**CHARLES UNIVERSITY IN PRAGUE**
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# LIVER GANGLIOSIDES IN ESTROGEN-INDUCED CHOLESTASIS

*Summary of Doctoral Thesis*

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## CONTENTS

## INTRODUCTION

### Intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis

Intrahepatic cholestasis of pregnancy (ICP), also referred as obstetric cholestasis, is a cholestatic condition during pregnancy. ICP adversely affects well-being of the mother and in particular can also impact neonatal outcome. ICP is characterized by pruritus of various degrees in the mother which commonly occurs in the second or third trimester of pregnancy. Blood tests can reveal elevated aminotransferases and total bile acid concentration (>10mmol/l), severe enough to affect quality of life; jaundice is uncommon in ICP, but may be present. All these symptoms and signs spontaneously relief till three weeks after delivery. Consequences of ICP can be premature delivery, fetal distress, preterm delivery and fetal loss in the infant through negative effects of retained bile acids [1-3]. Intrahepatic cholestasis of pregnancy is treated by administration of hydrophilic bile acid - ursodeoxycholic acid (10-20 mg/kg/d) [4].

ICP and contraceptive-induced cholestasis (CIC) are classified as acquired forms of cholestasis. ICP and CIC symptoms normally disappear when levels of female sex hormones decrease at their normal concentration, suggesting their important role in the ICP and CIC development [5]. In addition, onset of ICP symptoms usually occurs in the third trimester of pregnancy when serum levels of female sex hormones, estrogens and progesterone, reach their maxima [6,7].

### Liver cells and metabolism in ICP and ICI

Bile acid homeostasis is tightly regulated by nuclear receptors including FXR (farnesoid X receptor), PXR (pregnane X receptor), CAR (constitutive androstane receptor) and LXR (liver X receptor). The receptors act complementary to protect livers against bile acid induced injury constituting distinct layers of protection during cholestasis and modulate cholestatic state [8]. ICP and ICI differ in levels of metabolites of bile acids, indicating possible activation of their alternative elimination [9].

Alterations in bile salt transport have been found associating with ICP and impartment of membrane bile salt transporters in pathogenesis of ICP and probably CIC. Carriers of impaired BSEP [7], MRP2 [10] and MDR3 gene [11] were found to be more susceptible for ICP.

The huge amount of liver functions is carried out by liver parenchymal cells (Hepatocytes) and nonparenchymal cells (Kupffer cells, stellate cells,



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## ABBREVIATIONS

| | |
|---|---|
| **ALP** | alkaline phosphatase |
| **BSA** | bovine serum albumin |
| **EE** | 17α-ethinylestradiol |
| **PD** | 1,2-propanediol |
| **GSL** | glycosphingolipids |
| **PBS** | phosphate buffered saline |
| **TBA** | total bile acids |
| **DEAE** | diethylaminoethyl |
| **EDTA** | ethylendiamintetraacetic acid |
| **GSL** | glycosphingolipides |
| **HPLC** | high performance liquid chromatography |
| **HPTLC** | high performance thin layer chromatography |
| **Cer** | ceramide |
| **ChtB** | choleratoxin B-subunit |

Ganglioside symbols, according to Svennerholm [44,45], with IUPAC-IUB [90] nomenclature in parentheses

| | |
|---|---|
| **GM3** | ($II^3$NeuAc-LacCer) |
| **GM2** | ($II^3$NeuAc-$GgOse_3$Cer) |
| **GM1** | ($II^3$NeuAc-$GgOse_4$Cer) |
| **GD3** | ($II^3(NeuAc)_2$-Lac-Cer) |
| **GD2** | ($II^3(NeuAc)_2$-$GgOse_3$Cer) |
| **GD1a** | ($IV^3$NeuAc,$II^3$NeuAc$GgOse_4$Cer) |
| **GD1b** | ($II^3(NeuAc)_2$-$GgOse_4$Cer) |
| **GT1b** | ($IV^3$NeuAc,$II^3(NeuAc)_2$-$GgOse_4$Cer) |



cholangiocytes and sinusoidal endothelial cells). The phenotypic changes of liver cells are mediated by paracrine interplay and are involved in various disease processes [12]. The surrounding heterogeneity is important for short-term and also long-term regulation of metabolism. Hepatocytes of the periportal and perivenous zones markedly differ in their contents and activities of many subcellular structures, enzymes and other proteins; however, there are no sharp boundaries [13].

It is well established that administration of exogenous estrogens over a period of days impair hepatic excretory capacity, decreases bile flow in the rat and impairs hepatic excretory function in rats as well as in humans [14,15]. The cholestatic effect of estrogens is mediated by glucuronidation at the 17β-OH position [16]. The enzyme responsible for glucuronidation, UDP-glucuronosyltransferase UGT2B7, catalyzes exclusively conversion of estriol and estradiol to glucuronidated metabolites at the 17β-OH position, yielding the cholestatic steroid D-ring glucuronides [17]. EE binds to nuclear estrogen receptor alpha (ER-α), enters the nucleus, activating transcription of EE-regulated genes. It is generally believed that glucuronidation blocks activity of estrogens. It is important to note that there is no data on possible interaction among E2-17G and ER-α. However, ER-α knockout mice were resistant to EE induction of cholestasis and did not develop any of the hepatotoxic phenotypes such as hepatomegaly, elevation in serum bile acids, increase of alkaline phosphatase activity, liver degeneration, and inflammation [18].

### Plasmatic membrane fluidity in cholestasis

It was described more than two decades ago that erythrocytes from patients with cholestasis exhibited decreased membrane fluidity [19]. Next, Smith and Gordon analyzed rat livers with EE-induced cholestasis and found an increase in cholesterol ester and sphingomyelin content, as well as an increase in the cholesterol/phospholipid ratio, but an unaltered fatty acids composition [20] but GSL were not analyzed, though a body of evidence that GSL influence membrane fluidity exists [21-23]. Furthermore, Rosario et al. [24] examined the effect of EE administration on protein and lipid composition as well as membrane fluidity using purified sinusoidal and canalicular membrane fractions and found that EE administration selectively decreased sinusoidal membrane fluidity, again, complex GSL were not analyzed [25]. In addition, altered membrane fluidity was also described in cholestasis induced with lithocholate [26], as well as bile duct ligation, or phalloidin infusion [27,28].

## Gangliosides: Function and biosynthesis

The main lipid components of animal cell membranes are glycerophospholipids, sphingolipids, and cholesterol. Among these, sphingolipids are less abundant [29]. Gangliosides are sialic acid-containing glycosphingolipids (GSL), highly concentrated in the outer lipid bilayer of animal cells and are particularly abundant in the plasma membranes of neurons. They show a strong amphiphilic character due to their constitution. They constitute of a hydrophobic ceramide part and a structurally variable hydrophilic oligosaccharide part protruding toward the extracellular environment. The high transition temperatures of saturated chains of the more rigid ceramide exclude them from unsaturated chains of gycerophospholipids with a lower transition temperature [30]. In glycosphingolipids at the interface of water and membrane lipids are heteroatoms of the ceramide part. This rigid system comprises hydrogen bonds of donors and acceptors enabling formation of a stable net of hydrogen bonds. In contrast, the more abundant other membrane lipids, glycerophospholipids do not posses this property due to lack of hydroxyl as well as amide group [31]. In other words, gangliosides are not homogeneously distributed on the cell surface. The driving forces form the membrane areas with a distinct lipid composition leading to a liquid-ordered phase environment with attached interacting membrane proteins. This segregated membrane microdomains “lipid rafts” can consisted of cholesterol, gangliosides and glycosylphosphatidyl inositol-anchored proteins, and other sphingolipids [30-32].

Gangliosides have various functions. They act as receptor molecules and, together with the other GSL, are generally believed to protect cells against harmful environmental factors by forming a mechanically stable and chemically resistant outer leaflet of the cell membrane [32]. Besides stabilizing cell membranes, gangliosides also seem to be involved in cell-to-cell interactions and recognition [33], endocytosis and trafficking [34], as well as an important role in signal transduction the regulation of various receptor tyrosine kinases in the plasma membrane including the receptors of epidermal growth factor [35], nerve growth factor [36], insulin [37] and even the modulation of memory protection [38].

The biosynthesis of gangliosides starts with synthesis of the ceramide part. The ceramide part of ganglioside consists of a sphingoid base and a fatty acid. Sphingosine is biosynthesized by serine palrnitoyltransferase (SPT) by the condensation reaction of serine and palmitoyl-CoA to 3-ketosphinganine. This resulting base may be modified or used to ceramide synthesis. Ceramide is then glycosylated to glycosphingolipids or used for





**ABSTRACTS ON THE TOPICS OF THE DOCTORAL THESIS**

**14th United European Gastroenterology Week, Oct 21-25, 2006, Berlin, Germany**
Changes in the spektrum of gangliosides in the hepatocyte sinusoidal membrane in experimental cholestatis.
Mareček Zdeněk, Jirkovská Marie, Šmídová Jaroslava, Marečková Olga, Majer Filip, Trnka Ladislav, Vítek Libor, Šmíd František
*Gut* – an International Journal of Gastroenterology and Hepatology, Suppl No V, Vol 55, A314, Oct 2006

**Morphology 2006, 43rd International Congress on Anatomy, 43rd Lojda Symposium on Histochemistry, Sept 3-6, 2006, Prague, Czech Republic**
The content and localization of GM1 ganglioside in normal and cholestatic rat liver.
Jirkovská Marie, Šmídová Jaroslava, Šmíd František, Majer Filip, Vítek Libor, Mareček Zdeněk

**31st FEBS Congress, June 24-29, 2006, Istanbul, Turkey**
Estrogen induced cholestasis leads to increase of b-series gangliosides and a redistibution of GM1 in the rat liver
Majer Filip, Jirkovská Marie, Vítek Libor, Mareček Zdeněk, Šmíd František
The FEBS Journal, Vol 273, Suppl 1, June 2006, page 264

**ESCI 2006, 40th Annual Scientific Meeting of the European Society for Clinical Investigation March 15 – 18, 2006, Prague, Czech Republic**
Localization of GM1 ganglioside in hepatocyte membrane of normal liver and in experimental cholestasis
Jirkovska Marie, Šmídová Jaroslava, Majer Filip, Vítek Libor, Mareček Zdeněk, Šmíd František
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Estrogen induced cholestasis results in dramatic increase of b-series gangliosides in rat liver and in localisation of GM1 ganglioside
Mareček Zdeněk, Jirkovská Marie, Šmídová Jaroslava, Marečková Olga, Majer Filip, Trnka Ladislav, Vítek Libor, Šmíd František
*Canadian Journal of Gastroenterology* 0835-7900, Vol 19, Suppl C, R.0981

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Marked shift of GM1 ganglioside from intracellular localisation into cellular membranes might protect cholestatic hepatocytes against increased lvels of bile acids.
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Estrogen induced cholestasis resluts in dramatic increase of b-series gangliosides in rat liver.
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**XXXIII. Májové hepatologické dny, 4-6.5.2005, Karlovy Vary, Česká republika**
1) The change of ganglioside spectrum in the rat liver in ethinylestradiol-induced cholestasis.
Majer Filip, Trnka Ladislav, Šmíd František, Jirkovská Marie, Vítek Libor, Mareček Zdeněk
2) Increased GM1 ganglioside content in the hepatocyte’s sinusoidal membrane in experimental cholestasis
Jirkovská Marie, Majer Filip, Šmídová Jaroslava, Vítek Libor, Mareček Zdeněk, Šmíd František
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**8. Studentská vědecká konference 1. lékařské fakulty UK, 22.5.2007, Praha, Česká republika**
Úloha gangliosidů u estrogeny indukované cholestázy
Majer Filip, Šmíd František

## PUBLICATIONS ON THE TOPICS OF THE DOCTORAL THESIS

**Changes in GM1 ganglioside content and localisation in cholestatic rat liver**
Jirkovská Marie, Majer Filip, Šmídová Jaroslava, Stříteský Jan, Shaik Gouse M, Dráber Petr, Vítek Libor, Mareček Zdenek, Šmíd František
*Glycoconjugate Journal*, Vol 24, Issue 4-5, Jul 2007, 231-41. Epub 2007 Feb 27
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**Estrogen–induced cholestasis results in a dramatic increase of b-series gangliosides in the rat liver**
Majer Filip, Trnka Ladislav, Vítek Libor, Jirkovská Marie, Mareček Zdeněk, Šmíd František
*Biomedical Chromatography*, Vol 21, Issue 5, May 2007, 446-50.
IF 2007 = 1.663

## OTHER PUBLICATIONS

**Identification of bilirubin reduction products formed by Clostridium perfringens isolated from human neonatal fecal flora**
Vítek Libor, Majer Filip, Muchová Lucie, Zelenka Jaroslav, Jirásková Alena, Branný Pavel, Malina Jiří, Karel Ubik
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**Photodynamic therapy of non-melanoma skin cancer with topical *Hypericum perforatum* extract: A pilot study**
Denisa Kacerovská, Karel Pizinger, Filip Majer, František Šmíd
*Photochemistry and Photobiology*, Vol 84, May-June 2008, 779-785.
IF 2007 = 2.061

**Structure-based specificity mapping of secreted aspartic proteases of *Candida parapsilosis*, *Candida albicans*, and *Candida tropicalis* using peptidomimetic inhibitors and homology modeling**
Majer Filip, Pavlíčková Libuše, Majer Pavel, Hradilek Martin, Dolejší Elena, Hrušková-Heidingsfeldová Olga, Pichová Iva
*Biological Chemistry*, Vol 387, Issue 9, Sept 2006, 1247-1254.
IF 2006 = 2.752

**Two aspartic proteinases secreted by the pathogenic yeast Candida parapsilosis differ in expression pattern and catalytic properties**
Hrušková-Heidingsfeldová Olga, Dostál Jiří, Majer Filip, Havlíková Jana, Hradilek Martin, Pichová Iva
*Biological Chemistry*, Published Online: 2009 Jan 23.
IF 2007 = 2.840



synthesis of sphingomyelin [39,40]. Complex oligosaccharide part originate from lactosylceramide and its sialylated derivatives GM3, GD3, GT3 which serve in distal Golgi as precursors of 0-, a-, b- and c- pathways of biosynthesis (Figure 1) [41,42].

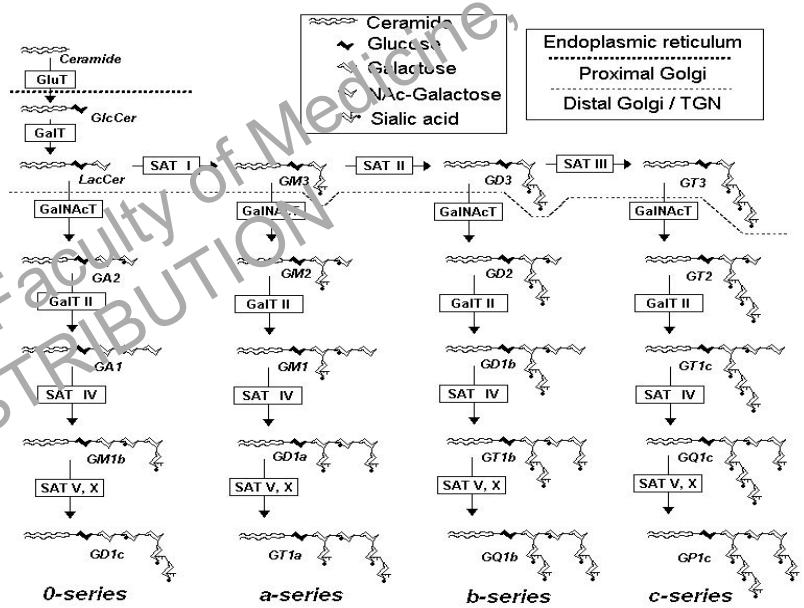


Figure 1. Scheme of the de novo biosynthesis of the oligosaccharide moieties of gangliosides of 0, a, b and c series. Abbreviations of enzymes catalyzing indicated reactions are as follows: SAT I, II, and III - sialyl(NeuAc)-transferases I, II, and III; GalTII - galactosyl(Gal)-transferase; GalNAcT - N-acetylgalactosaminyl(GalNAc)-transferase; SAT IV, V, and X - sialyl(NeuAc)-transferase IV, V and X. [41-43]. Abbreviations of gangliosides according to Svennerholm [44,45]

The biosynthesis of gangliosides takes place on intracellular membranes (endoplasmic reticulum and Golgi apparatus). The biosynthesis is catalysed by membrane-bound enzymes. Transport of de novo synthesized gangliosides to the plasma membrane is carried out by sorting vesicles toward the membrane and consequential fusion. Degradation of gangliosides take place in secondary lysosomes by lysosomal acidic hydrolytic enzymes (predominantly soluble). The hydrolysis is associated

with binding to activator proteins (e.g. GM2 activator protein) and negatively charged lipids (e.g. dolichol phosphate) [46].

The composition of gangliosides in the cellular membranes varies, depending on different cell types, and also changes with cell differentiation during both embryonic development [47] and malignant transformation [48,49]. The formation of the GSL variety is controlled at the transcriptional and post-transcriptional levels [41]. GSL also show polarized distribution as evidenced by studies with hepatocyte-derived HEP-G2 cells incubated with fluorescent dye-labeled glucosylceramide and sphingomyelin [50]. Composition of microdomains was shown to be crucial in cellular trafficking in polarized cells [51,52]. Nevertheless, the distribution of GSL microdomains in the canalicular and basolateral membranes of hepatocytes has not been investigated in situ.

Some bacterial toxin binds to gangliosides (GM2 [53], GM1 [54], GD1a [55], GD1b and GT1b [56], GT1b [57], GQ1b and GT1b and GD1a [58]) with high binding specificity [59]. Together with low molecular weight and non-toxicity (usage of fragments or toxin subunits retaining GSL binding activity) they are predetermined being used for specific detection of gangliosides like antibodies.

The mostly used toxin for detection is cholera toxin. Cholera toxin consist of one 27kDa A-subunit and five 12kDa B-subunits. The B-subunit (ChtB) retains the GM1 ganglioside binding ability and is widely used for detection of lipid rafts [60,61]. A question is whether gangliosides are accessible enough for the detection agents. Since the lipid bilayer consists of much bigger molecules such as glycoproteins. It also undoubtedly depends on cell cycle, cell type and shape of involved molecules [62].

## AIM

The aim of this study was to reveal whether ganglioside composition is changed in EE-induced cholestasis not analyzed in [20].

Consequent aims were:

1. Analysis of ganglioside content and their changes after cholestasis induction by EE-treatment in the liver

2. To determine changes in ganglioside localization in the rat liver after estrogen induction of cholestasis.



## CONCLUSIONS – SUMMARY

The thesis focuses on changes in ganglioside content and localization in the rat liver in 17α-ehtinylestradiol induced cholestasis. In this study we analyzed ganglioside content in the liver homogenate by TLC. We aimed to undertake changes in localization of gangliosides in cholestatic rat liver with ChtB as a specific probe.

### Changes in ganglioside composition in 17α-ehtinylestradiol induced cholestasis.

(1) The total amount of gangliosides was found higher in cholestatic livers as evidenced by increase in total lipid sialic acid.
(2) The distribution of gangliosides of b-series significantly increased in cholestatic livers.
(3) There are also changes in amount of GD1a and GM1 ganglioside when compared to controls.

### Changes in localization of gangliosides.

(1) Zonation of GM1 in the liver acinus has not been described so far.
(2) We observed significant GM1 positivity in sinusoids of the liver lobule.
(3) Even thought we did not manage to reveal the changes in sinusoidal surface in detail.

Thought we did not exactly identify exact localization of gangliosides at the cellular level, this study brought new findings regarding GM1 histochemistry. Association of changes in localization of liver gangliosides requires further investigation to testify protective hypothesis.

densitometric analysis under high magnification, (a) namely the increase of sin/cyt ratio due to increased staining of sinusoidal surface in zone III, and (b) the increase of sin/cyt ratio in zone I due to a decrease in GM1 staining in cytoplasm, correspond with the increased TBA blood concentration in EE-treated animals. According to previous reports, a shift in lipid content occurs within hepatocyte membranes during cholestasis contributing to the modulation of membrane fluidity [20,26]. The results of this study prove that GSL redistribution might be responsible for these changes. This conclusion is also corroborated by the known fact that ganglioside molecules are more rigid due to the conformation of ceramide and oligosaccharide parts of the molecule. The ceramide portion adopts a rigid conformation with two closely packed, parallel hydrocarbon chains [86,87], while the oligosaccharide portion is conformationally dependent on the high stability of sialic acid [88] and some other factors [89]. It is well established that the presence of gangliosides reduces the membrane fluidity [21]. The study on histological changes documents for the first time that GM1 ganglioside appears in enhanced levels in sinusoidal membranes of hepatocytes during cholestasis. It further demonstrates that microscopic methods remain an important analytical tool, especially in those situations where biochemical and genetical approaches reach their limits.





## MATERIALS AND METHODS

### Animals and treatments, collection of samples, analysis of serum markers

Female Wistar rats were housed under controlled temperature and a natural 12 h light–dark cycle. The cholestasis was induced in the experimental group by subcutaneous injections of EE in a dose 5 mg/kg b.wt. dissolved in 1,2-propanediol (PD) applied daily for 18 consecutive days. The control group of animals was without any treatment and the PD subgroup was treated with PD daily.

Blood samples were collected after laparotomy under ether anaesthesia, and livers were flushed blood-free with heparin and weighed. For further histological analyses, the livers stored at -80°C. For quantitative histochemical analysis of GM1 ganglioside, the liver specimens were collected using systematic uniform random sampling method. For Real-Time PCR analysis liver pieces were submerged in RNAlater and stored at -80°C. The pieces of liver tissues were stored at -20°C until processed biochemical analysis of ganglioside composition.

In order to determine the degree of cholestasis and liver injury, the following serum levels were assessed: total bile acids (TBA), total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT). TBA were determined enzymatically, while all other markers were quantified on an automatic analyzer.

### Isolation and TLC analysis of liver gangliosides

Liver gangliosides were analyzed by a densitometric method after TLC separation and detection with resorcinol-HCl reagent, as described in [63]. Homogenization of the rat livers was performed in methanol then gangliosides were isolated by Folch partitioning [64] with Suzuki's modification increasing yield of polysialogangliosides [65]. In the lower organic phase from Folch partitioning cholesterol and phospholipids as lipid inorganic phosphate were determinated [66-68]. The isolated gangliosides were purified according to Yu and Leeden [69] on a small silica-gel column. Total lipid sialic acid was determined in samples by spectrophotometry with resorcinol–HCl reagent [70]. Samples of isolated gangliosides were dissolved in chloroform–methanol 1:1 (v/v) and applied on an HPTLC silica-gel plate (Merck, Darmstadt, Germany) using a CAMAG LINOMAT IV sampler (Camag, Switzerland). Gangliosides were separated in a solvent system (chloroform–methanol–0.2% aqueous $CaCl_2$,

55:45:10, v/v/v), and detected with resorcinol–HCl reagent. The chromatograms were evaluated by densitometry in reflectance mode at 580 nm, using a TLC Scanner II, CAMAG (Muttenz, Switzerland) and 'Cats' 3.20 software. The sialic acid content corresponding to individual ganglioside fractions was calculated on the basis of the calibration of the GM1 ganglioside standard (Sigma, USA) as used previously by Chigorno et al. (1982). The amount of Fuc-GM1 was assayed in monosialoganglioside fractions [71] of the liver homogenate from control and EE-treated groups of animals by TLC with resorcinol detection and by TLC immunostaining.

## Light microscopy

**Morphological analysis**: Small tissue blocks were embedded in LR Gold resin (Sigma, USA). Semi-thin sections were cut, stained with toluidine blue and examined for autofluorescence of pigment granules.

**Alkaline phosphatase histochemistry**: The catalytic activity of ALP was demonstrated in thin 6 μm cryostat sections using simultaneous azo-coupling method [72], counterstained with Mayer´s hematoxylin and mounted in glycerin jelly.

**GM1 histochemistry**: Frozen 6 μm sections were fixed first in dry cold acetone and then in freshly prepared paraformaldehyde. Endogenous peroxidase activity was blocked by incubation in hydrogen peroxide in PBS supplemented with sodium azide. Endogenous biotin was blocked by means of avidin and biotin kit (DAKO, Denmark). In order to block nonspecific binding, sections were treated with BSA in PBS. Cholera toxin was used for GM1 ganglioside detection [73]. Sections were incubated with biotinylated cholera toxin-B-subunit (List Biological Laboratories, USA), then the sections were labeled with Streptavidin-peroxidase polymer Ultrasensitive (Sigma, USA) and the peroxidase activity was visualized with DAB. Sections were counterstained with Mayer´s hematoxylin and mounted in glycerine jelly. Two control tests were included in each series. First, fixed sections were extracted with chloroform–methanol mixture 2:1, second, the cholera toxin B-subunit was omitted in negative control tests.

**Quantitative study on the distribution of GM1 ganglioside in the hepatic lobule**: In this study, EE-treated animals and untreated control animals were used. From each liver, 6 pieces of randomly sampled tissue blocks were taken. One frozen 6 μm thick section was used from each block, and all sections were stained simultaneously using the above described histochemical reaction. The hematoxylin counterstaining was omitted. The images of whole sections were photographed at the objective magnification 5× (NA=0.12). The quantity of reaction product was



its nearly complete absence in liver parenchyma nearby the central vein. In the liver tissue after EE treatment, where the product of histochemical reaction was typically found in all lobular zones, an apparent change of GM1 ganglioside distribution in hepatic lobule was observed. Differences in optical brightness in separate lobular zones III and I, also supported by linear regression analysis, confirmed link between GM1 ganglioside redistribution and cholestasis (Fig. 4-10). These findings might be associated with lobular blood flow and liver zonation.

As blood flows in liver sinusoids from the periphery to the center of the lobule, numerous substances including bile acids are eliminated by hepatocytes. Considering the well known fact that the higher presence of GSL increases membrane rigidity, we conclude that the shift of GM1 ganglioside localized in the central zone of the liver lobule of EE-treated animals can be a response of the hepatocytes to the harmful effects of bile acids accumulated in sinusoidal blood during cholestasis.

The data on mRNA analysis of GalTII are in accordance with biochemical analysis of ganglioside content by TLC. The different distribution of GM1 gangliosides in zones I and III in the EE-treated animals, compared to control samples, and the same level (content) of GM1 and mRNA for GalTII (GM1-synthase) in both groups suggest that not higher biosynthesis in the whole liver but rather locally confined differences in biosynthesis in the central zone might be responsible for the observed differences in increased GM1 expression in sinusoidal surface of central zone in EE-treated animals. It should also be noted that the increase in GD3, observed by TLC does not seem to be due to an increased expression of SATII, as can be inferred from the results of RT-PCR analysis. Further studies, e.g. determination of sialyltransferase activity are necessary for explanation of the increase in b-branch gangliosides. Detailed densitometry at high magnification of the sinusoidal membrane in peripheral zone I did not show any significant increase of GM1 staining in EE-treated animals when compared with those treated with PD or those with no treatment (see data in Table 4-11b and i). These data suggest that GM1 concentration in sinusoidal membrane in peripheral lobular zone is present to a limited extent even in control animals. It thus seems that hepatocytes in peripheral zone I are attacked by harmful molecules even in untreated animals. Very surprising is observation of decreased GM1 staining in subsinusoidal area of peripheral zone cytoplasm. One possible explanation might be that the GM1 is rapidly transported by endosomes from cytoplasm into membranes at limited GM1 biosynthesis. This conclusion is supported by TLC densitometry data [77] and the results of RT-PCR. The two main findings of

associated with the activation of NF-κB [81,82]. In the study of hepatic content of GD3 ganglioside was found to be substantially increased in cholestatic rats. It is capable of inactivation of NF-κB, indicating another possible explanation for ganglioside-mediated hepatocyte cytoprotection. In fact, GD3 has been demonstrated to selectively repress NF-κB dependent gene expression by preventing the translocation of active NF-κB complexes to the nuclei [83], and thus regulating substantially cell apoptosis. The dramatic increase of b-series gangliosides might contribute to the protection of hepatocytes from the deleterious effects of cholestasis.

Several approaches may be used to study GSL in cholestatic liver membranes. Biochemical approach may be appropriate for analysis of GSL in membranes isolated by gradient ultracentrifugation; however, it is difficult to obtain canalicular and basolateral membrane fractions in sufficient quantity and purity. Considering that ALP activity serves as a marker of purity of canalicular membrane fraction isolated by gradient ultracentrifugation, the changes in ALP activity localization observed in EE-administered rats question the possibility to achieve a satisfactory and reliable purity of membrane fractions.

Unlike in normal rat liver, where weak ALP activity was restricted just to the canalicular hepatocyte membrane of peripheral lobular zone, the ALP activity in EE-administered animals was spread over the whole hepatocyte cell membrane except the sinusoidal part in all lobular zones. These results are in agreement with the study showing that the ALP activity in EE-treated rats extends to the central lobular zone [84], whereas a weak activity is confined only to the canalicular hepatocyte membrane at the periphery of the lobule in normal rat [85]. The spreading of ALP from canalicular to lateral membranes explains why ALP is an unreliable marker of canalicular membranes in their isolation from cholestatic livers.

Due to the above mentioned problems in isolation of membranes by ultracentifugation, selective biochemical approach based on specific binding of cholera toxin B subunit to GM1 was used in the histological study. The specificity of this binding has been carefully tested on several occasions. Wu and Ledeen [73] confirmed that, among different gangliosides, only GM1 binds cholera toxin B-subunit with very high affinity. In another study, Parton [34] demonstrated that the only components capable of binding cholera toxin B-subunit are glycolipids and not glycoproteins. The histological study is the first *in vivo* study demonstrating the localization of gangliosides in normal and cholestatic liver. The findings in normal rat liver tissue clearly detects GM1 localized on both the canalicular and sinusoidal surfaces in lobular zones I and II, and



determined as mean optical brightness of marked area using the image analysis program ACC 6.0 (SOFO, Czech Republic) and ImageJ (USA). Two ways were used for the quantification. First, whole section was marked and its mean optical brightness was evaluated. Second, areas of liver parenchyma in peripheral (periportal; zone I) intermediate (zone II) and central (zone III) zones of hepatic lobules were marked and their mean optical brightness was determined separately.

**Densitometric analysis of the amount of GM1 ganglioside in areas of sinusoidal membrane and of adjacent cytoplasm:** Animals from EE-treated, PD-treated and from control group were taken to this analysis. Six liver specimens were used from each animal. One section from each specimen was used for GM1 ganglioside detection with cholera toxin B-subunit histochemical procedure described above. On each section, four hepatic lobules with clearly discernible central vein were selected. In each lobule, one measuring frame in central lobular zone III and one measuring frame in corresponding peripheral lobular zone I were selected for analysis. In each frame, 15 areas of sinusoidal surface and 15 areas of adjacent hepatocyte cytoplasm were selected by the stratified random sampling method [74] and marked out. The reaction product was quantified as mean optical density of analyzed areas as determined by the densitometric program CUE 2 (Olympus) at objective magnification 40× (NA=0.7).

## RNA extraction, cDNA synthesis and real-time PCR

RNA was extracted from livers of control or EE-treated animals using acid guanidinium thiocyanate-phenol-chloroform mixture (RNA Blue reagent, Top-Bio, Czech Republic). Purity and quantity of RNA was checked by Nanodrop spectrophotometer (NanoDrop Technologies, USA). Singlestrand cDNA synthesis was performed using MMLV reverse transcriptase (Top-Bio, Czech Republic), according to manufacturer's instructions; a mixture of isolated RNA and random hexamers was used per reaction. Semi-quantitative real-time PCR based on SYBR-Green fluorescence was performed using Mastercycler ep realplex (Eppendorf, Germany). PCR products corresponding to the following genes were monitored: β-actin, GalTII (galactosyl(Gal)-transferase II, GM1-synthase) and SATII (sialyl(NeuAc)-transferase II, GD3-synthase). To ensure that correct DNA fragments were amplified in PCR, a melting curve of PCR products was obtained. Furthermore, the size of all PCR products was controlled by agarose electrophoresis. The final real-time-PCR data were expressed as the $C_T$ values of GalTII and SATII normalized with the $C_T$ of β-actin of each sample [75].

## RESULTS

### Induction of cholestasis - Biochemical analyses

EE treatment for 18 days resulted in the development of severe cholestasis, as evidenced by a marked elevation of serum bilirubin and TBA (Table 4-1a and 4-1b), as well as pronounced histological changes in livers typical of severe cholestasis (see below). Besides these effects, as compared with the controls, a significant loss of body weight was recorded in EE-treated animals (293 ± 32 vs 212 ± 43 g, p = 0.004). Treatment with 1,2-propanediol, a vehicle for EE, did not result in any change in cholestatic markers (data not shown).

**Table 4-1a.** Serum levels of cholestatic markers in control and experimental group in study of changes in ganglioside content.

| Marker | Controls (n = 6) | EE treated rats (n = 6) | p value |
|---|---|---|---|
| ALT-L [μkat/L] | 0.87 ± 0.14 | 1.16 ± 0.20 | 0.026 |
| AST-L [μkat/L] | 1.41 ± 0.28 | 1.85 ± 0.96 | 0.374 |
| ALP [μkat/L] | 2.04 ± 0.40 | 2.75 ± 1.50 | 0.394 |
| Bilirubin [μmol/L] | 2.10 ± 0.20 | 26.0 ± 17.0 | 0.002 |
| TBA [μmol/L] | 39 ± 25 | 589 ± 185 | 0.002 |





## DISCUSSION

Our study on changes of ganglioside content in the rat liver in Biomedical chromatography [77] is the first report on changes of the ganglioside pattern in rat liver in EE-induced cholestasis, an experimental model of cholestasis of pregnancy and oral contraceptive-induced. The control Wistar rats, used in the experiment, showed significant changes in liver ganglioside content. The total lipid sialic acid was almost twice as high as compared to control rats (p=0.002). The TLC analysis of GM1 ganglioside in rat livers showed only slight insignificant increase in EE-treated rats when compared with controls in total liver extract GM1 is not enhanced in EE-treated animals. However, gangliosides of b-branch of biosynthesis GD3, GD1b and GT1b are increased significantly (Fig.4-2) when in control livers high predominance of a-pathway gangliosides occurred. To conclude changes in liver ganglioside content, in cholestatic livers, we detected significant increases of concentrations of total lipid sialic acid, a-biosynthetic pathway ganglioside GD1a, and particularly the b-pathway gangliosides (GD3, GD1b and GT1b). These changes in ganglioside content and composition were much more highly expressed in this model of cholestasis compared with the bile duct ligation-induced cholestasis, published by Senn [78]. The observations in changes of ganglioside content are consistent with the results reported by Mesaric and Decker [79], who studied the influence of sex steroid hormones on the activities of GM3 and GD3 synthases in isolated hepatocytes. Progesterone, β-estradiol and testosterone all activated GD3 (but not GM3) synthase when added directly to hepatocytes cultured in modified William's E medium.

The increase of the b-pathway in EE-treated liver might be due to GD3 synthase (sialyltransferase II) overexpression/activation, leading to the increased synthesis of GD3 from GM3. Other mechanisms, however, might also contribute. Senn et al. [78] observed a marked increase of GD3 content in bile duct ligation-induced cholestasis, despite low hepatic activities of GD3 synthase. As the majority of gangliosides are concentrated in cell membranes, the histological findings suggest that b-series gangliosides might contribute to a cytoprotective resistance of hepatocytes, against the deleterious effects of bile acids intracellularly accumulated during cholestasis. This assumption was corroborated with data by Kasai et al. [80] on LEC rats almost completely lacking b-series gangliosides. These rats regularly develop severe hepatic pathology, suggesting the importance of b-series gangliosides for protection against various harmful factors. In addition, it was demonstrated that experimental cholestasis is commonly

increase of sinusoidal GM1 was found between zone III and zone I in both EE-treated (Table 4-11k, p=0.007) and PD-treated rats (Table 4-11m, p=0.0002).

### Analysis of gangliosides and expression levels of mRNA of GM1-synthase and GD3-synthase

To clarify the biochemical basis of the observed differences between EE- and PD-treated animals we further analyzed the content of gangliosides in whole liver. Data presented in Fig. 4-15a shows a general scheme of ganglioside biosynthesis, consisting of 0, a, b and c branch.

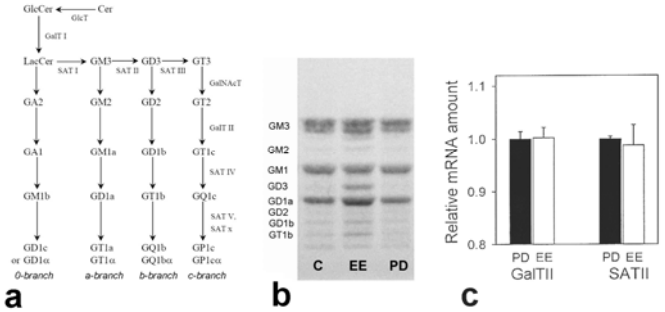


**Fig. 4-15.** (a) Scheme of ganglioside biosynthesis with branches *0*, *a*, *b* and *c*. Abbreviations of enzymes catalyzing the indicated reactions: SAT I, II, and III - sialyl(NeuAc)-transferases I, II, and III; GalTII - galactosyl(Gal)-transferase; GalNAcT - *N*-acetylgalactosaminyl (GalNAc)-transferase, SAT IV, V, and X - sialyl(NeuAc)-transferase IV, V and X. (b) TLC of gangliosides in untreated control (C), EE-treated (EE) and propanediol-treated (PD) rat liver shows an increase in *b*-branch gangliosides and GD1a *a* branch , but GM1 is not significantly increased. (c) Real-time PCR analysis of GalTII and SATII mRNA in liver cells. Total RNA was isolated from liver cells of control and EE-treated animals and subjected to real-time-PCR using primers for actin, GalTII and SATII. Results shown are representative of three independent experiments performed in triplicates.

TLC shows a significant increase in GD3 and b-branch gangliosides. To determine differences in the expression of enzymes involved in synthesis of GM1 or b-branch gangliosides, expression levels of mRNA for GalTII (GM1-synthase) and SATII (GD3-synthase) were estimated using real-time PCR. Data presented in Fig. 4-15c reveal that there are no differences between control and EE-treated animals.



**Table 4-1b.** Biochemical markers of control and EE-treated rats Controls EE-treated in study of histological localization.

| | Controls (n = 7) | EE-treated rats (n = 11) | p-value |
|---|---|---|---|
| **Weight of rats [g]** | 296 ± 26 | 219 ± 34 | **0.00001** |
| **Weight of liver [g]** | 10.7 (9.6 - 11.2) | 9.6 (8.6 - 11.9) | 0.63 |
| **TBA [µmol/l]** | 45 (21 - 53) | 382 (256 - 619) | **0.00001** |
| **Bilirubin [µmol/l]** | 2.0 (1.9 - 2.4) | 7.5 (5.5 - 27.8) | **0.00001** |
| **ALT [µkat/l]** | 0.81 (0.75 - 1.04) | 0.92 (0.81 - 1.20) | 0.22 |
| **AST [µkat/l]** | 1.28 (1.17 - 1.61) | 1.18 (1.00 - 1.77) | 0.76 |
| **ALP [µkat/l]** | 2.12 (1.68 - 2.44) | 2.42 (1.78 - 3.13) | 0.38 |

*Data are presented as mean ± SD, or median (25%-75%) when not normally distributed*

Changes in cholesterol and lipid phosphorus were also observed in the same sense as stated in the literature [20].

### Total lipid sialic acid and TLC analysis of ganglioside content

The mean total lipid sialic acid in livers of EE-treated rats was 79.1 ± 9.0 nmol/g of wet weight of liver tissue when compared with 44.3 ± 15.2 nmol/g in the control animals ($p < 0.01$), i.e. ratio 1.8:1. Hepatic ganglioside distribution in EE-treated and control samples obtained from TLC (Fig. 4-2) analysis and densitometry (Fig. 4-3) is shown in Table 4-4. Although induction of cholestasis leads to more than a 3-fold increase of GD1a (a marker of the a-biosynthetic pathway) in this particular experiment, the most pronounced effect was detected in gangliosides of the b-pathway, with 1–2 orders of magnitude higher amounts of GD3, GD1b and GT1b in the cholestatic livers. In total, all a-series gangliosides increased 1.3-fold in cholestatic livers, whereas b-series gangliosides were up-regulated 54-fold.

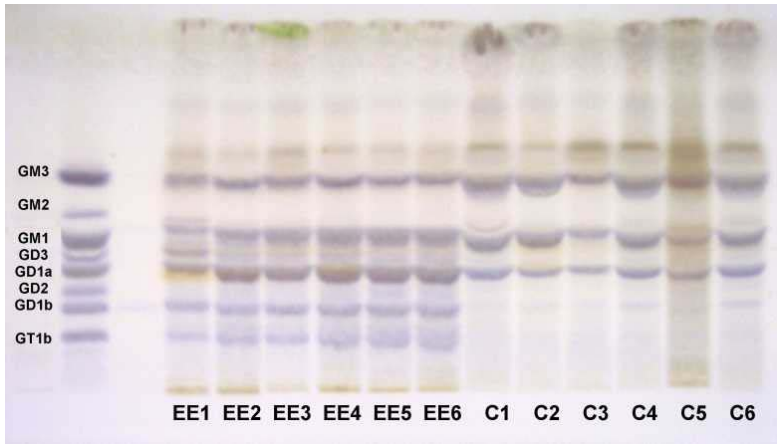


**Fig. 4-2.** Marked increase of b-pathway gangliosides GD3, GD1b and GT1b isolated from cholestatic (EE1–EE6) and control (C1–C6) rat livers.. Final densitometrical evaluation (Table 4-4) was based on the calibration with GM1.

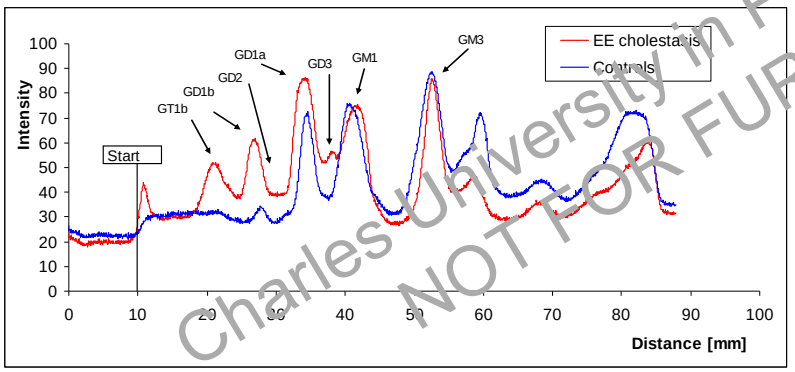


**Fig. 4-3.** Mean of scans of control and EE-treated group.

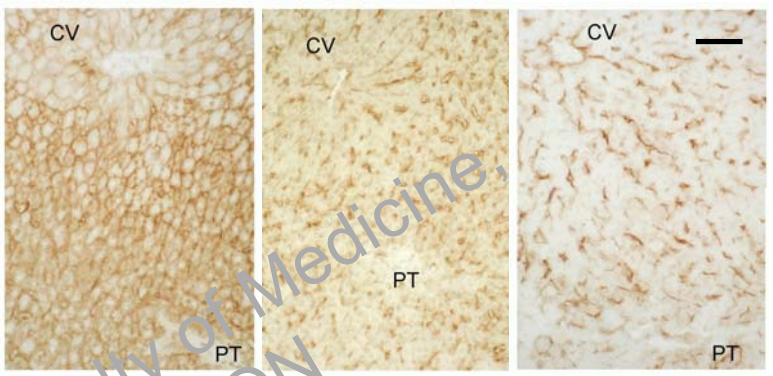


**Fig. 4-13.** (left) GM1 staining in cytoplasm of PD treated rats decreased rapidly in cytoplasm of EE treated rats (middle and right) especially of peripheral zone I. Serum concentration of TBA were 46, 366 and 868 μmol/l. Bar = 50 μm. Objective magnification in photography 20x (NA=0.5). CV = central vein. PT = portal area.

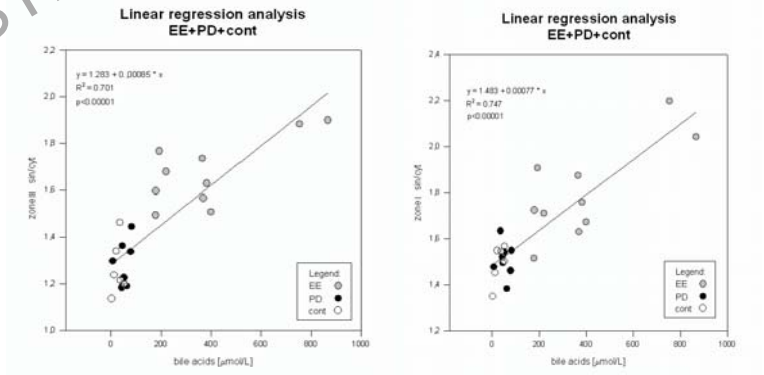


**Fig. 4-14.** (left) Linear regression analysis between serum TBA concentration and difference in GM1 ganglioside content between sinusoidal membrane and subsinusoidal area of cytoplasm in **zone III**. (right) Linear regression analysis between serum TBA concentration and difference in GM1 ganglioside content between sinusoidal membrane and subsinusoidal area of cytoplasm in **zone I** of hepatic lobules.

Linear regression analysis confirmed that higher degree of cholestasis expressed as TBA concentration correlate with increasing difference between sin/cyt staining (Fig. 4-14e) in zone I. Finally, an

(Table 4-11g). Therefore, the increase of sin/cyt ratio seems to be due to increased sinusoidal staining in EEtreated animals (Table 4-11h, p=0.00002) at a stable level of staining in sub-sinusoidal areas of cytoplasm. Linear regression analysis confirmed that widening difference between sin and cyt staining correlated well (p=0.00001) with higher degree of cholestasis as indicated by higher serum TBA concentration (see Fig. 4-14d).

### *The effect of EE-treatment on GM1 staining in peripheral lobular zone I*

Data presented in Table 4-11i show no significant difference in GM1 staining in sinusoidal surfaces areas in zone I between EE- and PD-treated animals (p=0.12). In contrast, the decrease of GM1 staining in the adjacent cytoplasmic area was found highly significant (Table 4-11j, p=0.0008). Consequently, the difference in ratios sin/cyt EE to sin/cyt PD is also highly significant (Table 4-11k, p=0.002). Examples of staining in cytoplasm of hepatocytes in periportal zone PD and EE-treated livers are shown in Fig. 4-13b and d.

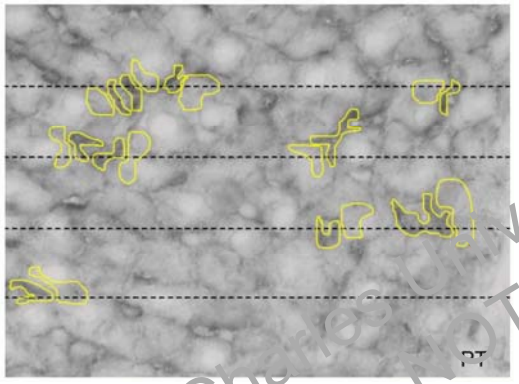


**Fig. 4-12.** Image analysis of GM1 in rat liver examined at high magnification (40x, NA=0.7). The demonstration of selection of areas in individual measuring frame. Usually several measuring frames are necessary in each field to select 15 areas of sinusoidal membranes and 15 sub-sinusoidal areas of cytoplasm by stratified random sampling method in central zone III and the same was done for neighboring peripheral areas.



**Table 4-4.** Ganglioside distribution in liver tissue of control and EE-treated rats.

| Ganglioside biosynthetic pathway | Ganglioside | Controls (n = 6) [nmol/g liver] | EE treated rats (n = 6) [nmol/g liver] | p value |
|---|---|---|---|---|
| a-series | GM3 | 11.4 ± 2.2 | 7.1 ± 0.6 | 0.001 |
| | GM1 | 7.8 ± 2.4 | 10.5 ± 3.0 | 0.146 |
| | GD1a | 3.6 ± 1.0 | 11.8 ± 3.0 | 0.001 |
| b-series | GD3 | 0.08 ± 0.03 | 2.0 ± 1.2 | 0.002 |
| | GD2 | 0.02 ± 0.03 | 0.32 ± 0.34 | 0.041 |
| | GD1b | 0.10 ± 0.06 | 5.4 ± 1.6 | 0.002 |
| | GT1b | 0.06 ± 0.03 | 6.4 ± 2.6 | 0.002 |

The densitometric method used in our study gave comparable results to the photometric method published by Suzuki (1964); and an equal amount of mono-, di- and trisialogangliosides gave densitometric areas in the ratio of 1:2:3, according to the content of sialic acid in the molecule. The samples gave a linear calibration up to 0.4 μmol of ganglioside, (i.e. up to approximately 6 nmol of sialic acid; [63].

Cholera toxin B-subunit can bind also Fuc-GM1 ganglioside but with one half of the GM1 affinity [76]. Therefore, in the histological study we needed to know if Fuc-GM1 is present. In the isolated monosialoganglioside fraction from the control rat liver pooled from 6 control animals (C) and cholestatic rat liver pooled from 6 EE-treated animals (EE) we found that the amount of Fuc-GM1 is lower than 2ng/g tissue weight and much lower than GM1. Thus interference of Fuc-GM1 in GM1 histochemistry (see section GM1 histochemistry) we concluded as not significant.

### Light microscopy – histopathological and morphological changes

Morphological signs of cholestasis were found in the liver of EE-treated rats (Fig. 4-6a), even though individual liver samples differed in the degree of pathological changes. More or less conspicuous degree of feathery degeneration of hepatocytes occurred mainly in peripheral areas of lobules, though it was found occasionally in hepatocytes of all lobular zones (Fig. 4-6b, 4-6d). Liver samples of EE-treated animals displayed also

variable amounts of intracellular bile pigments, while no pigment depositions were found in bile canaliculi (Fig. 4-6c). In some cases, fatty degeneration with numerous lipid droplets in hepatocytes was observable particularly in the zone I and zone II of the lobule (Fig. 4-6b). Liver cells displaying features of apoptosis or necrosis were evenly spread throughout the liver parenchyma (Fig. 4-6d).

### Alkaline phosphatase histochemistry

EE-induced cholestasis was also reflected in ALP catalytic histochemical analysis. In the control group, ALP was confined just to the canalicular membrane of hepatocytes in peripheral zones (zone I) of hepatic lobules and the enzyme activity expressed by the amount of reaction product was very low (Fig. 4-6e). The sections from EE-influenced liver specimens showed a markedly higher amount of the reaction product lining the canalicular and basolateral hepatocyte membranes in all zones of hepatic lobules. Only those parts of hepatocyte membrane directly adjacent to blood sinusoids displayed no enzymatic activity (Fig. 4-6f).



### *Differences in GM1 staining between samples obtained from untreated and PD-treated animals*

A possible influence of PD on GM1 staining was investigated by comparing the PD-treated animals and those without treatment. A slight increase of sinusoidal GM1 staining in zone III was observed (Table 3a; p=0.02).

**Table 4-11.** Results of densitometric quantification of GM1 ganglioside in areas of sinusoidal surface (sin) and of adjacent cytoplasm (cyt). C = untreated controls.

| | | | | Mean ± SD | Mean ± SD | p value |
|---|---|---|---|---|---|---|
| a | zone III sin PD | vs. | zone III sin C | 0.184 ± 0.012 | 0.164 ± 0.015 | **0.02** |
| b | zone I sin PD | vs. | zone I sin C | 0.283 ± 0.019 | 0.296 ± 0.036 | 0.57 |
| c | zone III cyt PD | vs. | zone III cyt C | 0.141 ± 0.013 | 0.135 ± 0.012 | 0.47 |
| d | zone I cyt PD | vs. | zone I cyt C | 0.189 ± 0.016 | 0.200 ± 0.019 | 0.30 |
| e | zone I sin C | vs. | zone III sin C | 0.296 ± 0.036 | 0.164 ± 0.013 | **0.003** |
| f | zone III sin EE | vs. | zone III sin PD | 0.228 ± 0.023 | 0.184 ± 0.012 | **0.0002** |
| g | zone III cyt EE | vs. | zone III cyt PD | 0.138 ± 0.020 | 0.141 ± 0.014 | 0.75 |
| n | zone III sin/cyt EE | vs. | zone III sin/cyt PD | 1.675 ± 0.144 | 1.313 ± 0.108 | **0.00002** |
| i | zone I sin EE | vs. | zone I sin PD | 0.264 ± 0.028 | 0.283 ± 0.020 | 0.13 |
| j | zone I cyt EE | vs. | zone I cyt PD | 0.149 ± 0.023 | 0.189 ± 0.017 | **0.0008** |
| k | zone I sin/cyt EE | vs. | zone I sin/cyt PD | 1.803 ± 0.204 | 1.511 ± 0.074 | **0.002** |
| l | zone I sin EE | vs. | zone III sin EE | 0.264 ± 0.028 | 0.228 ± 0.023 | **0.007** |
| m | zone I sin PD | vs. | zone III sin PD | 0.283 ± 0.019 | 0.184 ± 0.011 | **0.0002** |



For this reason, PD-treated animals were used as controls in the study of EE influence on GM1 staining. Significant changes were found neither in sinusoidal GM1 staining in zone I (Table 4-11b, p=0.57) nor in cytoplasm of both zones I and III (Table 4-11c, d). It should be noted that in untreated controls, significantly higher GM1 staining on sinusoidal surfaces was found in zone I compared to zone III (Table 4-11e, p=0.03).

### *The effect of EE-treatment on GM1 staining in areas of sinusoidal surface of central lobular zone III*

Data in Table 4-11f show a significant increase of GM1 centrolobular sinusoidal surface areas of EE-treated rats when compared with those treated with PD (p=0.0002). No significant difference in GM1 staining was found between EE- and PD-treated animals in cytoplasmic areas in zone III

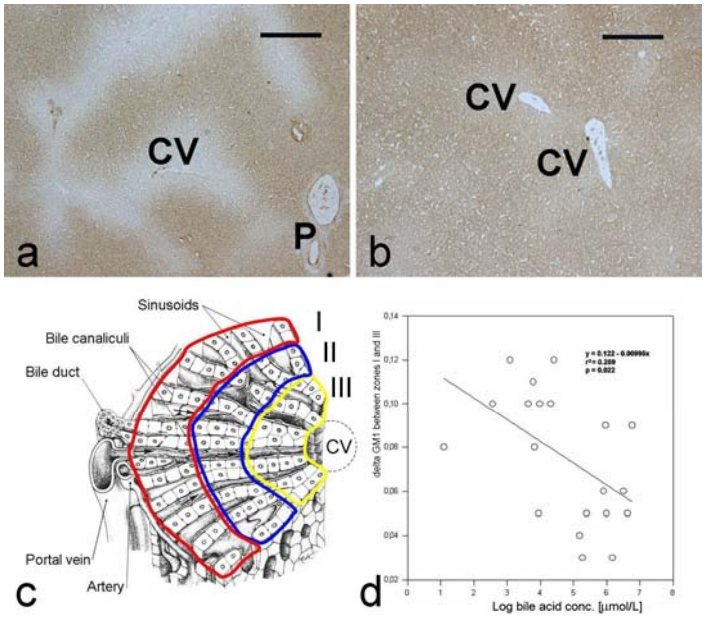


**Fig. 4-10.** Histochemical localization of GM1 ganglioside in rat liver as seen at low magnification. (a) In control liver, the reaction product occurs in lobular zones I and II and is nearly or completely absent in zone III. (b) In EE-treated liver, the difference in the amount of reaction product between peripheral and central areas of the lobule is hardly observable. cv = central vein, p = portal area; bars = 500 µm. (c) Schematic representation of the liver lobule with marked areas of measurement. Red = peripheral zone (zone I), blue = intermediate zone (zone II), yellow = central zone (zone III). d) Linear regression analysis between serum TBA concentration and the difference in GM1 ganglioside content between lobular zones I and III.

### Comparison of the amount of GM1 ganglioside in areas of sinusoidal membrane and of adjacent cytoplasm

The effect of EE on localization of GM1 was analyzed under high magnification. The results are summarized in Table 4-11.



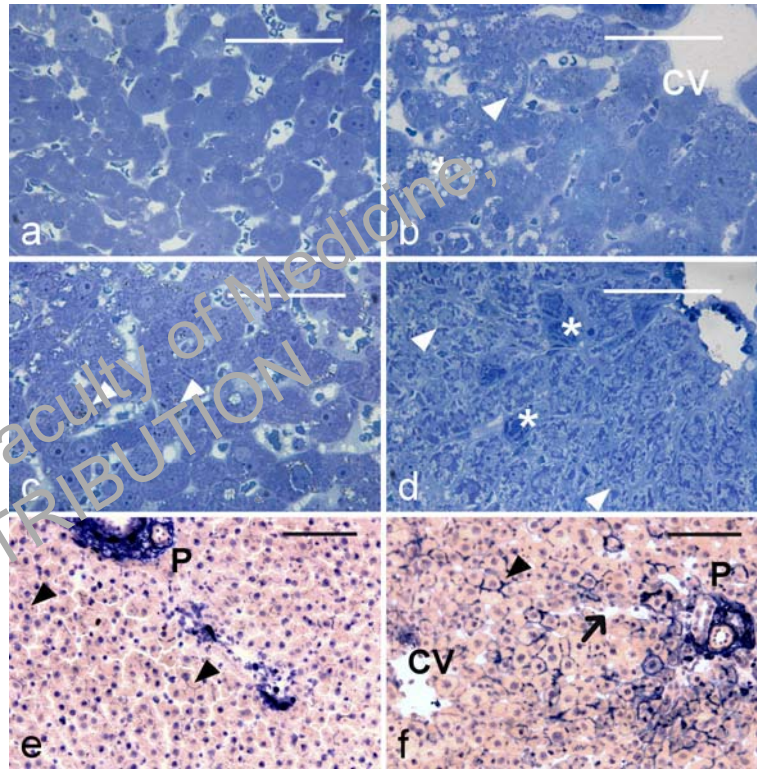


**Fig. 4-6.** Histopathological analysis of cholestatic rat livers. Light microscopy of the liver tissue of control and EE-treated animals. (a) Normal liver morphology, (b, c, d) EE-treated animals stained with toluidine blue. Pathological changes of hepatocytes were classified as follows: (b) steatosis (*) and some degree of feathery degeneration (arrowhead) in the central lobular zone (c) abundant intracellular localization of bile pigments (arrowheads), as revealed by light refraction. The pigment granules displayed no autofluorescence; (d) massive feathery degeneration (arrowheads) of hepatocytes and sparsely spread cells displaying some features of apoptosis and necrosis (*); (e, f) ALP histochemistry of liver tissue in EE-treated and control animals. (e) Control liver shows only mild ALP activity on the canalicular hepatocyte membrane at the periphery of hepatic lobule (arrowheads). (f) Except for the sinusoidal pole of hepatocyte membrane (arrow), the EE-treated liver displays ALP activity in canalicular

and basolateral hepatocyte membranes in all zones of hepatic lobules (arrowhead); p = portal area, cv = central vein, bars = 100 μm.

### GM1 histochemistry

In the liver specimens of all control animals, a small amount of reaction product of GM1 ganglioside histochemistry was distributed on both the sinusoidal and canalicular membranes except for the central lobular zone (zone III), where almost no reaction occurred (Fig. 4-7a). In all EE-treated animals, the GM1 ganglioside was nearly uniformly distributed in all lobular zones from the portal areas up to the central veins (Fig. 4-7b). Sinusoidal surfaces of hepatocytes were conspicuously lined with the reaction product, whereas the reaction on canalicular surfaces was rather weak.

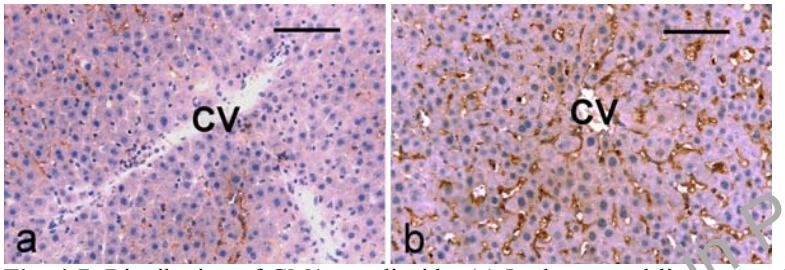


**Fig. 4-7.** Distribution of GM1 ganglioside. (a) In the control liver, a small amount of reaction product occurs in canalicular and sinusoidal hepatocyte membrane in peripheral and intermediate lobular zones (zone I and II) whereas the central zone is nearly free of it. (b) In the EE-treated liver, the reaction product is found in canalicular and sinusoidal hepatocyte membranes in all lobular zones; cv = central vein; bars = 100 μm.

No GM1 staining was observed in both control blank experiments in which either the GM1 was extracted or the cholera toxin was omitted in staining procedure.



### Quantiative study on the distribution of GM1 ganglioside in hepatic lobule

The distribution pattern of GM1 ganglioside in liver sections is demonstrated under low microscopic magnification (Fig. 4-10). The amount of GM1 ganglioside expressed as optical brightness of whole sections showed no significant difference between EE-treated and control groups (Table 4-9).

**Table 4-9.** Densitometric quantification of GM1 ganglioside in control and EE-treated livers.

| | Controls (n = 7) | EE-treated rats (n = 11) | p value |
|---|---|---|---|
| **Mean optical brightness measured in whole sections** | 0.61±0.04 | 0.61±0.04 | 0.923 |
| **Differences of optical brightness between lobular zones I and III** | 0.097 (0.079-0.107) | 0.055 (0.034-0.058) | **0.0035** |

This finding corresponds to the insignificant difference of GM1 concentration determined by biochemical analysis. In contrast to the results obtained in whole sections, the differences of the GM1 content between peripheral and central lobular zones were significantly higher in controls (Table 4-9). The linear regression analysis demonstrated that the higher degree of cholestasis, measured by TBA concentration, correlated well with the lower difference of the GM1 content between lobular zone I and III.