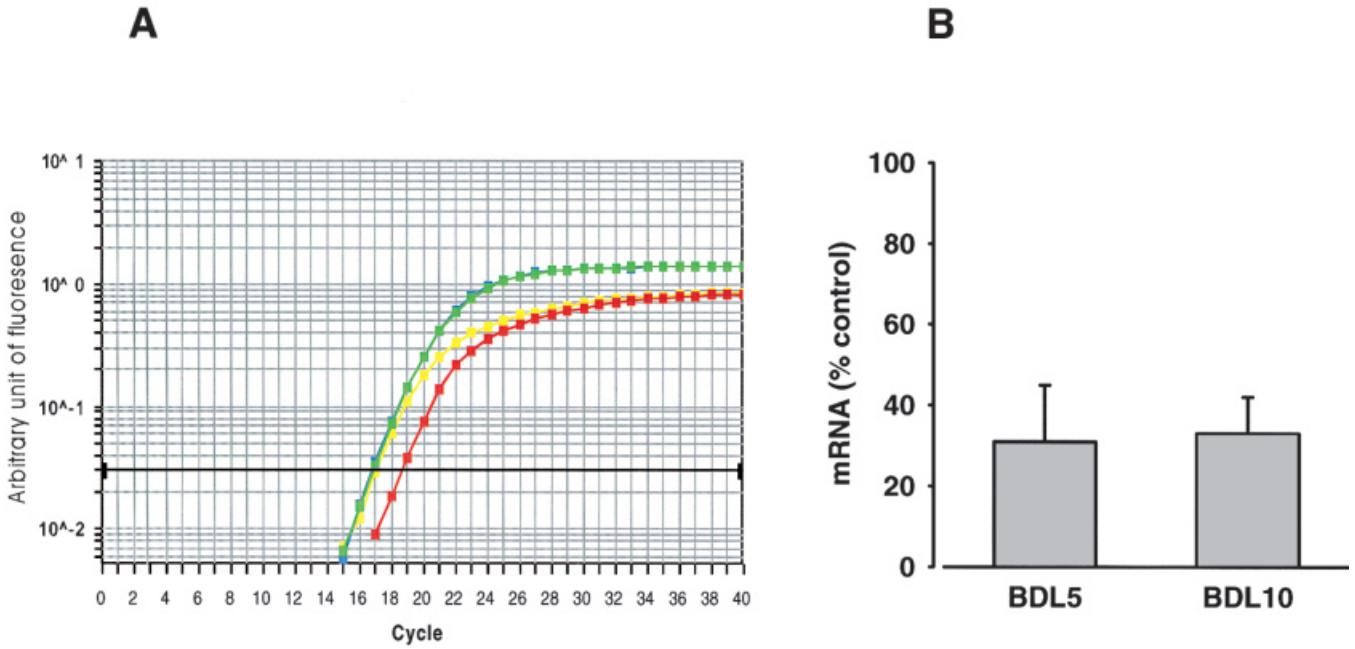

Fig. 2. Analysis of hepatic HMG-CoA synthase mRNA expression by real-time quantitative PCR. Rats studied were either bile duct ligated (BDL) or sham-operated (CON). Control rats were pair fed with BDL rats, and all animals were starved for 24 h before the final experiments. A: Representative amplification 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 (B) (control rats are set at 100%). Data are given as means ± SD. * $P < 0.05$ BDL versus CON rats.

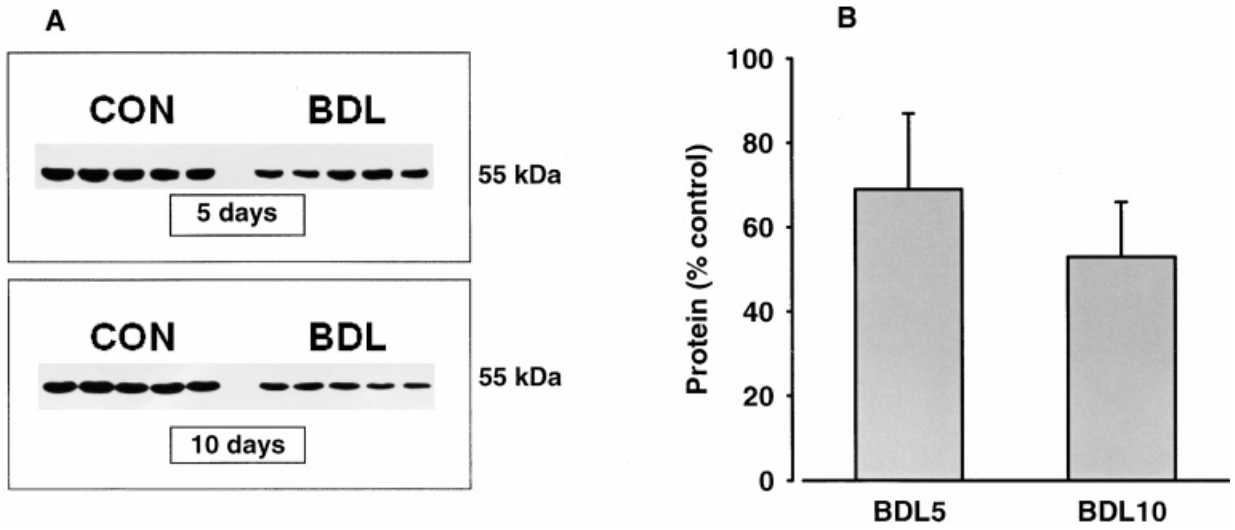

Fig. 3. Effect of bile duct ligation on mitochondrial HMG-CoA synthase protein. Rats studied were either bile duct ligated (BDL) or sham operated (CON). Control rats were pair fed with BDL rats, and all animals were starved for 24 h before the final experiments. Rats were decapitated and their livers were quickly removed and frozen in liquid nitrogen and processed for electrophoresis and Western transfer, which was followed by incubation with a specific antibody for mitochondrial HMG-CoA synthase. Blots were quantitatively analyzed with a luminescent image analyzer. The autoradiographs for both time points studied are shown in (A) and the relative quantification is shown in (B) (control rats are set at 100%). Data are given as means ± SD. * $P < 0.05$ BDL versus CON rats.

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 (Fig. 1). 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, $^{14}CO_2$ production from [^{14}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 Fig. 1) and the substrate for the HMG-CoA pathway and the Krebs cycle, was not lower in BDL as compared with 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). Because disrupted mitochondria form only acetoacetate but not β-hydroxybutyrate from acetyl-CoA (19), a deficiency of β-hydroxybutyrate dehydrogenase could not be the reason for 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. Because of the high activity of this enzyme (see Table 4), 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 enzyme 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 therefore cannot 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 of 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 that upregulate transcription include starvation, long-term exercise, a 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 (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 firm conclusions to be drawn about the mechanisms responsible for the suspected decrease in HMG-CoA synthase transcription. Because BDL rats have generally increased glucagon but unchanged insulin concentrations in comparison with control rats (1), a hormonal cause for this finding is unlikely. Similarly, nutritional factors are also an unlikely cause of impaired transcription of HMG-CoA synthase in acute cholestasis, because control rats were pair fed with 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 determine 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 fate 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 very low density lipoprotein particles into the blood.

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

Manuscript received 2 June 2000 and in revised form 15 August 2000.

REFERENCES

1. Krähenbühl, L., C. Talos, J. Reichen, and S. Krähenbühl. 1996. Progressive decrease in tissue glycogen content in rats with long-term cholestasis. *Hepatology.* **24:** 902–907.
2. Krähenbühl, S., C. Talos, and J. Reichen. 1994. Mechanisms of impaired hepatic fatty acid metabolism in rats with long-term bile duct ligation. *Hepatology.* **19:** 1272–1281.
3. Krähenbühl, S., and E. P. Brass. 1991. Fuel homeostasis and carnitine metabolism in rats with secondary biliary cirrhosis. *Hepatology.* **14:** 927–934.
4. Ramsay, R. R., and A. Arduini. 1993. The carnitine acyltransferases and their role in modulating acyl-CoA pools. *Arch. Biochem. Biophys.* **302:** 307–314.
5. McGarry, J. D., and N. F. Brown. 1997. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur. J. Biochem.* **244:** 1–14.
6. Indiveri, C., V. Iacobazzi, N. Giangregorio, and F. Palmieri. 1997. The mitochondrial carnitine carrier protein: cDNA cloning, primary structure and comparison with other mitochondrial transport proteins. *Biochem. J.* **321:** 713–719.
7. Koyama, K., S. Kashimura, H. Yamauchi, Y. Takagi, Y. Owada, and T. Sato. 1975. Metabolism of short-chain fatty acids in rat liver in biliary obstruction. *Tohoku J. Exp. Med.* **117:** 335–341.
8. Krähenbühl, S., J. Stucki, and J. Reichen. 1992. Reduced activity of the electron transport chain in liver mitochondria isolated from rats with secondary biliary cirrhosis. *Hepatology.* **15:** 1160–1166.
9. Krähenbühl, L., M. Schäfer, and S. Krähenbühl. 1998. Reversibility of hepatic mitochondrial damage in rats with long-term cholestasis. *J. Hepatol.* **28:** 1000–1007.
10. Wächter, S., L. Krähenbühl, M. Schäfer, and S. Krahenbuhl. 1999. The liver carnitine pool reflects alterations in hepatic fatty acid metabolism in rats with bile duct ligation before and after biliodigestive anastomosis. *J. Hepatol.* **30:** 242–248.
11. Ludwig, J. 1987. New concepts in biliary cirrhosis. *Semin. Liver Dis.* **7:** 293–301.
12. Clements, W. D., T. Diamond, D. C. McCrory, and B. J. Rowlands. 1993. Biliary drainage in obstructive jaundice: experimental and clinical aspects. *Br. J. Surg.* **80:** 834–842.
13. Rege, R. V. 1995. Adverse effects of biliary obstruction: implications for treatment of patients with obstructive jaundice. *Am. J. Roentgenol.* **164:** 287–293.
14. Olsen, C. 1971. An enzymatic fluorometric micromethod for the determination of acetoacetate, β-hydroxybutyrate, pyruvate and lactate. *Clin. Chim. Acta.* **33:** 293–300.
15. Hoppel, C. L., J. P. DiMarco, and B. Tandler. 1979. Riboflavin and rat hepatic cell structure and function. Mitochondrial oxidative metabolism in deficiency states. *J. Biol. Chem.* **254:** 4164–4170.
16. Gornall, A. G., G. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:** 751–766.
17. Krahenbuhl, S., M. Chang, E. P. Brass, and C. L. Hoppel. 1991. Decreased activities of ubiquinol:ferricytochrome *c* oxidoreductase (complex III) and ferrocytochrome *c*:oxygen oxidoreductase (complex IV) in liver mitochondria from rats with hydroxycobalamin[c-lactam]-induced methylmalonic aciduria. *J. Biol. Chem.* **266:** 20998–21003.
18. Fréneaux, E., G. Labbe, P. Letteron, T. Le Dinh, C. Degott, J. Genève, D. Larrey, and D. Pessayre. 1988. Inhibition of the mitochondrial oxidation of fatty acids by tetracycline in mice and man: possible role in microvesicular steatosis induced by this antibiotic. *Hepatology.* **8:** 1056–1062.
19. Chapman, M. J., L. R. Miller, and J. A. Ontko. 1973. Localization of the enzymes of ketogenesis in rat liver mitochondria. *J. Cell Biol.* **58:** 284–306.
20. Quant, P. A., P. K. Tubbs, and M. D. Brand. 1989. Treatment of rats with glucagon or mannoheptulose increases mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase activity and decreases succinyl-CoA content in liver. *Biochem. J.* **262:** 159–164.
21. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. Dufour, J. F., M. Luthi, M. Forestier, and F. Magnino. 1999. Expression of inositol 1,4,5-triphosphate receptor isoforms in rat cirrhosis. *Hepatology.* **30:** 1018–1026.
23. Serra, D., N. Casals, G. Asins, T. Royo, C. J. Ciudad, and F. G. Hegardt. 1993. Regulation of mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A synthase protein by starvation, fat feeding and diabetes. *Arch. Biochem. Biophys.* **307:** 40–45.
24. Krahenbuhl, S., J. Stucki, and J. Reichen. 1989. Mitochondrial function in carbon tetrachloride-induced cirrhosis in the rat. Qualitative and quantitative defects. *Biochem. Pharmacol.* **38:** 1583–1588.
25. Zimmermann, T., H. Franke, M. Peuker, and R. Dargel. 1992. Quantitative studies on fatty acid metabolism in isolated parenchymal cells from normal and cirrhotic livers in rats. *J. Hepatol.* **15:** 10–16.
26. Nozu, F., N. Takeyama, and T. Tanaka. 1992. Changes of hepatic

fatty acid metabolism produced by chronic thioacetamide administration in rats. *Hepatology.* **15:** 1099–1106.
27. Hegardt, F. G. 1999. Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis. *Biochem. J.* **338:** 569–582.
28. Krähenbühl, L., J. Reichen, C. Talos, and S. Krähenbühl. 1997. Benzoic acid metabolism reflects mitochondrial function in rats with long-term extrahepatic cholestasis. *Hepatology.* **25:** 278–283.
29. Williamson, D. H., M. W. Bates, and H. A. Krebs. 1968. Activity and intracellular distribution of enzymes of ketone-body metabolism in rat liver. *Biochem. J.* **108:** 353–361.
30. Dashti, N., and J. A. Ontko. 1979. Rate-limiting function of 3-hydroxy-3-methylglutaryl-coenzyme A synthase in ketogenesis. *Biochem. Med.* **22:** 365–374.
31. Ayté, J., G. Gil-Gomez, P. F. Marrero, D. Haro, and F. G. Hegardt. 1990. Rat mitochondrial and cytosolic 3-hydroxy-3-methylglutaryl-CoA synthases are encoded by two different genes. *Proc. Natl. Acad. Sci. USA.* **87:** 3874–3878.
32. Quant, P. A., P. K. Tubbs, and M. D. Brand. 1990. Glucagon activates mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase *in vivo* by decreasing the extent of succinylation of the enzyme. *Eur. J. Biochem.* **187:** 169–174.
33. Lowe, D. M., and P. K. Tubbs. 1985. Succinylation and inactivation of 3-hydroxy-3-methylglutaryl-CoA synthase by succinyl-CoA and its possible relevance to the control of ketogenesis. *Biochem. J.* **232:** 37–42.
34. Casals, N., N. Roca, M. Guerrero, G. Gil-Gomez, J. Ayté, C. Ciudad, and F. G. Hegardt. 1992. Regulation of the expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene. Its role in the control of ketogenesis. *Biochem. J.* **283:** 261–264.