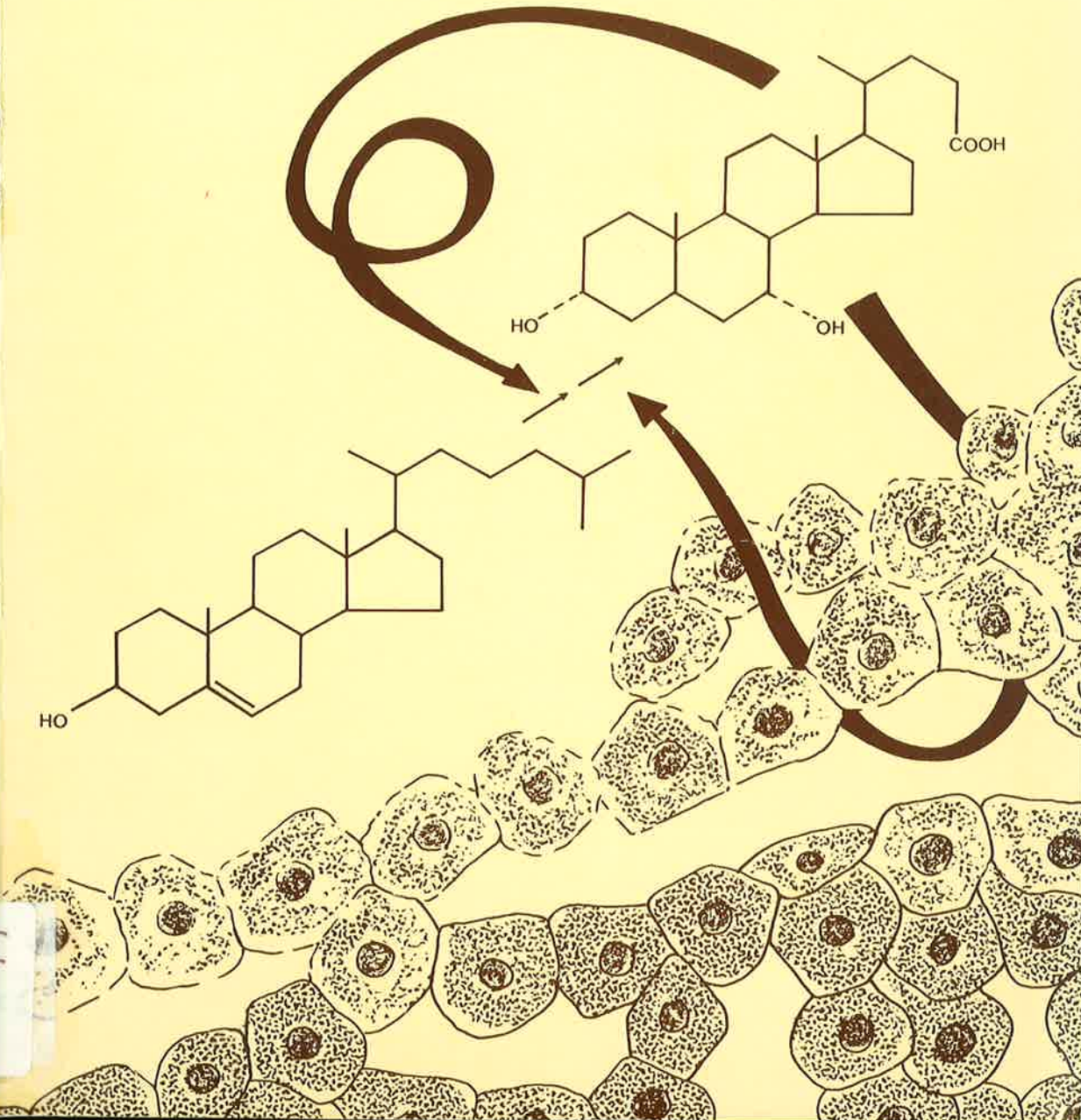
Regulation of bile acid synthesis in cultured hepatocytes

J. Kwekkeboom



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REGULATION OF BILE ACID SYNTHESIS IN CULTURED HEPATOCYTES

REGULATIE VAN GALZUURSYNTHESE IN HEPATOCYTEN IN KWEEK

Proefschrift

ter verkrijging van de graad van Doctor aan de Rijksuniversiteit te Leiden, op gezag van de Rector Magnificus Dr. J.J.M. Beenakker, hoogleraar in de faculteit der Wiskunde en Natuurwetenschappen, volgens het besluit van het college van dekanen te verdedigen op woensdag 11 april 1990 te klokke 15.15 uur

door

Jacob Kwekkeboom

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Stellingen

behorende bij het proefschrift van Jaap Kwekkeboom, getiteld: "Regulation of bile acid synthesis in cultured hepatocytes".

1. "Feedback" remming van galzuursynthese door galzuren geschiedt door een rechtsreeks effekt van galzuren op de leverparenchymcellen.
Dit proefschrift.

2. Bakteriële transformatie van galzuren in de darm is geen onmisbare schakel in de "feedback" regulatie van galzuursynthese.
Dit proefschrift.
Gustafsson, BE, Angelin, B, Einarsson, K en Gustafsson, J (1978) J Lipid Res 19, 972-977.
Gustafsson, BE, Angelin, B, Björkhem, I, Einarsson, K en Gustafsson, J (1981) Lipids 16, 228- 233.

3. Bij onderzoek naar het effekt van lipoproteïnen op galzuursynthese door hepatocyten in vitro, dient rekening te worden gehouden met de mogelijke aanwezigheid van galzuren in de geïsoleerde lipoproteïne frakties.
Kwekkeboom, J et al, niet gepubliceerde resultaten.
Carey, MC (1982) in The liver, Biology and Pathobiology (Arias, IM, Popper, H, Schachter, D en Shafritz, DA, eds) Raven Press, New York, pp 429-466.
Hedenborg, G, Norman, A en Ritzen, A (1988) Scand J Clin Lab Invest 48, 241-245.

4. De relatieve bijdrage van de leverparenchymcellen aan de opname en degradatie van Low Density Lipoproteins door de lever, vertoont bij de mens veel grotere interindividuele variatie dan bij de rat; deze variatie wordt mogelijk bepaald door de voedingstoestand.
Harkes, L en Van Berkel, TJC (1984) Biochem J 224, 21-27.
Nagelkerke, JF, Bakkeren, HF, Kuipers, F, Vonk, RJ en van Berkel, TJC (1986) J Biol Chem 261, 8908-8913.

Kleinherenbrink-Stins, M. Thesis, Leiden 1990.

5. Het is, gezien de concentratie gradiënt van galzuren over de leveracini, onjuist om op basis van galzuurconcentraties in leverhomogenaten uitspraken te doen over intracellulaire galzuurconcentraties in levercellen.
Blitzer, BC en Boyer, JC (1982) Gastroenterology 82, 346-357.
Coleman, R (1987) Biochemical J 244, 249-261.

Jones, AL, Hradek, GT, Renston, RH, Wong, KY, Karlaganis, G en Paumgartner, G (1980) Am J Physiol 238, G233-G237.
Groothuis, GMM, Hardonk, MJ, Keulemans, KPT, Nieuwenhuis, P en Meijer, DKF (1982) Am J Physiol 243, G455-G462.

6. Het is onwaarschijnlijk dat het recent ontdekte LDL receptor gerelateerde eiwit geen andere funkties heeft dan die van chylomicron remnant receptor.
Herz, J, Hamann, U, Rogne, S, Myklebost, O, Gausepohl, H en Stanley, KK (1988) EMBO J 7, 4119-4127.

7. Uit de door Miller, NE gekonstateerde negatieve korrelatie tussen de fraktionele klaringssnelheid van apolipoproteïne B en de leeftijd bij de mens, mag niet zonder meer worden gekonkludeerd dat de LDL receptor aktiviteit in het menselijk lichaam afneemt bij het ouder worden.
Miller, NE, The Lancet 1984, 263-266.

8. De verklaring van Darlington et al voor de door hen gevonden diskrepantie tussen het initiële effekt van monocyt gekonditioneerd medium op de hoeveelheid albumine mRNA en de albumine synthese in HEP 3B2 cellen, namelijk de relatieve stabiliteit van het albumine mRNA, is in strijd met hun eigen waarnemingen.
Darlington, GJ, Wilson, DR en Lachman, LB (1986) J Cell Biology 103, 787-793.

9. De aanname van Stern et al dat de binding van polyclonaal antiserum aan deletie mutanten van weefsel plasminogeen aktivator (tPA) gelijk is aan de binding aan de wild-type vorm is aanvechtbaar met door anderen gepubliceerde gegevens; derhalve zijn hun konklusies m.b.t de specifieke aktiviteiten van tPA mutanten onvoldoende onderbouwd.
Stern, A, Mattes, R, Buckel, P en Weidle, UH (1989) Gene 79, 333-344.

Larsen, GR, Henson, K en Blue, Y (1988) J Biol Chem 263, 1023-1029.

10. De huisvrouw verdient meer waardering in de samenleving.

11. Overheidsdiensten die belast zijn met het kunstbeleid dienen kunstwerken te onderscheiden van kunststukjes en subsidies alleen te bestemmen voor de eerste kategorie.

12. Bedrijven die hun personeelsdienst aanduiden met "human resources department" wekken de indruk onvoldoende oog te hebben voor de verschillen tussen mensen en delfstoffen.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 MOTIVATION OF THE STUDY

Coronary and peripheral vascular diseases are major health problems in Western Society. In 1986, 42% of all deaths in The Netherlands were attributable to these diseases (1). Vascular diseases are mainly caused by atherosclerosis. One of the important risk factors for coronary heart disease morbidity and mortality is an elevated serum cholesterol concentration (2). In blood, cholesterol is transported in lipid-protein complexes, called lipoproteins. These lipoproteins can be divided into four major classes according to their density: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Epidemiological studies have established that LDL cholesterol is the main contributor to the relationship between serum cholesterol concentration and coronary heart disease, while HDL cholesterol levels are inversely related to the incidence of this disease (3,4).

The only quantitatively significant way by which the body can excrete cholesterol is via the bile, either by excretion of the sterol in unchanged form or after its conversion into bile acids. The secretion of cholesterol into bile is coupled to the secretion of bile acids (5). Consequently, remedies which accelarate bile acid synthesis should lead to enhanced removal of cholesterol, in the form of bile acids and of the steroid itself, from the body. Such tools can therefore be expected to be effective in lowering serum cholesterol, and to be valuable for the prevention and/or treatment of atherosclerosis. The validity of this concept has been proven in studies in which bile acid synthesis in man was augmented by interruption of the enterohepatic circulation of bile acids, either by ileal bypass surgery or by treatment with a resin which binds bile acids in the intestine (such as cholestyramine). As a result, plasma LDL levels dropped, while HDL levels remained unchanged (6). A large clinical trial with cholestyramine has shown that this treatment indeed resulted in a reduction of the incidence of coronary heart disease mortality and non-fatal myocardial infarction (7).

However, the magnitude of the effect of cholestyramine on serum LDL level is limited (usually in the range of 20-35% reduction (6,8)) and the drug has a poor patient acceptability. Possible side effects are constipation, heartburn and nausea and the drug is is unpalatable (6,7,8). More detailed knowledge of the cellular mechanisms of the regulation of bile acid synthesis might reveal new possibilities for the development of pharmaca, which accelarate the catabolism of cholesterol to bile

acids. Therefore, a study of the factors which regulate bile acid synthesis at the cellular level was undertaken. For this purpose, monolayer cultures of pig hepatocytes were used.
In the following sections the current knowledge of bile acid formation and its regulation are briefly reviewed and the progress in the use of isolated hepatocytes to study this liver function is discussed.

1.2 BILE FORMATION AND LIVER MORPHOLOGY

Bile acids are synthesized exclusively in the liver and secreted together with cholesterol, phospholipids and electrolytes into bile. The bile is delivered via the hepatic and common bile ducts into the duodenum. In some species (such as man and pig but not the rat), bile is stored in the gallbladder interdigestively and discharged into the intestine postprandially. In the intestine, bile acids play a role in the emulsification and absorption of lipids. The majority of bile acids (93 - 99%) is reabsorbed from the intestine and subsequently transported to the liver via the portal vein (9). The cyclical movement of bile acids from liver to intestine and back is termed the enterohepatic circulation.
The liver is composed of polygonal lobuli (10), with at the corners portal canals consisting of small branches of the portal vein, the hepatic artery and the bile duct (fig. 1). In a lobule, the parenchymal liver cells (the main liver cell type, also denoted as hepatocytes) are arranged in plates which are disposed radially to the terminal branches of the hepatic vein. These plates are interconnected and form a three dimensional network. On either side of the plates, blood is flowing through sinusoids from the portal canal to the central vein (the terminal branch of the hepatic vein). The wall of these sinusoids consists of fenestrated endothelium. In these sinusoids are located Kupffer cells, which belong to the mononuclear phagocyte system, while fatstoring cells are found in the spaces between the sinusoids and the parenchymal cells (the perisinusoidal spaces).
Bile acids are synthesized in the parenchymal cells, and are secreted into bile canaliculi which run between each adjacent pair of hepatocytes. These canaliculi are formed by the membranes of the adjacent parenchymal cells and are sealed off from the sinusoidal blood by tight junctions. They form a network around the parenchymal cells and are connected, via bile ductules and bile ducts, to the hepatic duct.

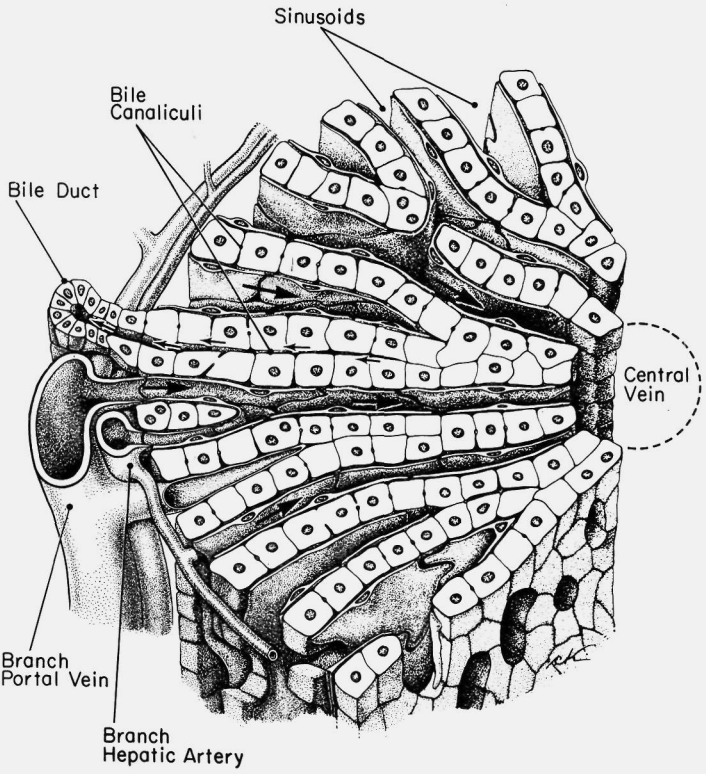

Fig. 1. Diagrammatic representation of the radial disposition of the parenchymal cell plates and sinusoids around the central vein, showing the centripetal blood flow from the branches of the portal vein and hepatic artery to the central vein and the centrifugal flow of bile to the small bile duct (from ref. 10).

1.3 THE BIOSYNTHESIS OF BILE ACIDS

The reaction sequence.

Bile acids are formed by a sequence of metabolic reactions in the liver parenchymal cells from cholesterol. The main pathway for bile acid synthesis in the rat was established by the late 1960's (11) and is depicted in fig. 2. The numbering of the carbon atoms in cholesterol and other steroids is shown in fig. 3. Experimental work in humans in vivo and with preparations from human liver has shown that pathways of bile acid synthesis in man are qualitatively similar to those in rat.

The first step in bile acid formation is the 7α-hydroxylation of cholesterol, which is catalyzed by the enzyme cholesterol 7α-hydroxylase. This enzyme is located in the endoplasmatic reticulum (12). The product of this reaction, 5-cholestene-3β,7α diol (7α-hydroxycholesterol), is converted to 7α-hydroxy-4-cholestene-3-one. Cytosolic oxido-reductases saturate the Δ4-double bond and the 3-oxo group is reduced into a

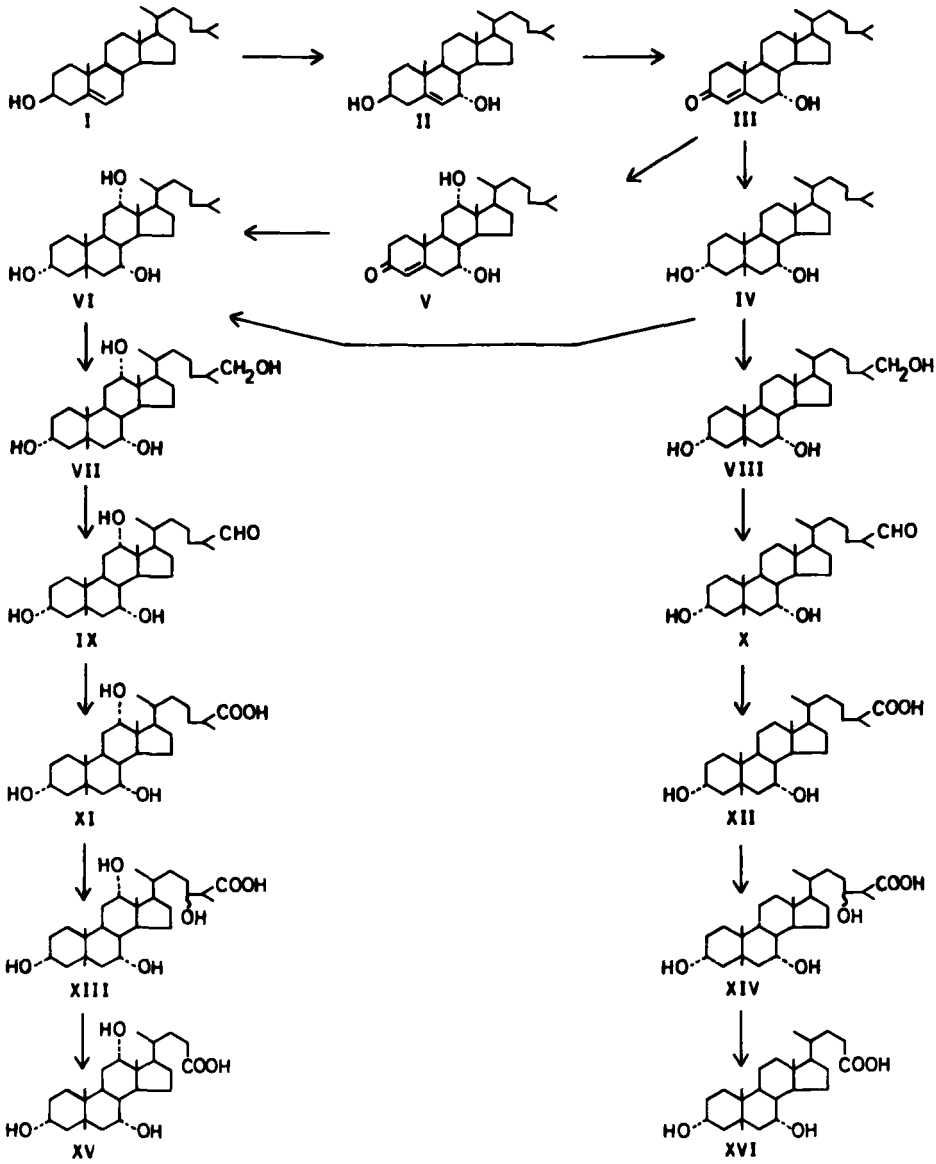

Fig. 2. Major pathways for biosynthesis of primary bile acids. I, cholesterol; II, 5-cholestene-3β,7α-diol; III, 7α-hydroxy-4-cholesten-3-one; IV, 5β-cholestane-3α,7α-diol; V, 7α,12α-dihydroxy-4-cholesten-3-one; VI, 5β-cholestane-3α,7α,12α-triol; VII, 5β-cholestane-3α,7α,12α,26-tetrol; VIII, 5β-cholestane-3α,7α,26-triol; IX, 3α,7α,12α-trihydroxy-5β-cholestan-26-al; X, 3α,7α-dihydroxy-5β-cholestan-26-al; XI, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; XII, 3α,7α-dihydroxy-5β-cholestanoic acid; XIII, 3α,7α,12α,24ϵ-tetrahydroxy-5β-cholestanoic acid; XIV, 3α,7α,24ϵ-trihydroxy-5β-cholestanoic acid; XV, cholic acid; XVI, chenodeoxycholic acid (from ref. 18).

3α-hydroxyl group. In human liver are synthesized the bile acids chenodeoxycholic acid and cholic acid. The latter contains a 12α-hydroxyl group which can be introduced in both 7α-hydroxy-4-cholestene-3-one and 5β-cholestane-3α,7α-diol (fig. 2). Side chain cleavage starts with 26-hydroxylation, which occurs in the mitochondria. The 26-hydroxylated products are oxidized to the corresponding acids and the side chain is degraded by a β-oxidation process, which mainly takes place in the peroxisomes (13-16). This results in the formation of bile acids with 24 C-atoms and in the elimination of propionic acid. Detailed descriptions of all these reactions can be found in recent reviews (17,18).

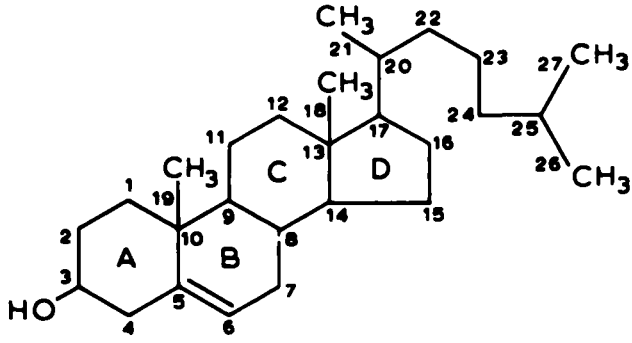

Fig. 3. The numbering of the carbon atoms of cholesterol.

An alternative mechanism for side chain degradation has been proposed by Shefer et al (19), involving 25-hydroxylation and elimination of acetone instead of propionate. Recent investigations on the production of radioactive acetone from [26-^{14}C]cholesterol both in rat and man have, however, led to the conclusion that this pathway accounts for no more than 5% of total bile acid synthesis (20,21).
Furthermore, an alternative pathway has been described involving initial 26-hydroxylation instead of 7α-hydroxylation (22,23). Labelled 26-hydroxycholesterol can indeed be converted into bile acids in rat (24), hamster (24), rabbit (25) and man (26-28), but the relative importance of this pathway has not been definitely established. Based on comparison of the conversion of radiolabelled 26-hydroxy- and 7α-hydroxycholesterol to bile acids (27,28), it is generally thought that it plays under normal conditions only a minor role.

A number of mammalian species synthesize bile acids not found in man. In rat and mouse, α- and β-muricholic acids are formed by 6β-hydroxylation of chenodeoxycholic acid (18). The pig synthesizes hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid) by 6α-hydroxylation of chenodeoxycholic acid. (29,30).
Prior to their secretion into bile, the carboxyl group of the bile acids is conjugated with either glycine or taurine. Additional forms of bile acid conjugation are sulfation (of the hydroxylgroups) (31) and glucuronidation (of the hydroxylgroups and possibly also of the carboxylgroup (32,33)). These conjugation reactions are of minor importance under non-pathological conditions.
The bile acids which are synthesized in the liver are termed primary bile acids. By the action of intestinal bacteria, secondary bile acids are formed from the primary ones. The major bacterial transformation yielding secondary bile acids is 7α-dehydroxylation, which gives deoxycholic acid from cholic acid and lithocholic acid from chenodeoxycholic acid. Besides, bile acids are in part deconjugated by intestinal bacteria.

The enzyme cholesterol 7α-hydroxylase.
Cholesterol 7α-hydroxylase is considered to be the main site of regulation of bile acid synthesis (see paragraph 1.4). This enzyme belongs to the cytochrome P-450 superfamily and is a mixed function monooxygenase (34). These enzymes catalyze oxidative reactions in which one atom of a molecule of O_2 is introduced into the substrate, the other atom being reduced to water (35). Cholesterol 7α-hydroxylase uses NADPH as electron donor; the electrons are transferred to the cytochrome P-450 by NADPH-cytochrome P-450 reductase (34). The enzyme has been purified to near homogeneity (36,37). The apparent molecular weight upon polyacrylamide gel electrophoresis was reported to be 52-53 kD. Purification of the enzyme was, however, hampered by considerable loss of activity (36). During the time the study described in this thesis was performed, no antibodies against cholesterol 7α-hydroxylase were available, so that the enzyme could only be measured by activity determinations. Likewise, no DNA coding for the enzyme was cloned. Only very recently, a specific polyclonal antibody against rat cholesterol 7α-hydroxylase has been obtained (38). By the use of this antibody preparation, a cDNA clone encoding for the enzyme was isolated from a cDNA library prepared from liver poly (A^+)RNA of cholestyramine-treated rats (38). The isolated clone contained a 1509 base pairs open reading frame encoding for 503 amino acid residues (Mr = 56880). The low similarity of the amino acid sequence, as deduced from the nucleotide sequence, to other P-450's, indicates that the enzyme constitues a novel cytochrome P-450 family.

The substrate for bile acid synthesis.
The possible sources of substrate for bile acid synthesis include cholesterol which is

newly synthesized in the hepatocyte, and pre-existing cholesterol delivered by lipoproteins to the hepatocyte. Based on experiments with radioactive precursors and inhibitors of cholesterol synthesis, it was concluded that in rats with a bile fistula 50 to 70% of the bile acids are formed from de novo synthesized cholesterol (39,40). However, this is probably not the case in animals with an intact enterohepatic circulation, since shortly after creation of a bile-fistula, the greater part of bile acids originates from pre-existing cholesterol (41,42). Moreover, the opposite acinar localization of the rate-limiting enzyme of cholesterol synthesis, HMG-CoA reductase (periportal) (43) and the first enzyme in the bile acid synthesis pathway, cholesterol 7α-hydroxylase (perivenous) (44) in rats with an intact enterohepatic circulation, probably restricts direct linkage between the two processes to a small zone of the acinus.

Although, in man at least, acceleration of bile acid synthesis leads to a lowering of plasma LDL cholesterol level (as described above), the nature of the coupling between lipoprotein uptake by the liver and bile acid synthesis is as yet unresolved. Studies in which human (45,46) and rat (47) LDL was labelled in the cholesterol ester moiety, have revealed that in the rat uptake of LDL is not efficiently coupled to bile acid secretion. Excretion of the label into bile was slow as compared to hepatic uptake. However, induction of LDL receptors on the parenchymal cells by administration of ethinyl-oestradiol, led to an accelerated appearance of radioactivity in biliary bile acids (45,46). Likewise, studies in man suggested that lipoprotein cholesterol esters make only a small contribution to bile acid synthesis (48,49). Studies on the fate of labelled unesterified cholesterol in lipoproteins were hampered by rapid exchange of the unesterified cholesterol between lipoprotein classes and membrane surfaces, but suggested that in both man and rat HDL unesterified cholesterol may be used preferentially to LDL unesterified cholesterol for the formation of bile acids (50,51).

1.4 THE REGULATION OF BILE ACID SYNTHESIS

Feedback regulation.

The synthesis of bile acids is in some way inhibited as a consequence of the enterohepatic circulation of bile acids. In the 1950's it was already recognized that in rats diversion of bile via a bile fistula leads to a marked stimulation of the rate of bile acid synthesis (34). Reported increases vary from 2 to 13 times (17,52-54). From experiments showing that bile acid synthesis in bile-diverted rats could be restored to a normal rate by intraduodenal (52) and intravenous (55) infusions of taurocholate, it was concluded that the bile acids returning to the liver are responsible for the

inhibition in animals with intact enterohepatic circulation.

By three different approaches it was shown that the 7α-hydroxylation of cholesterol is the major site of feedback regulation:

a. Biliary drainage and cholestyramine treatment were found to stimulate cholesterol 7α-hydroxylase activity 5 to 8 fold, while the activities of enzymes catalyzing subsequent steps in bile acid formation were either much less or completely not affected (56,57).
b. Incorporation of radiolabelled mevalonate and cholesterol, but not of 7α-hydroxycholesterol, into bile acids was increased in perfused livers of cholestyramine treated rabbits, as compared with untreated rabbits (58).
c. Conversion of radiolabelled mevalonate and cholesterol, but not of 7α-hydroxycholesterol, into bile acids in bile-diverted rats, was inhibited by intraduodenal infusions of taurocholate (59).

It was concluded that no steps beyond the 7α-hydroxylation of cholesterol are subject to feedback inhibition by bile acids. These experiments do, however, leave open the possibility that HMG-CoA reductase, which is also inhibited by bile acids (17,60) may be a secondary regulation site of bile acid synthesis. However, the different acinar localization of HMG-CoA reductase and cholesterol 7α-hydroxylase, as mentioned above, seems to exclude that bile acids regulate bile acid synthesis mainly by modulation of HMG-CoA reductase activity.

In man too, bile acid synthesis is thought to be regulated by a feedback suppression (61,62), which is exerted on cholesterol 7α-hydroxylase (63).

The concept of feedback control of bile acid synthesis by bile acids returning to the liver via the portal vein was generally accepted, until it was reported by three different research groups that bile acids, in concentrations even exceeding those found in portal blood, failed to inhibit bile acid synthesis in rat hepatocytes in suspension (64) and monolayer culture (65,66). This prompted sevaral research groups to re-evaluate the effects of intravenous and intraduodenal bile acid infusions on bile acid synthesis in bile-diverted rats. These experiments were for the greater part performed with taurocholic acid and are summarized in table 1. The results are quite puzzling. Some authors reported suppression of bile acid synthesis upon intraduodenal or intravenous taurocholate infusions, while others found no effect. The reasons for these discrepancies are not clear. Negative results cannot be explained by too low bile acid infusion rates, since absence of inhibition was also observed in experiments with infusion rates which were within the range of physiological bile acid fluxes over the liver. The differences in results may be related to the fact that it is technically difficult to measure endogenous bile acid synthesis in the presence of an infusion of exogenous bile acids. In such experiments endogenous bile acid synthesis may account for only 3 - 20% of the total biliary bile acid output (67-69). Furthermore, bile acids

Table 1. Effect of intravenous and intraduodenal bile acid infusions on bile acid synthesis in the rat.

first author	infusion rate (μmol/kg/h)	infused bile acid	inhibition
		intraduodenal infusions	
Shefer (52)	180	taurocholic acid	+
Shefer (59)	210-260	taurocholic acid	+
Davis (67)	200	taurocholic acid	-
Spady (68)	100-300	taurocholic acid	+
Stange (69)	1-300	taurocholic acid	-
Stange (70)	1-300	taurocholic acid	-
	100	taurochenodeoxycholic acid	-
	15-50	(tauro)deoxycholic acid	+
		intravenous infusions	
Pries (55)	30-100	taurocholic acid	+
Davis (67)	110-250	taurocholic acid	-
Duane (71)	100	taurocholic acid	-
Spady (68)	100-300	taurocholic acid	+
Heuman (72)	240-360	taurocholic acid	+
Stange (69)	0-300	taurocholic acid	-
physiological bile acid flux over liver (53,72,74)	250-480		

infused into another vein than the portal vein, may have, because of dilution in the circulation, less pronounced effects on the liver than bile acids reaching the liver only via the portal vein. This possible explanation is supported by a report on the effect of construction of a porto-caval anastomosis. This treatment resulted in an increase of cholesterol 7α-hydroxylase activity (75). Finally, it has been suggested that the actual regulators of bile acid synthesis are not the primary bile acids (such as taurocholic acid), but secondary bile acids. Recently, Stange et al (70) reported that in the rat intraduodenal infusion of deoxycholic and taurodeoxycholic acids, even at low rates (15 - 50 μmol/kg/h), significantly inhibited bile acid synthesis, while infusion of taurocholic acid had no effect in his experiments. Furthermore, Hall et al (73) found that in the rabbit, oral administration of cholic or deoxycholic acids failed to down-regulate bile acid synthesis, while intravenous injection of lithocholic or 3β-hydroxy-5-cholenoic acids led to lower endogenous bile acid synthesis.

Thus, although there is no doubt that diversion of bile components by bile duct canulation or administration of cholestyramine results in increased bile acid synthesis, it is presently a matter of discussion whether the inhibition with intact enterohepatic circulation is exerted by a direct effect of bile acids on the liver or whether this inhibitory effect is mediated by other factors.

Besides a dispute on the mechanism of feedback inhibition, quite different hypotheses are found in the literature with regard to the relative effectiveness of bile acids in regulating bile acid synthesis. It has been suggested that secondary bile acids are the actual regulators (as mentioned above). Moreover, it has been stated that the hydrophobicity of bile acids is determining their inhibitory potential (72,76). This hypothesis was supported by data showing that oral administration of bile acids which are relatively hydrophobic, as defined by their mobility in reverse phase high-performance liquid chromatography (cholic, chenodexoycholic and deoxycholic acids), resulted in an inhibition of cholesterol 7α-hydroxylase in rats, while the enzyme was not suppressed upon administration of more hydrophilic bile acids (ursocholic, ursodeoxycholic, hyocholic and hyodeoxycholic acids) (76).

Diurnal variation of bile acid synthesis.

In the rat, the rate of bile acid synthesis and the activity of cholesterol 7α-hydroxylase varies diurnally, reaching a maximum during the night and falling to a minimum during the day (34). In man, bile acid synthesis does not peak during the night but during the day (9 a.m.), probably related to an opposite waking and feeding pattern in man as compared to the rat (77). The diurnal variation persists after bile diversion or cholestyramine administration in the rat (78), and after oral bile acid administration in man (79) and thus seems to be regulated independently from feedback suppression. It has been proposed that the diurnal rhythm of bile acid synthesis is regulated by glucocorticoids, since the observed variation follows the circadian pattern of plasma corticosterone levels in the rat (34). Three authors have indeed reported (partial or total) loss of the diurnal rhythm of cholesterol 7α-hydroxylase activity in rats after bilateral adrenalectomy (34) but this could not be confirmed by other investigators measuring bile acid synthesis in adrenalectomized rats with a bile fistula (80,81).

Regulation by hormones.

Insight into the role of the endocrinological system in the regulation of bile acid synthesis is limited. As mentioned above, there is some evidence suggesting that glucocorticoids may play a role in the regulation of the diurnal rhythm of cholesterol 7α-hydroxylase. Administration of corticosteroids to rats did indeed lead to a rise of cholesterol 7α-hydroxylase activity (34,81). Recently, Princen et al (82) demonstrated that glucocorticoids stimulate bile acid synthesis and cholesterol 7α-hydroxylase acitivity in cultured rat hepatocytes. Furthermore, it has been suggested that these hormones may play a role in the postnatal development of bile acid synthesis. Cholesterol 7α-hydroxylase activity is low in suckling rats. It increases after weaning and the diurnal variation is established in this period. This developmental pattern

coincides with the postnatal development of a diurnal rhythm in glucocorticoid secretion (83).
It has been reported that thyroxine administration stimulates cholesterol 7α-hydroxylase in rats (34). However, the rate of bile acid synthesis is normal in hypothyroid (84,85) and even diminished in hyperthyroid humans (86). The activity of cholesterol 7α-hydroxylase was found to be increased in diabetic rats and humans and to be returned to normal upon insulin administration (87,88). Conversely, glucagon administration resulted in a higher rate of bile acid synthesis in rats (89) and this hormone slightly stimulated bile acid synthesis in suspensions of rat hepatocytes (90). Pharmacological doses of ethinyl-oestradiol were found to inhibit bile acid synthesis and cholesterol 7α-hydroxylase activity in rats (91), but this hormone had no direct effect on bile acid synthesis in cultured fat hepatocytes (82).

Regulation by substrate availability.
There is some evidence that delivery of lipoprotein cholesterol to the liver can induce bile acid synthesis. Supplementation of cholesterol to the diet has been found to stimulate bile acid synthesis (34) and cholesterol 7α-hydroxylase activity (78, 92) in the rat. Furthermore, bile acid synthesis in cultured rat hepatocytes was reported to be susceptible to supply of lipoprotein cholesterol. This is discussed more extensively in paragraph 1.5. The effect of dietary cholesterol on bile acid synthesis in man is not yet conclusively determined. Both stimulation (93) and absence of effect (34,94) have been reported.
De novo synthesis of cholesterol may also influence bile acid synthesis in the rat. Inhibition of cholesterol synthesis leads to reduced bile acid synthesis (40,67) and cholesterol 7α-hydroxylase activity (95). Conversely, infusion of mevalonic acid stimulates cholesterol 7α-hydroxylase activity (96). Similar results were obtained in experiments using rat hepatocytes in suspension and monolayer culture (see paragraph 1.5).
The idea of regulation of bile acid synthesis by cholesterol supply is an attractive one from the viewpoint that the hepatocyte controls cholesterol homeostasis in the body, but at present the insight in its mechanism and physiological significance is limited.

Molecular basis of the regulation of cholesterol 7α-hydroxylase activity.
Since the increase of activity of cholesterol 7α-hydroxylase after interruption of the enterohepatic circulation and during the dark period could be prevented by actinomycin D (34,97), it is thought that these changes are mediated by regulation of synthesis of the enzyme. Due to the fact that specific antibodies against the enzyme were not available until very recently, this idea has not been investigated by enzyme protein determinations. Very recently, it was shown by the use of an antibody directed

against another cytochrome P-450 species, but cross-reacting with cholesterol 7α-hydroxylase, that cholestyramine treatment of rats resulted in the induction of a 54 kD protein in liver microsomes (98). On the basis of the molecular weight of the induced protein, it was concluded that feedback regulation indeed involves modulation of the amount of cholesterol 7α-hydroxylase protein.

Quite another mechanism of feedback regulation was proposed by Davis et al (99) to account for the absence of a direct effect of infused taurocholate on bile acid synthesis in bile-diverted rats in their experiments. They suggested that bile acids regulate bile acid synthesis by modulation of availability of substrate for cholesterol 7α-hydroxylase. They reasoned as follows: since bile acids are required for intestinal cholesterol absorption, their presence in the intestine promotes production of chylomicrons, which in turn inhibit HMG-CoA reductase activity in the liver. This leads to a shortage of newly synthesized cholesterol, which is the preferred substrate for bile acid synthesis. This idea was substantiated by the observation of a significant correlation between liver microsomal unesterified cholesterol concentrations and cholesterol 7α-hydroxylase activities in control and bile-diverted rats (99). However, these observations could not be confirmed by Einarsson et al (100), who found no changes in microsomal cholesterol concentrations upon bile diversion. Moreover, Björkhem et al (101) reported that the increase of cholesterol 7α-hydroxylase activity after biliary drainage was not affected by infusion of cholesterol-enriched Intralipid, which depressed HMG-CoA reductase activity. Apparently, upregulation of bile acid synthesis after bile diversion is not simply a consequence of increased supply of newly synthesized cholesterol to cholesterol 7α-hydroxylase, but rather mediated by induction of the amount of cholesterol 7α-hydroxylase protein.

A similar dispute is found in the literature with regard to the mechanism by which lipoprotein cholesterol modulates bile acid synthesis. Some authors feel that this regulation is a consequence of differences in supply of substrate to cholesterol 7α-hydroxylase (78,102,103) while others argue that modulation of the amount of enzyme plays a role (92,101).

As a third mechanism it has been proposed that modulation of cholesterol 7α-hydroxylase activity can be brought about by physico-chemical changes in the membrane in which the enzyme is embedded. Such a mechanism was suggested to play a role in the non-competitive inhibition of cholesterol 7α-hydroxylase activity by ethinyl oestradiol (91).

Finally, there is some evidence from studies with microsomal preparations and partially purified enzyme preparations that the activity of cholesterol 7α-hydroxylase can be modulated by alterations in its phosphorylation state (104-106). Other investigators could, however, not confirm these observations (107-109) and it is not known whether this mechanism plays any role in the intact parenchymal cell.

1.5 THE USE OF ISOLATED HEPATOCYTES FOR THE STUDY OF BILE ACID SYNTHESIS

Since 1969, when Berry and Friend (110) published a method for the isolation of rat liver parenchymal cells in high yield, many hepatocyte functions have been studied in isolated hepatocyte preparations. For the study of bile acid synthesis such an in vitro system has potentially several advantages. Firstly, bile acid production by the hepatocytes can be determined directly, which is difficult in vivo, due to interference of bile acids from the enterohepatic circulation. Secondly, the direct effects of regulatory agents on the parenchymal cell can be examined in isolation from the influences of other cells or factors. Thirdly, many experiments can be performed with cells from one animal, avoiding interfering interindividual differences. A disadvantage is the loss of tissue architecture which forms in the case of hepatocytes the basis for the segregation of the biliary and the sinusoidal compartments.

In 1979, Botham et al (111) began a study of cholesterol 7α-hydroxylase activity in isolated rat hepatocytes in suspension culture. The results were not encouraging: although activity of the enzyme in freshly isolated hepatocytes was comparable with that in liver microsomes, it decreased to 50% of its original value within 2 hours of incubation.

Subsequently, a number of research groups (112,113), including our own (114), determined bile acid synthesis in suspensions of rat hepatocytes and found that production rates during the first 1 to 3 hours were within the range of those in vivo in the rat, but declined rapidly afterwards. However, changes induced by in vivo manipulations of bile acid synthesis were retained in the isolated hepatocytes. Thus, interruption of the enterohepatic circulation in the donor rats resulted in higher bile acid production in the suspension cultures (64,112-115) and production rates in hepatocytes isolated at different points in the diurnal cycle varied according to the circadian variation in vivo (64,113,115).

Kempen et al (116) showed that in hepatocytes, isolated from cholestyramine treated rats, bile acids were mainly synthesized from pre-existing cholesterol during the first hour of incubation, while during the second and third hour of incubation newly synthesized cholesterol became a quantitatively significant substrate.

Bile acid synthesis and cholesterol 7α-hydroxylase activity in these short-term hepatocyte suspensions were not affected by addition of bile acids to the incubation medium (64,111). On the other hand, bile acid synthesis was found to be slightly stimulated by dibutyryl cAMP (117,118). In response to glucagon, intracellular cAMP level increased and this rise correlated significantly with the increase in bile acid synthesis (90). Therefore, it was suggested that cAMP might act as a second messenger in the stimulation of bile acid synthesis by glucagon.

Since parenchymal liver cells in suspension are short-lived, this experimental system can be only used for study of rapid regulatory phenomena. To investigate long-term regulatory mechanisms, such as those resulting in changes of transcription or translation rate, monolayer cultures in which hepatocytes can be maintained for several days, are theoretically more suitable. Unfortunately, published rates of bile acid synthesis in hepatocytes isolated from untreated rats (65,119,120), and cultured in serum-free media, appeared to account for only 3 to 8% of the in vivo bile acid synthesis rates in the rat (see chapter 4, table IV). Hepatocytes isolated from cholestyramine treated rats synthesized, in monolayer culture, bile acids at 3 to 10 fold higher rates (65,120,121). Cholesterol 7α-hydroxylase acitvities in microsomes isolated from these cultured parenchymal cells (121) were, however, reported to be about 50 times lower than the activity of the enzyme in microsomes directly prepared from livers of cholestyramine treated rats (122). Thus under the applied culture conditions, rat hepatocytes apparently lose a great part of their original capacity to synthesize bile acids. Probably, this phenomenon has to do with the cytochrome P-450 nature of cholesterol 7α-hydroxylase. Loss of individual and total cytochrome P-450 activities is a well known problem in the use of cultured hepatocytes for the study of cytochrome P-450 enzymes (123).

Two research groups reported that bile acid synthesis in their rat hepatocyte culture systems was not suppressed by exogenous bile acids (65,66), thereby casting doubt upon the concept of feedback inhibition of bile acid synthesis. On the other hand, bile acid synthesis in cultured rat hepatocytes was found to be susceptible to supply of lipoprotein cholesterol and manipulation of de novo cholesterol synthesis. Stimulation was observed after addition of mevalonic acid (119, 124), a lipoprotein fraction from cholesterol fed rats containing VLDL and chylomicron remnants (119), apo E rich HDL (125) and HDL2 (126). Administration of an inhibitor of HMG-CoA reductase led to decreased bile acid synthesis (65,119).

Recently, Princen et al (82) showed that bile acid synthesis in cultured rat hepatocytes was induced by glucocorticoids. These hormones specifically stimulated cholesterol 7α-hydroxylase activity in the cells. Furthermore, Princen et al initiated the use of rat hepatocyte cultures for investigation of the effects of clinically used drugs on bile acid synthesis; bile acid synthesis in cultured rat hepatocytes was found to be inhibited by the antimycoticum ketoconazole (127).

1.6 INTENT OF THIS STUDY

The current knowledge of the regulation of bile acid synthesis, as summarized in the previous paragraphs, points to bile acids, hormones and supply of cholesterol (either by lipoproteins or by de novo synthesis in the hepatocyte) as possible factors

regulating bile acid synthesis. However, the present insights in the regulation by hormones and cholesterol supply are limited and with regard to feedback regulation a lot of conflicting data and hypotheses are found in the literature. It is particularly unclear which of these factors affect bile acid synthesis by a direct attack on the liver parenchymal cells. Therefore, in 1980, in the Gaubius Institute a research programme was initiated aimed to investigate at the cellular level which factors are able to modulate bile acid synthesis directly. For that purpose, suspension and monolayer cultures of liver parenchymal cells were used. When such regulation factors have been identified, the availability of in vitro systems may make it possible to investigate subsequently the molecular mechanisms of the regulation processes. Ultimately, the in vitro sytems can be used for the development and screening of agents which mimic the effects of stimulatory or antagonize the effects of inhibitory factors. Such agents may have the potential of lowering serum cholesterol.

In the study presented in this thesis, as an alternative for rat hepatocytes, the suitability of cultured pig hepatocytes for investigating the regulation of bile acid synthesis was examined. Particularly, for the study of the link between lipoprotein metabolism and bile acid synthesis, pig hepatocytes might offer a model which properties correspond better with those of the human system than rat hepatocytes do. Firstly, as in man, the plasma LDL level in swine decreases after treatment with cholestyramine (128). This does not occur in the rat (129). Moreover, both in pig and man LDL is a major lipoprotein species, while it is only present in a low amount in the rat (130). Thirdly, hepatocytes from neonatal pigs in monolayer culture, have been shown to express receptor mediated LDL uptake and degradation (131,132) comparable with that observed in cultured human hepatocytes (133-135).

The work described in this thesis was concentrated on the following subjects:

1. The development of a in vitro system of cultured pig hepatocytes, suitable for the study of regulation of bile acid synthesis and cholesterol 7α-hydroxylase. Attention had to be paid to the maintenance of the activity of cholesterol 7α-hydroxylase under the in vitro conditions, since published data pointed to a rapid loss of activity of this enzyme in cultured hepatocytes.
2. The establishment of sensitive methods for the determination of the mass of bile acids produced by cultured hepatocytes and for the measurement of the activity of cholesterol 7α-hydroxylase in cultured hepatocytes.
3. The study of feedback regulation of bile acid synthesis in the cultured hepatocytes. Particulary, the following questions were studied: Do bile acids have a suppressive effect on bile acid synthesis in cultured pig hepatocytes? If yes, which is the site of inhibition? Are there any differences in the relative effectiveness of bile acids in regulating bile acid synthesis and can a relation be found between bile acid structure and the efficacy of inhibition?

REFERENCES

1. Centraal Bureau voor de Statistiek (1988). Statistisch Zakboek. Staatsuitgeverij, pp 94.
2. Martin, MJ, Hulley SB, Browner, WS, Kuller, LH and Wentworth, D (1986) The Lancet, 933-936.
3. Rhoads, GG, Gulbrandsen, CL and Kagan, A (1976) New Engl J Med 294, 293-298.
4. Gordon, T, Castelli, WP, Hjortland, MC, Kannel, WB and Dawber, TR (1977) Am J Med 62, 707-714.
5. Turley, SD, Dietschy, JM (1982) in The Liver, biology and pathobiology (Arias, IM, Popper, H, Schachter, D and Schafritz, DA, eds) pp 467-492, Raven Press, New York.
6. Packard, CJ and Shepherd, J (1982) J Lipid Res 23, 1081-1098.
7. The Lipid Research Clinics Coronary Primary Prevention Trial Results (1984) J Am Med Ass 251, 351-364.
8. Brown, MS and Goldstein, JL (1985) in The pharmacological basis of therapeutics (Gilman, AF, Goodman, LS, Rall, TW and Murad, F, eds) pp 827-845, Macmillan Publishing Company, New York.
9. Carey, MC (1982) in The Liver, biology and pathobiology (Arias IM, Popper, H, Schachter, D and Schafritz, DA, eds) pp 429-466, Raven Press, New York.
10. Bloom, W and Fawcett, DW (1975) A textbook of Histology, WB Saunders Company, Philadelphia, USA.
11. Danielsson, H, Einarsson, K (1969) in The biological basis of medicine (Bitten, EE and Bittar, N, eds) pp 279-314, Acad. Press.
12. Mitropoulos, KA, Venkatesan, S, Balasubramaniam, S and Peters, TJ (1978) Eur J Biochem 82, 419-429.
13. Kase, F, Björkhem, I and Pedersen JI (1983) J Lipid Res. 24, 1560-1567
14. Prydz, K, Kase, BF, Björkhem, I and Pedersen, JI (1986) J Lipid Res 27, 622-628.
15. Kase, BF, Prydz K, Björkhem, I and Pedersen, JI (1986) Biochim Biophys Acta 877, 37-42.
16. Kase, BF, Pedersen, JI, Strandvik, B and Björkhem, I (1985) J Clin Invest 76, 2393-2402.
17. Björkhem, I (1985) in Sterols and Bile Acids (Danielsson, H and Sjövall, J, eds) pp 231-278, Elsevier Science Publishers, Amsterdam.
18. Danielsson, H and Wikvall, K (1986) in Cholesterol 7α-hydroxylase (7α-monooxygenase) (Fears, R and Sabine, JR, eds) pp 9-19, CRC Press, Boca Raton, USA.
19. Shefer, S, Cheng, FW, Dayal, B, Hauser, S, Tint, GS, Salen, G and Mosbach, EH (1976) J Clin Invest 57, 897-903.
20. Duane, WC, Björkhem, I, Hamilton, JN and Mueller, SM (1988) Hepatology 8, 613-618.
21. Duane, WC, Pooler, PA and Hamilton, JN (1988) J Clin Invest 82, 82-85.
22. Mitropoulos, KA and Myant, NB (1967) Biochem J 103, 472-479.
23. Mitropoulos, KA, Avery, MD, Myant, NB and Gibbons, GF (1972) Biochem J 130, 363-371.
24. Wachtel, N, Emerman, S and Javitt, NB (1968) J Biol Chem 243, 5207-5212.
25. Ayaki, Y, Kok, E and Javitt, NB (1989) J Biol Chem 264, 3818-3821.
26. Anderson, KE, Kok, E and Javitt, NB (1972) J Clin Invest 51, 112-117.
27. Swell, L, Gustafsson, J, Schwartz, CC, Halloran, LG, Danielsson, H and Vlahcevic, ZR (1980) J Lipid Res 21, 455-466.
28. Goldman, M, Vlahcevic, ZR, Schwartz, CC, Gustafsson, J and Swell, L (1982) Hepatology 2, 59-66.
29. Bergström, S, Danielsson, H and Göransson, A (1959) Acta Chem Scand 13, 776-783.
30. Boström, H (1986) J Lipid Res 27, 807-812.
31. Palmer, RH (1967) Proc Natl Acad Sci USA 58, 1047-1050.
32. Radominska-Pyrek, A, Zimniak P, Chari, M, Golunski E, Lester, R and Pyrek, JS (1986) J Lipid Res 27, 89-101.
33. Zimniak, P, Radominska A, Zimniak, M and Lester, R (1988) J Lipid Res 29, 183-190.
34. Myant, NB and Mitropoulos, KA (1977) J Lipid Res 18, 135-152.
35. Nebert, DW and Gonzalez, FJ (1987) Ann Rev Biochem 56, 945-993.
36. Hansson, R and Wikvall, K (1986) in Cholesterol 7α-hydroxylase (7α-Monooxygenase) (Fears, F and Sabine, JR, eds) CRC Press, Boca Raton, Florida, USA pp 51-66.
37. Ogishima, T, Deguchi, S and Okuda, K (1987) J Biol Chem 262, 7646-7650.
38. Noshiro, M, Nishimoto, M, Morohashi, K and Okuda, K (1989) FEBS Lett. 257, 97-100.
39. Björkhem, I and Lewenhaupt, A (1979) J Biol Chem 254, 5252-5256.
40. Kempen, HJM, de Lange, J, Vos-van Holstein, MPM, van Wachem, P, Havinga, R and Vonk, RJ (1984) Biochim Biophys Acta 794, 435-443.
41. Long, TT, Jakoi, L, Stevens, R and Quarfordt, S (1978) J Lipid Res 19, 873-878.

42. Stange, EF, Spady, DK and Dietschy, JM (1985) in Enterohepatic circulation of bile acids and sterol metabolism (Paumgartner, G, Stiehl, A and Gerok, W, eds) pp 29-36, MTP Press Ltd, Lancaster.
43. Singer, II, Kawka, DW, Kazazis, DM, Alberts, AW, Chen, JS, Huff, JW and Ness, GC (1984) Proc Natl Acad Sci USA 81, 5556-5560.
44. Ugele, B, Kempen, HJM, Gebhart, P, Meier, P, Burger, HJ and Princen, HMG (1989) in Trends in bile acid research (Paumgartner, G, Stiehl, A and Gerok, W, eds) pp 53-62, Kluwer Academic Publishers, Lancaster.
45. Nagelkerke, JF, Bakkeren, HF, Kuipers, F, Vonk, RJ and van Berkel, TJC (1986). J Biol Chem 261, 8908-8913.
46. Kuipers, F, Nagelkerke,JF, Bakkeren, H, Havinga, R, van Berkel, TJC, and Vonk, RJ (1989) Biochem J 257, 699-704.
47. Bhattacharya, S, Balasubramaniam, S and Simons, LA (1986) Biochim Biophys Acta 876, 413-416.
48. Schwartz, CC, Berman, M, Vlahcevic, ZR, Halloran, LG, Gregrory, DH and Swell, L (1978) J Clin Invest 61, 408-423.
49. Schwartz, CC, Vlahcevic, ZR, Halloran, LG and Swell, L (1981) Biochim Biophys Acta 663, 143-162.
50. Halloran, LG, Schwartz, CC, Vlahcevic, ZR, Nisman RM and Swell, L (1978) Surgery 84, 1-7.
51. Bravo, E, Cantafora, A and Argiolas, L (1989) Biochim Biophys Acta 1003, 315-320.
52. Shefer, S, Hauser, S, Bekersky, I and Mosbach, EH (1969) J Lipid Res 10, 646-655.
53. Kuipers, F, Dijkstra, R, Havinga, R, van Asselt, W and Vonk, RJ (1985) Gastroenterology 88, 403-411.
54. Dowling, RH, Mack, E, Small, DM and Picott, J (1970). J Clin Invest 49, 232-242.
55. Pries, JM, Gustafson, A, Wiegand, D and Duane WC (1983) J Lipid Res 24, 141-146.
56. Danielsson, H, Einarsson, K and Johansson, G (1967) Eur J Biochem 2, 44-49.
57. Johansson, G (1970) Eur J Biochem 17, 292-295.
58. Mosbach, EH Rotschild, MA, Bekersky, I, Oratz, M and Mongelli, J (1971) J Clin Invest 50, 1720-1730.
59. Shefer, S, Hauser, S, Bekersky, I and Mosbach, EH (1970) J Lipid Res 11, 404-411.
60. Shefer, S, Hauser, S, Lapar, V and Mosbach, EH (1973). J Lipid Res. 14, 573-580.
61. Einarsson, K, Hellström, K and Kallner, M (1973) Metabolism 12, 1477-1483.
62. Danziger, RG, Hofmann, AF, Thistle, J and Schoenfield, LJ (1973) J Clin Invest 52, 2809-2821.
63. Einarsson, K, Angelin, B, Ewerth, S, Nilsell, K and Björkhem, I (1986) J Lipid Res 27, 82-88.
64. Botham, KM, Lawson, ME, Beckett, GJ, Percy-Robb, IW and Boyd, GS (1981) Biochim Biophys Acta 666, 238-245.
65. Davis, RA, Highsmith, WE, Malone-McNeal, M, Archambault Schexnayder, J and Kuan, JCW (1983) J Biol Chem 258, 4079-4082.
66. Kubaska, WM, Gurley, EC, Hylemon, PB, Guzelian, PS and Vlahcevic, ZR (1985) J Biol Chem 260, 13459-13463.
67. Davis, RA, Musso, CA, Malone-McNeal, M, Lattier, GR, Hyde, PM, Archambault-Schexnayder, J and Straka, M (1988) J Lipid Res 29, 202-211.
68. Spady, DK and Dietschy, JM (1987) in Bile Acids and the Liver (Paumgartner, G, Stiehl, A and Gerok, W, eds) pp 15-24 MTP Press Ltd, Lancaster.
69. Stange, EF, Scheibner, J, Lutz, C and Ditschuneit, H (1988) Hepatology 8, 879-886.
70. Stange, EF, Scheibner, J and Ditschuneit, H (1989) J Clin Invest 84, 173-180.
71. Duane, WC, McHale, AP and Hamilton, JN (1988) J Lipid Res 29, 212-214.
72. Heuman, DM, Hernandez, CR, Hylemon, PB, Kubaska, WM, Hartman, C and Vlahcevic, ZR (1988) Hepatology 8, 358-365.
73. Hall, R, Kok, E and Javitt, NB (1988). FASEB J 2, 152-156.
74. Weiss, EE and Barth, CA (1978) J Lipid Res 19, 856-862.
75. Boyd, GS and Lawson, ME (1976) FEBS Lett. 64, 435-439.
76. Heuman, DM, Vlahcevic, ZR, Bailey, ML and Hylemon, PB (1988) Hepatology 8, 892-897.
77. Duane, WC Levitt, DG and Mueller, SM (1983) J Clin Invest 72, 1930-1936.
78. Mitropoulos, KA, Balasubramaniam, S and Myant, NB (1973) Biochim Biophys Acta 326, 428-438.
79. Pooler, PA and Duane, WC (1988) Hepatology 8, 1140-1146.
80. Ho, K and Drummond, JL (1975) Am J Physol. 229, 1427-1437.
81. Duane, WC, Gilberstadt, ML and Wiegand DM (1979) Am J Physiol 236, R175-R179.
82. Princen, HMG, Meier, P and Hofstee, B (1989) Biochem J 262, 341-348.
83. Van Cantfort, MJ (1972) C R Acad Sc Paris, 274, 2593-2595.
84. Abrams JJ and Grundy, SM (1981) J Lipid Res 22, 323-338.
85. Angelin, B, Einarsson, K and Leijd, B (1983) Eur J Clin Invest 13, 99-106.

86. Pauletzki, J, Stellaard, F and Paumgartner, G (1989) Hepatology 9, 852-855.
87. Subbiah, MTR and Yunker, RL (1984) Biochim Biophys Res Commun 124, 896-902.
88. Bennion, LJ and Grundy, SM (1977) N Engl J Med 296, 1365-1371.
89. Guettet, C, Mathe, D, Riottot, M and Lutton, C (1988) Biochim Biophys Acta 963, 215-223.
90. Botham, KM, Suckling, KE and Boyd, GS (1984) FEBS Lett 168, 317-320.
91. Davis, RA, Elliott, TS and Lattier, GR (1986) Biochemistry 25, 1632-1636.
92. Björkhem, I, Buchmann, MS and Skrede, S (1985) Biochim Biophys Acta 835, 18-22.
93. Lin, DS and Connor, WE (1980) J Lipid Res 21, 1042-1052.
94. Nestel, PJ and Poyser, A (1976) Metabolism 25, 1591-1599.
95. Björkhem, I (1986) Biochim Biophys Acta 877, 43-49.
96. Mitropoulos, KA, Balasubramaniam, S, Venkatesan, S and Reeves, BEA (1978) Biochim Biophys Acta 530, 99-111.
97. Einarsson, K and Johansson, G (1968) FEBS Lett. 1, 219-222.
98. Eldredge, ER, Jackson, B, Suckling, KE and Wolf, CR (1989) Biochem. J 262, 91-95.
99. Davis, RA, Musso, CA and Lattier, GR (1985) in Enterohepatic circulation of bile acids and sterol metabolism (Paumgartner, G, Stiehl, A and Gerok, W, eds) pp 37-45, MTP Press Ltd, Lancaster, England.
100. Einarsson, K, Akerlund, JE and Björkhem, I (1987) J Lipid Res 28, 253-256.
101. Björkhem, I and Akerlund, JE (1988) J Lipid Res 29, 136-143.
102. Shefer, S, Cheng, FW, Hauser, S, Batta, AK and Salen, G (1981) J Lipid Res 22, 532-536.
103. Shefer, S, Hauser, S, Salen, G, Zaki, FG, Bullock, J, Salgado, E and Shevitz, J (1984) J Clin Invest 74, 1773-1781.
104. Shangvi, A, Grassi, E, Warty, V, Diven, W, Wight, C and Lester, R (1981) Biochim Biophys Res Commun 103, 886-892.
105. Goodwin, CD, Cooper, BW and Margolis, S (1982) J Biol Chem 257, 4469-4472.
106. Tang, PM and Chiang, JYL (1986) Biochim Biophys Res Commun 134, 797-802.
107. Lidström-Olsson, B (1985) FEBS Lett 189, 124-128.
108. Berglund, L, Björkhem, I, Angelin, B and Einarsson, K (1986) Acta Chem Scand 40, 457-461.
109. Danielsson, H, Kalles, I and Wikvall, K (1984) J Biol Chem 259, 4258-4262.
110. Berry, MN and Friend, DS (1969) J Cell Biol 43, 506-520.
111. Botham, KM and Boyd, GS (1979) Eur J Biochem 95, 533-542.
112. Whiting, MJ and Edwards, AM (1979) J Lipid Res 20, 914-918.
113. Botham, KM, Beckett, GJ, Percy-Robb, IW and Boyd, GS (1980) Eur J Biochem 103, 299-305.
114. Kempen, HJM, Vos-van Holstein, MPM and de Lange, J (1982) J Lipid Res 23, 823-830.
115. Botham, KM, Lawson, ME, Beckett, GJ, Percy-Robb, IW and Boyd, GS (1981) Biochim Biophys Acta 665, 81-87.
116. Kempen, HJM, Vos-van Holstein, M and de Lange, J (1983) J Lipid Res 24, 316-323.
117. Botham, KM and Boyd, GS (1983) Eur J Biochem 136, 313-319.
118. Sundaram, GS, Rothman, V and Margolis, S (1983) Lipids 18, 443-447.
119. Davis, RA, Hyde, PM, Kuan, JCW, Malone-McNeal, M and Archambault-Schexnayder, J (1983) J Biol Chem 258, 3661-3667.
120. Ford, RP, Botham, KM, Suckling, KE and Boyd, GS (1985) Biochim Biophys Acta 836, 185-191.
121. Hylemon, PB, Gurley, EC, Kubaska, WM, Whitehead, TR, Guzelian, PS and Vlahcevic, ZR (1985) J Biol Chem 260, 1015-1019.
122. Shefer, S, Hauser, S and Mosbach, EH (1968) J Lipid Res 9, 328-333.
123. Sirica, AE and Pitot, HC (1980) Pharmacol Rev 31, 205-228.
124. Sampson, WJ, Suffolk, RA, Bowers, P, Houghton, JD, Botham, KM and Suckling, KE (1987) Biochim Biophys Acta 920, 1-8
125. Mackinnon, AM, Drevon, CA, Sand, TM and Davis ,RA (1987) J Lipid Res 28, 847-855.
126. Ford, RP, Botham, KM, Suckling, KE and Boyd GS (1985) FEBS lett 179, 177-180.
127. Princen, HMG, Huijsmans, CMG, Kuipers, F, Vonk, RJ and Kempen, HJM (1986) J Clin Invest 78, 1064-1071.
128. Huff, MW, Telford, DE, Woodcraft, K and Strong, WLP (1985) J Lipid Res 26, 1175-1186.
129. Spady, DK, Turley, SD and Dietschy, JM (1985) J Lipid Res 26, 465-472.
130. Chapman, MJ (1986) in Methods in Enzymology, vol 128 (Segrest, JP and Alberts JJ, eds) pp 70-143, Academic Press Inc, New York.
131. Pangburn, SH, Newton, RS, Chang, C, Weinstein, DB and Steinberg, D (1981) J Biol Chem 256, 3340-3347.
132. Bachorik, PS, Franklin, FA, Virgil, DG and Kwiterovich, PO (1982) Biochemistry 21, 5675-5684.

133. Kosygh, VA, Preohrazhensky, SN, Ivanov, VO, Tsibulsky, VP, Repin, VS and Smirnov, VN (1985) FEBS Lett 183, 17-20.
134. Edge, SB, Hoeg, JM, Triche, T, Schneider, PD and Brewer, HB (1986) J Biol Chem 3800-3806.
135. Havekes, LM, Verboom, H, De Wit, E, Yap, SH and Princen, HMG (1986) Hepatology 6, 1356-1360.

CHAPTER 2

RESULTS AND CONCLUSIONS

RESULTS

In this chapter, the results of and conclusions from the study are presented. The complete experimental work with all details is described in the chapters 3 - 8.

Development of a method to to determine cholesterol 7α-hydroxylase activity in homogenates of cultured hepatocytes.

For the determination of cholesterol 7α-hydroxylase activity in microsomal preparations of cultured hepatocytes, large amounts of cells (40 - 70 million) are needed (1). A method to determine the activity of this enzyme in homogenates of cultured hepatocytes, without subcellular fractionation, would allow determinations in smaller amounts of cells and therefore facilitate studies on the regulation of this enzyme in cultured hepatocytes. For this reason, an assay method was developped, initially for rat hepatocytes, based on the measurement of the amount of radioactive cholesterol converted into 7α-hydroxycholesterol by hepatocyte homogenates, in the presence of a NADPH-regenerating system (chapter 3). Blank values were determined in parallel incubations without the NADPH-regenerating system. The reaction products were separated from cholesterol by thin-layer chromatography. Two products were formed NADPH-dependently, 7α-hydroxycholesterol and 26-hydroxycholesterol. Furthermore, cholesterol was oxidized NADPH-independently to 25-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 3β,5α,6β-trihydroxycholestane and a small amount of 7α-hydroxycholesterol. No further conversion of the formed 7α-hydroxycholesterol was noticed under the experimental conditions. Optimal assay conditions were established for rat (chapter 3) and subsequently for pig hepatocytes (chapter 4). To saturate the enzyme, 105 μM exogenous cholesterol (solubilized in Tween 80) was added to the assay mixture. From the mass of 7α-hydroxycholesterol formed (measured by quantitative acetylation with [^{3}H]acetic anhydride) it was concluded that, under the applied conditions, besides the exogenous cholesterol, approximately 70% of the unesterified endogenous cholesterol participated in the reaction. Therefore, for calculation of the enzyme activity in terms of picomoles formed 7α-hydroxycholesterol, the amount of exogenous cholesterol plus 0.7 times the amount of endogenous cholesterol was considered as substrate. By this assay method, activity of the enzyme could be measured in as little as 1 to 2 million parenchymal cells.

Activity of the enzyme in freshly isolated rat hepatocytes was slightly higher than activity in corresponding liver homogenates (2), which demonstrates that during hepatocyte isolation no loss of activity occurred. Rat hepatocytes, routinely cultured in Williams E medium supplemented with 10% fetal bovine serum, 50 nM dexamethasone and 135 nM insulin, lost 60% of their initial cholesterol 7α-hydroxylase activity during the first 24 hours of culture. Decline of cytochrome P-450 activity during culture is a well known problem in the use of cultured hepatocytes for the study of cytochrome P-450 enzymes (3). However, under the culture conditions applied in this study, the activity of cholesterol 7α-hydroxylase in rat hepatocytes was restored during the second culture day to the value found in freshly isolated hepatocytes. During the third culture day, activity of the enzyme declined again (-60%). This was the first report demonstrating cholesterol 7α-hydroxylase activities in cultured hepatocytes, which are comparable to those in vivo.

Development of a system of cultured pig hepatocytes for the study of regulation of bile acid synthesis.

Initial attempts to bring hepatocytes of four months old swine into culture failed, due to poor attachment of the cells to the culture dish plastic (chapter 4). More successful was the use of hepatocytes isolated from 2 to 7 weeks old piglets. As other groups have reported (4,5,6), it was possible to maintain these cells in monolayer culture for several days. A marked difference was observed in cholesterol 7α-hydroxylase activities between hepatocytes isolated from unweaned (2 to 3 weeks old) and newly weaned (7 to 8 weeks old) piglets. Activities of the enzyme in homogenates of freshly isolated hepatocytes from unweaned piglets were very low (on the average 18 times lower than in rat hepatocytes), approaching the detection limit of the assay method. Fortunately, activities of cholesterol 7α-hydroxylase in hepatocytes of newly weaned piglets were at the average 16 times higher than the values in unweaned piglet hepatocytes. The observed increase after weaning (which occurred at 4 weeks after birth) was confirmed by determinations of cholesterol 7α-hydroxylase activity in microsomes prepared from livers of 2 to 30 weeks old piglets (chapter 5). Microsomal cholesterol 7α-hydroxylase activity in 6 to 8 weeks old piglets was at the average 12 times higher than activity in 2 to 4 weeks old piglets. A simultaneous increase was observed in the activities of HMG-CoA reductase and squalene synthetase, two enzymes participating in cholesterol synthesis, the first being rate-limiting in this pathway. HMG-CoA reductase activity increased thirty-fold and squalene synthetase activity five-fold. It is suggested that the rise at weaning of the activities of the latter enzymes may be caused by the change from a cholesterol-rich diet (sow milk which contains 0.22% cholesterol) to a diet with low cholesterol content (pig chow, which

contained 0.016% cholesterol). The elevation of cholesterol 7α-hydroxylase activity may be due to a cholestyramine-like effect of the pig chow or may be related to the ontogeny of the activity of the hypothalamo-pituitary-adrenal axis. These hypotheses were not subjected to further research. Interestingly, activities of all three enzymes were decreased again in pubertal (16 to 30 weeks old) piglets to similar values as before weaning.

During the culture of hepatocytes from unweaned piglets in the same culture medium as used for rat hepatocytes, cholesterol 7α-hydroxylase activity remained low (chapter 4). Hepatocytes from weaned piglets, which immediately after isolation had a high cholesterol 7α-hydroxylase activity (12.6 ± 5.7 pmol/h/μg DNA), lost 90% of the initial activity during the first 24 hours in monolayer culture. However, activity was restored to the values found in freshly isolated hepatocytes during the next two culture days. Subsequently, during the fourth and fifth culture day, activity of the enzyme declined slowly (-50% in 48 hours). It appeared that the supplementation of fetal bovine serum to the culture medium was essential for the recovery of cholesterol 7α-hydroxylase activity in these hepatocytes. When the serum was omitted from the medium, enzyme activity was not restored. When the hormones dexamethasone and insulin were omitted from the culture medium, restoration of enzyme activity still occurred, but was not complete. In hormone-free medium, cholesterol 7α-hydroxylase activity was approximately two-fold lower during the second and third culture day as compared to the values in hepatocytes cultured in medium supplemented with these hormones. Moreover, in the absence of these hormones, enzyme activity declined more rapidly after the third culture day and was completely lost at the fifth day of culture. Possibly, the inductive effect of the hormone supplementation on cholesterol 7α-hydroxylase activity was owing to dexamethasone. Princen et al (7) recently showed that glucocorticoids stimulated the activity of this enzyme in cultured rat hepatocytes. Subsequently, bile acid synthesis by pig hepatocytes cultured in the complete medium was characterized and quantified. Hepatocytes from both unweaned and weaned piglets converted radiolabelled cholesterol (solubilized in fetal bovine serum) into 11 different water-soluble metabolites, of which 95% was found to be conjugated. The bile acids synthesized were not only conjugated with glycine and taurine but also for a part with sulphate and glucuronic acid. The most prominent product after deconjugation and solvolysis was identified as hyocholic acid, a bile acid specific for the pig. Five other metabolites migrated upon thin-layer chromatography as dihydroxy bile acids, among which chenodeoxycholic, hyodeoxycholic and murocholic acid were identified. Five metabolites migrated in the area of monohydroxy bile acids; one of these was identified as lithocholic acid.

In order to be able to determine rate of mass production of bile acids by cultured hepatocytes, a very sensitive capillary gas chromatographic method was set up.

Detection limit of this method was 1 ng. Mass production of bile acids by hepatocytes from weaned piglets, cultured in the complete medium mentioned above, increased during the second and third culture day, in accordance with the increasing cholesterol 7α-hydroxylase activity. Rate of bile acid synthesis during the third culture day amounted on the average 138 pmol/h/mg protein which is comparable to the in vivo rate in the pig (180 ± 54 pmol/h/mg protein) (8). This rate is 7 times higher than the reported bile acid production rates in rat hepatocytes cultured in serum-free media (9,10). On the other hand, when the fetal bovine serum was omitted from the culture medium, bile acid production in the pig hepatocytes was 7 times lower, in agreement with the the low cholesterol 7α-hydroxylase activities in serum-free medium, and similar to the synthesis rates reported for rat hepatocytes cultured in serum-free media.

Hepatocytes from unweaned piglets, cultured in the complete medium, synthesized bile acids at a 3 times lower rate than hepatocytes from weaned piglets. The rate of bile acid synthesis in cultured hepatoctyes from unweaned piglets was 4 times higher as would be predicted by their cholesterol 7α-hydroxylase activities, suggesting that part of the bile acids produced by these cells in culture may be synthesized by a route bypassing cholesterol 7α-hydroxylase. Possibly, a pathway starting with 26-hydroxylation of cholesterol accounted for part of the bile acid synthesis in these cells.

Because of their high cholesterol 7α-hydroxylase activities and high rates of bile acid production in culture, hepatocytes of young weaned pigs, cultured in the presence of fetal bovine serum, dexamethasone and insulin were judged to be most suitable for the study of feedback regulation of bile acid synthesis.

Feedback regulation of bile acid synthesis in hepatocytes from young weaned pigs in monolayer culture.

In order to investigate whether bile acid synthesis in cultured pig hepatocytes was susceptible to feedback inhibition by bile acids, the hepatocytes were incubated with bile acids (100 μM) during the second and third culture day, and bile acid synthesis from exogenous radiolabelled cholesterol was determined over the third culture day (chapter 6). Formation of radiolabelled bile acids was indeed found to be inhibited upon addition of taurocholic acid and several other amidated and non-amidated bile acids in a concentration of 100 μM to the culture medium. The concentration used is within the physiological range of bile acid concentrations of portal blood in the pig (60 - 134 μM) (11). The bile acids, in this concentration, did not affect plasma membrane integrity (as assessed by measurement of the release of lactate dehydrogenase to the medium) or the metabolic state of the cells (as assessed by ATP measurement). Upon the addition of taurocholic acid (50 or 100 μM) to the culture

medium, also mass production of bile acids was suppressed (65% during the second culture day and 85% during the third culture day) (chapter 7). There was no difference in suppressive effects of 50 and 100 μM exogenous taurocholate. The rate of formation of bile acids from radiolabelled 7α-hydroxycholesterol was not affected by taurocholic acid, indicating that the inhibitory effect probably was exerted on cholesterol 7α-hydroxylase. Measurements of the activity of this enzyme in homogenates of pig hepatocytes incubated for 48 hours with different concentrations of taurocholic acid, showed a concentration dependent suppression of the enzyme, which was maximally (-80%) at 50 to 100 μM of exogenous bile acid. Incubations of the hepatocytes with 100 μM taurocholic acid for different times revealed that the decline of enzyme activity followed first-order kinetics, with a half-life period of 11 hours. The observed suppression of cholesterol 7α-hydroxylase activity represented not merely a repression of the induction of activity by the hormones which were included in the culture medium, since the phenomenon was also observed in hormone-free cultures.

Addition of taurocholic acid to the cholesterol 7α-hydroxylase assay mixture did not affect the activity of the enzyme, suggesting that bile acids modulated either the synthesis or breakdown of the enzyme. The changes in cholesterol 7α-hydroxylase activity cannot be explained by changes in substrate availability, since activity of the enzyme was determined in an assay with saturating exogenous cholesterol concentrations.

To investigate whether bile acids differed in relative effectiveness in regulating cholesterol 7α-hydroxylase, hepatocytes were incubated with 9 different bile acids. Like taurocholic acid, glycochenodeoxycholic and glycohyodeoxycholic acids, unconjugated cholic, chenodeoxycholic, hyodeoxycholic and deoxycholic acid inhibited activity of cholesterol 7α-hydroxylase. On the other hand, hyocholic acid had no influence and ursodeoxycholic acid only weakly inhibited activity of the enzyme. Thus, the ability to suppress cholesterol 7α-hydroxylase is, in pig hepatocytes as least, not confined to secondary bile acids, as has been proposed by Stange et al (12,13). The results show some similarity with those obtained in rats in vivo by Heuman et al (14), who postulated that the ability of bile acids to inhibit cholesterol 7α-hydroxylase is related to their hydrophobicity. However, this hypothesis is not supported by the inhibitory effect of hyodeoxycholic acid, which is relatively hydrophilic, as defined by mobility in reverse phase high-performance liquid chromatography (15).

There are two possible explanations why feedback inhibition of bile acid synthesis was observed in this in vitro sytem of cultured pig hepatocytes, but could not be demonstrated by two other research groups in cultured rat hepatocytes (16,17). Firstly, in contrast to the rat hepatocytes used by these groups, the cultured pig hepatocytes expressed high cholesterol 7α-hydroxylase activities and rates of bile acid synthesis

which equalled those in the pig in vivo, owing to the presence of fetal bovine serum in the culture medium. Secondly, cultured rat hepatocytes rapidly lose the capacity to take up and accumulate exogenous bile acids intracellularly, while in pig hepatocytes the capacity to accumulate bile acids is retained for the greater part during the first few culture days. In a comparative study (chapter 8), maximal capacities to accumulate taurocholic acid intracellularly were assessed for pig, rat and human hepatocytes cultured for no more than 24 hours. Accumulation capacities were saturated at extracellular taurocholic acid concentrations of 100 - 500 μM. At these concentrations, hepatocytes of the three species concentrated taurocholic acid intracellularly to the same extent (13 to 17 nmol per mg cell protein). However, with proceeding culture age, the accumulation capacities of rat and human hepatocytes declined steeply (-80% and -60%, respectively during the second culture day). In contrast, pig hepatocytes had retained even 60% of their original capacity to concentrate taurocholic acid intracellularly after three days of culture. The intracellular bile acid accumulation was not due to an impaired capability to secrete it, since upon change to bile acid-free medium, pig hepatocytes secreted intracellular taurocholate almost completely within 60 min.

CONCLUSIONS

In this study, an in vitro system of pig hepatocytes in monolayer culture with high cholesterol 7α-hydroxylase activities and bile acid production rates has been realized. For that purpose, the following conditions were found to be important:

1. The use of young, newly weaned donor animals. Hepatocytes isolated from piglets of this age-group expressed a high initial cholesterol 7α-hydroxylase activity and attached well to the culture dish plastic.
2. The inclusion of fetal bovine serum into the culture medium, which resulted in a restoration of cholesterol 7α-hydroxylase activity in the cultured hepatocytes after an initial decline. Consequently bile acid production rates comparable to those in the pig in vivo were realized in vitro.

This in vitro system is suitable for the study of regulation of bile acid synthesis. By the use of this model, it was shown that bile acid synthesis is regulated by feedback inhibition by bile acids. The inhibition is exerted by a direct action of bile acids on the parenchymal cell, and not mediated by other factors. The feedback regulation is directed to one enzyme in the metabolic pathway from cholesterol to bile acids, namely to cholesterol 7α-hydroxylase. Most probably, bile acids regulate activity of this enzyme by modulation of the amount of enzyme protein. There are differences in the relative effectiveness of various bile acids in inhibition of cholesterol 7α-

hydroxylase activity, but a clear relation with bile acid structure could not be conclusively determined.

PERSPECTIVES

The in vitro system described in this thesis provides an excellent model to study the molecular mechanism of the inhibition of cholesterol 7α-hydroxylase by bile acids. For that purpose it is necessary to dispose of specific antibodies against this enzyme in order to be able to quantify the amount of enzyme protein, and of a DNA probe in order to be able to study gene expression. Insight into the cellular events coupled to this regulation process, might provide the opportunity of antagonizing feedback inhibition of bile acid synthesis. The described in vitro system is suitable for screening of agents expected to have such an effect. Such compounds may act in vivo as cholesterol lowering drugs and may be valuable for therapy of hypercholesterolemia. Furthermore, because of the resemblance of pig hepatocytes to human hepatocytes with respect to LDL cholesterol metabolism, the model may be suitable to study the link between lipoprotein metabolism and bile acid synthesis.

REFERENCES

1. Hylemon, PB, Gurley, EC, Kubaska, WM, Whitehead, TR, Guzelian, PS and Vlahcevic, ZR (1985) J Biol Chem 260, 1015-1019.
2. Ugele, B, Kempen, HJM, Gebhart, P, Meier, P, Burger, HJ and Princen, HMG (1989) in Trends in bile acid research (Paumgartner, G, Stiehl, A and Gerok, W, eds) pp 53-62, Kluwer Academic Publishers, Lancaster.
3. Sirica, AE and Pitot, HC (1980) Pharmacol Rev 31, 205-228.
4. Pangburn, SH, Newton, RS, Chang, C, Weinstein, DB and Steinberg, D (1981) J Biol Chem 256, 3340-3347.
5. Bachorik, PS, Franklin, FA, Virgil, DG and Kwiterovich, PO (1982) Biochemistry 21, 5675-5684.
6. Caperna, FJ, Failla, ML, Kornegay, ET, Richards, MP and Steele, NC (1985) J. Animal Sci 61, 1576-1586.
7. Princen, HMG, Meijer, P and Hofstee, B (1989) Biochem J 262, 341-348.
8. Kim, DN, Rogers, DH, Li, JR, Reiner, JM, Lee, KT and Thomas, WA (1977) Exp Mol Pathol 26, 434-447.
9. Davis, RA, Hyde, PM, Kuan, JW, Malone-McNeal, M and Archambault-Schexnayder, J (1983) J Biol Chem 258, 3661-3667.
10. Ford, RP, Botham, KM, Suckling, KE and Boyd, GS (1985) Biochim Biophys Acta 830, 185-191.
11. Legrand-Defretin, V, Juste, C, Corring, T and Rérat, A (1986) Am J Physiol 250, G295-G301.
12. Stange, EF, Scheibner, J, Lutz, C and Ditschuneit, H (1988) Hepatology 8, 879-886.
13. Stange, EF, Scheibner, J and Ditschuneit, H (1989) J Clin Invest 84, 173-180.
14. Heuman, DM, Vlahcevic, ZR, Bailey, ML and Hylemon, PB (1988) Hepatology 8, 892-897.
15. Heuman, DM (1989) J Lipid Res 30, 719-730.
16. Davis, RA, Highsmith, WE, Malone McNeal, M, Archambault Schexnayder, J and Kuan, JCW (1983) J Biol Chem 258, 4079-4082.
17. Kubaska, WM, Gurley, EC, Hylemon, PB, Guzelian, PS and Vlahcevic, ZR (1985) J Biol Chem 260, 13459-13463.

CHAPTER 3

ASSAY OF CHOLESTEROL 7α-HYDROXYLASE ACTIVITY IN RAT HEPATOCYTES IN PRIMARY MONOLAYER CULTURE

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SUMMARY

A sensitive and precise method is described to assay cholesterol 7α-hydroxylase activity in homogenates of rat hepatocytes cultured in monolayers for up to 76 h. The assay is based on measurement of the amount of radioactive cholesterol converted into 7α-[^{14}C]hydroxycholesterol. Since no subcellular fractionation was applied to measure enzyme activity, this method is rapid and can be performed with cell protein, corresponding to as little as 1 to 2 million hepatocytes. Optimal assay conditions were determined and the reproducibility of this cholesterol 7α-hydroxylase determination was established. Exogenous cholesterol (105 μM), solubilized in Tween 80, was added to saturate the enzyme, giving an apparent K_m of 56 μM. Under these conditions, 70% of the cholesterol present in the homogenates is directly accessible to the cholesterol 7α-hydroxylase. The detection limit of the assay was found to be about 10 pmol per incubation. A time course of the cholesterol 7α-hydroxylase activity in cultured hepatocytes revealed that after an initial loss of approximately 60% of the activity as compared with 287 pmol/h/mg for freshly isolated cells, the enzyme activity was increased to the initial level in hepatocytes cultured for 52 h. This result and the finding that the cholesterol 7α-hydroxylase activity was diminished by 94% after a 24-h incubation with 5 μM cycloheximide suggest that the enzyme activity is associated with *de novo* protein synthesis. Since the cholesterol 7α-hydroxylase activity was a good reflection of the bile acid synthesis rate in hepatocytes during 76 h in culture, we conclude that this method may be a valuable tool in the study of the regulation of bile acid synthesis.

INTRODUCTION

The liver plays an important role in the synthesis and catabolism of cholesterol (1). Conversion of cholesterol into bile acids takes place exclusively in the liver and represents the major pathway for elimination of cholesterol from the body. The

microsomal cholesterol 7α-hydroxylase (EC 1.14.13.17) is the first and major rate-limiting enzyme in the bile acid synthesis pathway (1-4). The 7α-hydroxylase activity is believed to be regulated by the amount of bile acids returning to the liver via the portal vein and by nutritional and hormonal factors (3,4). Other studies revealed that cytosolic proteins (5-8) and phosphorylation-dephosphorylation (7,9,10) may be involved in the regulation of the cholesterol 7α-hydroxylase activity. Most of the information concerning regulation of bile acid synthesis has been obtained from experiments using intact animals. In recent years, primary monolayer cultures of rat hepatocytes have been shown to be a valuable model for studying a variety of metabolic functions in the liver, including bile acid synthesis (11-14). This *in vitro* system offers the opportunity to investigate the effects of mediators thought to be involved in cholesterol and bile acid metabolism (e.g., bile acids (15,16), lipoproteins (11,17), or hormones) directly on hepatocytes in a controlled and chemically defined environment. Moreover, the (side-)effects of several drugs, currently applied in clinical practice, on the synthesis of bile acids may conveniently be determined using monolayer cultures of hepatocytes (14,15).

Most studies on the regulation of bile acid synthesis in hepatocyte monolayers, however, have been hampered by the difficulty in measuring the activity of the rate-limiting cholesterol 7α-hydroxylase in cultured cells. Although two reports (12,18) have been published, describing the measurement of enzyme activity in microsomes isolated from cultured rat hepatocytes, in these investigations large amounts of cells were needed (40-70 x 10^6) or hepatocytes were kept in suspension culture for only 5 h (18). In the present paper, we report a rapid and sensitive assay method for cholesterol 7α-hydroxylase in homogenates of 1-2 x 10^6 rat hepatocytes, cultured for 76 h, using measurement of ^{14}C radioactivity. In this work, the contribution of endogenous free cholesterol as substrate for the enzyme was also studied.

MATERIALS AND METHODS

Chemicals and animals.

[4-^{14}C]Cholesterol (60 mCi/mmol), [^3H]acetic anhydride (50 mCi/mmol), and Enhance spray were purchased from New England Nuclear (Boston, MA). Culture medium and fetal bovine serum were from Flow Laboratories (Irvine, Scotland). Collagenase type I, soybean trypsin inhibitor, bovine serum albumin, dexamethasone, pyruvic acid, dithiothreitol (DTT), butylated hydroxytoluene (BHT), and Tween 80 were obtained from Sigma Chemicals (St. Louis, MO). 7-Ketocholesterol (3β-hydroxy-5-cholesten-7-one), 7α- and 7β-hydroxycholesterol, and 3β,5α,6β-trihydroxycholestane were from Steraloids (Wilton, NH). Glucose 6-phosphate, NADP, glucose-6-phosphate

dehydrogenase, NADH, and lactate dehydrogenase (from rabbit muscle) were purchased from Boehringer (Mannheim, FRG). Insulin (Actrapid, 40 IU/ml) was from Novo Industri (Copenhagen, Denmark). Trypan blue was from BDH Chemicals (Poole, Dorset, England). Cholesterol, Hepes, TLC precoated silica gel 60, and aluminium oxide (type E) plates (0.25 mm thickness) and all salts and solvents were obtained from E. Merck (Darmstadt, FRG). X-Omat AR films for autoradiography were purchased from Eastman-Kodak Company (Rochester, NY).
Radioactive markers 7α- and 7β-[4-^{14}C]hydroxycholesterol were prepared from 7-[4-^{14}C]ketocholesterol, which is the most pronounced impurity in commercially available [4-^{14}C]cholesterol, by reduction with sodium borohydride (19). 26-Hydroxycholesterol was a gift from Dr. N.B. Javitt, Division of Hepatic Diseases, New York University Medical Center (New York, NY). Male Wistar rats 250-350 g were used throughout and maintained on standard chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. For preparation of hepatocytes animals were killed between 9 and 10 AM.

Rat hepatocyte preparation and culture.
Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously (20). Viability, as determined by trypan blue (0.11%) exclusion, was higher than 86%. The cells were seeded on 60-mm-diameter plastic tissue culture dishes (Costar, Cambridge, MA) at a density of 1 x 10^5 cells/cm^2 and were routinely maintained in Williams' E medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM *L*-glutamine, 20 mU/ml insulin, 50 nM dexamethasone, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2/95% air atmosphere. For each time point three to four dishes were plated. After a 4-h attachment period and every 24 h thereafter, medium was refreshed with 2.5 ml of culture medium. Hepatocytes were cultured for 3 days under these conditions and remained viable, as judged by trypan blue exclusion (more than 90% after 76 h) and leakage of the cytoplasmic enzyme lactate dehydrogenase to the culture medium. Lactate dehydrogenase activity (determined as described in Ref. (21)) in culture medium was less than 10% of cellular content and decreased during culture time, in agreement with others (22).

Cholesterol 7α-hydroxylase assay.
Under Results and Discussion we will describe the various experiments leading to the selection of optimal conditions for the cholesterol 7α-hydroxylase assay.
Immediately after isolation and cell count, a part of the hepatocyte suspension was centrifuged at 4°C for 5 min at 75*g*. Cells were resuspended and washed three times in cold Hanks' buffer without Ca^{2+} and Mg^{2+}, the cell pellet was then quickly frozen

in liquid nitrogen in portions of 5-10 x 10^6 cells, and stored at -80°C routinely (t = 0 h sample). Cultured hepatocytes were harvested at indicated time points in the above buffer, washed three times, scraped using a rubber policeman, and frozen as described for t = 0 h samples. For determination of the cholesterol 7α-hydroxylase activity cell pellets were thawed rapidly and resuspended in 250 μl of cold hypotonic buffer containing 20 mM potassium phosphate, pH 7.4, 5 mM DTT, and 1 mM EDTA. Cells were disrupted at 4°C by 25 downward passes in a hand-driven 2-ml Potter-Elvehjem homogenizer equipped with a Teflon pestle. One milliliter of 100 mM potassium phosphate buffer, pH 7.4, plus 5 mM DTT was added and the homogenate was homogenized a further 15 strokes. Samples were taken for determination of protein and cholesterol, which were measured according to Lowry et al (23) and Gamble et al (24), respectively. An aliquot of the homogenate, containing 1-2 mg of protein, was used to assay cholesterol 7α-hydroxylase activity. Homogenates in duplicates were preincubated for 10 min at 37°C in a medium containing 50 mM potassium phosphate, pH 7.4, 2.5 mM DTT, 105 μM [4-^{14}C]cholesterol (0.3 μCi, purified before use), and 1.5 mg/ml Tween 80. Enzyme activity at the end of the preincubation time was assayed by addition of a NADPH regenerating system containing 20 mM glucose 6-phosphate, 2 mM NADP, 4 mM $MgCl_2$, and 1.4 IU glucose-6-phosphate dehydrogenase to a final volume of 1.0 ml. After 30 min at 37°C in a shaking water bath with exclusion of light the reaction was stopped by addition of 10 ml chloroform/methanol 2:1 containing 0.01% BHT. The organic layer was washed with 2 ml 0.9% NaCl and evaporated under nitrogen. The residue was applied to a thin layer of silica which was twice developed with toluene/ethylacetate 2:3, using 7α- and 7β-[4-^{14}C]hydroxycholesterol as radioactive markers. Usually, plates were autoradiographed for 6 to 8 days at -80°C to visualize 7α-hydroxycholesterol and cholesterol; however, exposure times could be reduced to 2-3 days after spraying the plates with Enhance. For rapid determinations 30-50 μg 7α- and 7β-hydroxycholesterol and cholesterol were added as markers to the residue after evaporation and visualized with Rhodamine 6B. Spots containing 7α-hydroxycholesterol and cholesterol were scraped off and counted, using the [4-^{14}C]cholesterol input as recovery standard. Blank values, determined by running parallel incubations without a NADPH generating system, were subtracted before calculating enzyme activity.
Measurement of the mass of 7α-hydroxycholesterol formed was performed by the double isotope derivative procedure, as previously described by Mitropoulos and Balasubramaniam (25) and Shefer et al (26), using the conditions of Shefer et al.

Bile acid synthesis in cultured hepatocytes.
Synthesis of bile acids in primary cultures of hepatocytes was determined by measuring conversion of 0.15 μCi [4-^{14}C]cholesterol per 10 cm^2 of cells into bile acids,

accumulated during 24-h periods after 28, 52, or 76 h of incubation as previously reported (14).

RESULTS AND DISCUSSION

Properties of the enzyme system.

To achieve equilibration of exogenous with endogenous cholesterol, serving as a substrate for the enzyme, incubation mixtures were preincubated for various time intervals. Maximum enzyme activity was reached after a 10-min incubation, in agreement with others (7). Using this preincubation time, optimal assay conditions were determined. These are illustrated in Fig. 1. The rate of formation of 7α-hydroxycholesterol was linear with respect to total cell protein concentration up to 2.5 mg/ml (2.5 mg per assay, Fig. 1A). In this figure also the absolute requirement for NADPH in the reaction medium is shown. The reaction rate was proportional to the incubation time during the first 45 min after addition of the NADPH regenerating system (Fig. 1B). Therefore, in standard incubations the amount of protein did not exceed 2 mg and reactions were performed for 30 min to assure optimal assay conditions. Under similar conditions using rat liver microsomes, we observed that formation of 7α-hydroxycholesterol was linear for at least 90 min and up to 1.5 mg added microsomal protein (14).

As depicted in Fig. 1C endogenous cholesterol in hepatocytes (10.2-18.0 μg/mg, Table 2) was not sufficient to saturate the enzyme. In the presence of Tween 80, however, the enzyme appeared to be saturated at about 120 μM, a concentration well below the amount of cholesterol usually available to the enzyme in the standard assay (130-170 μM). With the graphical method of Lineweaver-Burk an apparent K_m for the enzyme of 56 μM was found. The difference between this value and the K_m for cholesterol in an assay with microsomes (105 μM, Ref. (14)) may be explained by the presence of cytosolic activators in the homogenates fortifying the formation of 7α-hydroxycholesterol (5-8). It has been suggested that one of these proteins, the cytosolic sterol carrier $protein_2$ facilitates introduction of substrate to the membrane-bound cholesterol 7α-hydroxylase (27,28).

Participation of endogenous cholesterol to the enzymatic reaction was determined by quantitative acetylation of the assay mixture or the biosynthetic 7α-hydroxycholesterol, separated on TLC after incubation, with [^{3}H]acetic anhydride to measure the mass of 7α-hydroxycholesterol formed (25,26).

Under the conditions applied, it was estimated that approximately 70% of cholesterol present in the homogenates was directly accessible to the enzyme. This value is comparable with the percentage of cholesterol in 9000*g* supernatants of rat liver

available as substrate for the enzyme (60-70%, Refs. (29,30)). For calculation of the amount of cholesterol participating in the enzyme reaction, we utilized the amount of endogenous cholesterol x 0.7 plus the amount of added exogenous cholesterol, both in nanomoles.

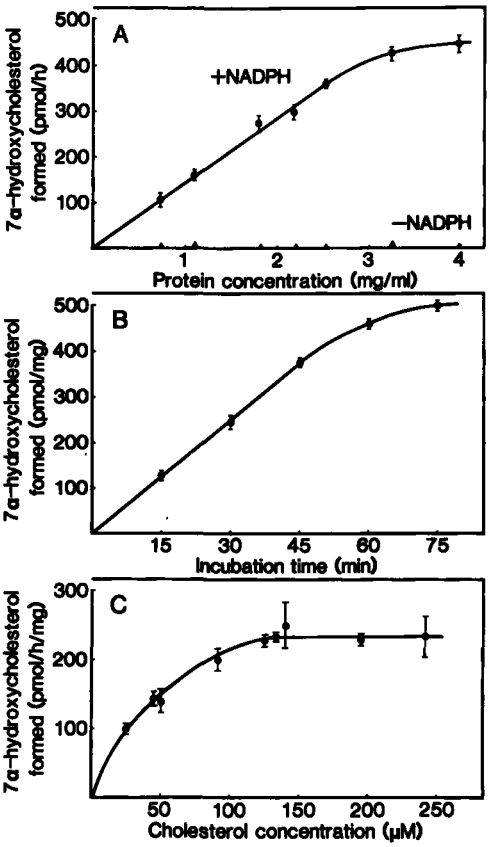

FIG. 1. Effect of increasing amounts of homogenate (A), time (B), and substrate concentration (C) on cholesterol 7α-hydroxylase activity in homogenates of hepatocytes after isolation (t = 0 h samples). Standard assay conditions were employed, except for protein concentration in (A), incubation time in (B), and substrate concentration in (C). Protein in (B) and (C) was 1.66 mg/ml. (A) Incubations in the presence (●) and absence (▲) of NADPH regenerating system (blank). Values of incubations without NADPH were corrected for nonspecific autoxidation as observed in incubation mixtures without homogenates (see Results and Discussion). Similar findings were observed with homogenates of hepatocytes cultured for various times.

Blank values, obtained by running parallel incubations without NADPH regenerating system, were 60 to 70 pmol 7α-hydroxycholesterol per 30-min incubation (under

standard assay conditions). Similar values were observed after incubation of exogenous cholesterol without homogenate or after addition of boiled homogenates to reaction mixtures supplied with NADPH generating system. This obervation indicates that 60 to 70 pmol of 7α-hydroxycholesterol was formed by nonspecific autoxidation of cholesterol and that no NADPH was present or biosynthesized in cell homogenates during the reaction. Subtraction of zero time blanks, as employed by several investigators, led to an overestimation of the reaction rate, since we found that 30 to 40 pmol of 7α-hydroxycholesterol originated during the 40-min preincubation and incubation. The sensitivity of the method is such that 10 pmol of 7α-hydroxycholesterol can be detected.

Cells were routinely cultured (14,32) in Williams' E medium supplemented with 10% FCS and insulin and dexamethasone. These hormones greatly increase cell viability in primary monolayer cultures of rat hepatocytes (33,34).

A representative autoradiograph of a TLC of extracts of 7α-hydroxylase assays with homogenates of cultured rat hepatocytes is shown in Fig. 2. By comparison of lanes containing metabolites of reactions carried out with or without NADPH, it can be

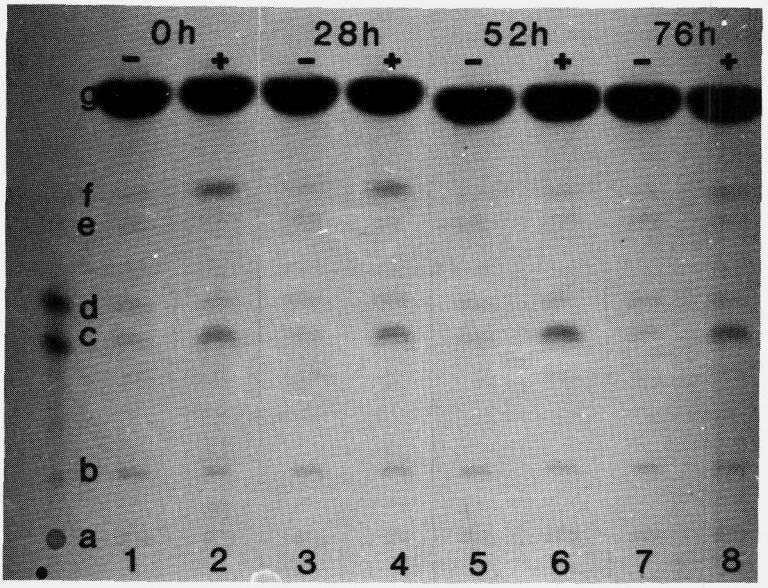

FIG. 2. Autoradiographs of thin-layer chromatograms of the extracts from cholesterol 7α-hydroxylase assays with homogenates of rat hepatocytes. Incubations were performed with homogenates of hepatocytes cultured for 0 h (lanes 1 and 2), 28 h (lanes 3 and 4), 52 h (lanes 5 and 6), and 76 h (lanes 7 and 8) with (+) or without (-) NADPH generating system (blanks). Enzyme activities in this experiment, in which one of a duplicate incubation is shown, were for t = 0 h 217 ± 3 pmol/h/mg, for t = 28 h 94 ± 11 pmol/h/mg, for t = 52 h 341 ± 11 pmol/h/mg, and for t = 76 h 140 ± 3 pmol/h/mg. The autoradiograph was exposed for 9 days without using Enhance spray. The positions of reference compounds (b-g) are indicated. a, start; b, 3β,5α,6β-trihydroxycholestane; c, 7α-hydroxycholesterol; d, 7β-hydroxycholesterol; e, 7-ketocholesterol; f, 25- and 26-hydroxycholesterol; and g, cholesterol.

seen that during incubation [4-^{14}C]cholesterol was autoxidized (5,25,30) giving the following products: 3β,5α,6β-trihydroxycholestane, 7β-hydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol as identified by TLC, and several unidentified nonpolar compounds. In addition, a small amount of 7α-[4-^{14}C]hydroxycholesterol was present in added [4-^{14}C]cholesterol and formed nonspecifically during the 40-min preincubation and incubation (see remarks above). However, two metabolites were NADPH-dependently synthesized, 7α-hydroxycholesterol and a product running together with 25-hydroxycholesterol. This compound was identified using another TLC system (hexane/ethylacetate/aceton/2-propanol 85:15:2:3.5) and GLC-MS as 26-hydroxycholesterol, thought to be formed by a mitochondrial 26-hydroxylase (1) in a NADPH-dependent manner. It is clear from this autoradiograph that no metabolites of 7α-hydroxycholesterol originate, as might be expected, since the next step in bile acid synthesis, the formation of 7α-hydroxy-4-cholesten-3-one, needs NAD^+ as a cofactor (1,4). Furthermore, no radioactivity was detected in the methanol-water layer after extraction.

Effect of storage, CO, and potassium fluoride on enzyme activity.

No cholesterol 7α-hydroxylase activity was lost during freezing and storage for 5 to 10 months, using the procedure described under Materials and Methods (Table 1).

Table 1. Cholesterol 7α-hydroxylase activities in hepatocytes after storage and after incubation under CO and with 50 mM KF.

	Cholesterol 7α-hydroxylase activity (pmol/h/mg)				
Time of storage:	0 h[a]	1 week	2 months	5 months	10 months
Experiment 1[b]	152 ± 13	143 ± 14	164 ± 11		
Experiment 2	296 ± 4		312 ± 17	299 ± 14	263 ± 2*
Experiment 3		457 ± 43		443 ± 31	435 ± 9
Experiment 4	217 ± 3		228 ± 5	232 ± 17	
Experiment 4[c]					
+ CO	15 ± 4		0		
+ 50 mM KF	201 ± 9		228 ± 7		

[a] Cholesterol 7α-hydroxylase activities were determined immediately after hepatocyte isolation. Freezing in liquid nitrogen and rapid thawing (as described under Materials and Methods) did not change activities.

[b] Experiments 1-4 were performed with t = 0 h samples. Similar results were obtained in several experiments with hepatocytes cultured for 28, 52, and 76 h.

[c] A representative experiment out of two (CO) or three (KF) different experiments is given. Values shown are means (± SD) of experiments with hepatocytes of one isolation with duplicate incubations.

* Significant difference ($P < 0.05$) between freshly isolated cells and cells stored for the indicated period.

Usually enzyme activity was determined within 2 weeks after hepatocyte isolation. Incubation under a CO atmosphere completely inhibited the formation of 7α-hydroxycholesterol. In agreement with others (8,31) no indication was obtained about involvement of phosphorylation-dephosphorylation regulation for cholesterol 7α-hydroxylase (7,9,10). Enzyme activity did not differ upon homogenization and incubation in the presence or absence of 50 mM KF (Table 1).

Cholesterol 7α-hydroxylase activity in rat hepatocytes.

Figure 2 also indicates that there is a difference in cholesterol 7α-hydroxylase activity in rat hepatocytes cultured for various times. Measured values of the specific rat hepatocyte culture shown in Fig. 2 and mean results of eight cultures are given in the legend of Fig. 2 and in Table 2, respectively. There was considerable variation in specific activity of the enzyme in hepatocytes from different animals, especially in t = 0 h samples. Similar variations have been reported by Hylemon et al (12) in microsomes of rat hepatocytes prepared immediately after isolation and by us in rat liver microsomes (35). It is possible that this variation represents interindividual differences, since no correlation was found between good and less optimal hepatocyte isolations (viability ranging from 86 to 95%). The existence of large interindividual

Table 2. Cholesterol 7α-hydroxylase activities, cellular cholesterol concentrations, and bile acid synthesis in cultured rat hepatocytes

	Age of culture		Cholesterol 7α-hydroxylase		Free cholesterol		Bile acid synthesis
	h	n	pmol/h/mg	% of t = 0	(μg/mg)	n	(dpm/24 h/mg)
Control							
	0	10	287 ± 153		10.2 ± 1.3		
	28	8	105 ± 46	37	13.4 ± 1.8	9	4870 ± 1390
	52	8	250 ± 67	87	17.1 ± 1.7	9	8690 ± 2010
	76	8	102 ± 46	36	18.0 ± 3.6	9	6250 ± 1800
Cycloheximide							
1 μM	52	2	69 ± 16		15.7 ± 2.9		
5 μM	52	3	15 ± 4		13.4 ± 1.4		

Note. Cholesterol 7α-hydroxylase activities and cellular free cholesterol concentrations were determined in cells harvested at the indicated times. Bile acid synthesis was measured by determination of radioactivity in bile acids, accumulated in cells and medium during the 24-h period preceding the indicated time. Cycloheximide was present during a 24-h period preceding the mentioned time. Values shown are means (± SD) of experiments with hepatocytes of n different rats with duplicate incubations and are expressed per milligram of total cell protein. Cholesterol 7α-hydroxylase activity in t = 0 h samples ranged between 153 ± 6 and 592 ± 51 pmol/h/mg.

variations in the rat of drug metabolism, particularly by the mixed-function oxidase system as a result of genetic factors, has been described (36,37). Since cholesterol 7α-hydroxylase is a cytochrome *P*-450-dependent enzyme (1,3,4) a similar phenomenon may occur with this enzyme.

Hepatocytes lost cholesterol 7α-hydroxylase activity during culturing for 28 h (63%). However, enzyme activity was restored to initial levels after 52 h and decreased afterwards. Loss of total cytochrome *P*-450 has been well established as one of the phenotypic changes associated with adaptation of hepatocytes to conditions of monoalyer culture (for a review, see Ref. (38)), but there may be marked heterogeneity in the rates at which individual *P*-450 forms are lost in culture (39). Here we report that, after an initial loss, there is again an increase of this specific cytochrome *P*-450 activity in primary monolayer cultures. A similar pattern has been found by Hylemon et al (12). This observation, together with the fact that after addition of 5 μM cycloheximide to the culture medium the 7α-hydroxylase activity dramatically decreased (Table 2), suggests that cholesterol 7α-hydroxylase activity results from *de novo* protein synthesis during the 76-h culture period.

Cholesterol 7α-hydroxylase activity perfectly paralelled bile acid synthesis as measured by conversion of [4-^{14}C]cholesterol into bile acids (Table 2). With respect to this, it should be realized, that measurement of enzyme activity gives an instantaneous reflection of bile acid synthesis rate, whereas determination of bile acids formed over a 24-h period is a cumulative process.

In conclusion, we have described an accurate and sensitive method to measure cholesterol 7α-hydroxylase activity in hepatocytes cultured for up to 76 h. In contrast with previously described methods (12,18), the procedure is rapid and can be performed with a small amount of cells (1 to 2 x 10^6), since enzyme activity is determined directly in homogenates.

Our results indicate that measurement of cholesterol 7α-hydroxylase in primary monolayers of cultured hepatocytes is a reliable method and, in addition to measuring bile acid biosynthetic rates, is a useful extension to the study of regulation of bile acid synthesis.

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REFERENCES

1. Björkhem, I (1985) in Sterols and Bile Acids: New Comprehensive Biochemistry (Danielsson, H and Sjövall, J, eds) Vol 12, pp 231-278, Elsevier, Amsterdam.
2. Shefer, S, Hauser, S and Mosbach, EH (1968) J Lipid Res 9, 328-333.
3. Myant, NB and Mitropoulos, KA (1977) J Lipid Res 18, 135-153.
4. Danielsson, H and Wikvall, K (1986) in Cholesterol 7α-Hydroxylase (7α-Monooxygenase) (Fears, R and Sabine, JR, eds) pp 10-19, CRC Press, Boca Raton, USA.
5. Mitton, JR, Scholan, NA and Boyd, GS (1971) Eur J Biochem 20, 569-579.
6. Spence, JT and Gaylor, JL (1977) J Biol Chem 252, 5852-5858.
7. Kwok, CT, Burnett, W and Hardie, IR (1981) J Lipid Res 22, 570-589.
8. Danielsson, H, Kalles, I and Wikvall, K (1984) J Biol Chem 259, 4258-4262.
9. Sanghvi, A, Grassi, E, Warty, V, Diven, W, Wight, C and Lester, R (1981) Biochem Biophys Res Commun 103, 886-892.
10. Goodwin, CD, Cooper, BW, and Margolis, S (1982) J Biol Chem 257, 4469-4472.
11. Davis, RA, Hyde, PM, Kuan, JW, Malone-McNeal, M and Archambault-Schexnayder, J (1983) J Biol Chem 258, 3661-3667.
12. Hylemon, PB, Gurley, EC, Kubaska, WM, Whitehead, TR, Guzelian, PS and Vlahcevic, ZR (1985) J Biol Chem 260, 1015-1019.
13. Ford, RP, Botham, KM, Suckling, KE and Boyd, GS (1985) Biochim Biophys Acta 836, 185-191.
14. Princen, HMG, Huijsmans, CMG, Kuipers, F, Vonk, RJ and Kempen, HJM (1986) J Clin Invest 78, 1064-1071.
15. Davis, RA, Highsmith, WE, Malone-McNeal, M, Archambault-Schexnayder, J and Kuan, JW (1983) J Biol Chem 258, 4079-4082.
16. Kubaska, WM, Gurley, EC, Hylemon, PB, Guzelian, PS and Vlahcevic, ZR (1985) J Biol Chem 260, 13,459-13,463.
17. Ford, RP, Botham, KM, Suckling, KE and Boyd, GS (1985) FEBS Lett 179, 177-180.
18. Botham, KM and Boyd, GS (1979) Eur J Biochem 95, 533-542.
19. Nimmannit, S and Porter, JW (1980) Arch Biochem Biophys 201, 533-543.
20. Princen, HMG, Moshage, HJ, De Haard, HJW, Van Gemert, PJL and Yap, SH (1984) Biochem J 220, 631-637.
21. Bergmeyer, HU and Bernt, E (1970) in Methoden der enzymatische Analyse (Bergmeyer, HU, ed) pp 533-538, Verlag-Chemie, Weinheim/Bergstrom.
22. Jaurequi, HO, Hayner, NT, Driscoll, JL, Williams-Holland, R, Lipsky, MH and Galleti, PM (1981) In Vitro 17, 1100-1110.
23. Lowry, OH, Rosebrough, NJ, Farr, AL and Randall, RJ (1951) J Biol Chem 193, 265-275.
24. Gamble, W, Vaughan, M, Kruth, MS and Avigan, J (1978) J Lipid Res 19, 1068-1071.
25. Mitropoulos, KA and Balasubramaniam, S (1972) Biochem J 128, 1-9.
26. Shefer, S, Nicolau, G and Mosbach, EH (1975) J Lipid Res 16, 92-96.
27. Seltman, H, Diven, W, Rizk, M, Noland, BJ, Chanderbhan, R, Scallen, TJ, Vahouny, G and Sanghvi, A (1985) Biochem J 230, 19-24.
28. Lidström-Olsson, B and Wikvall, K (1986) Biochem J 238, 879-884.
29. Balasubramaniam, S, Mitropoulos, KA and Myant, NB (1973) Eur J Biochem 34, 77-83.
30. Van Cantfort, J, Renson, J and Gielen, J (1975) Eur J Biochem 55, 23-40.
31. Berglund, L, Björkhem, I, Angelin, B and Einarsson, K (1986) Acta Chem Scand B 40, 457-461.
32. Sprengers, ED, Princen, HMG, Kooistra, T and Van Hinsbergh, VWM (1985) J Lab Clin Med 105, 751-758.
33. Bissell, DM and Guzelian, PS (1980) Ann N Y Acad Sci 349, 85-98.
34. Ichihara, A, Nakamura, T and Tanaka, K (1982) Mol Cell Biochem 43, 145-160.
35. Cohen, LH, Griffioen, AM, Wanders, RJA, Van Roermund, CWT, Huijsmans, CMG and Princen, HMG (1986) Biochem Biophys Res Commun 138, 335-341.
36. Breimer, DD and Danhof, M (1980) in Towards Better Safety of Drugs and Pharmaceutical Products (Breimer, DD, ed) pp 117-141, Elsevier/North-Holland, Amsterdam.
37. Conney, AH, Pantuck, EJ, Pantuck, CB, Fortner, JG, Alvares, AP, Anderson, KE and Kappas, A (1980) in Chemical Pharmacology and Therapeutics (Turner, P, ed) pp 51-62, MacMillan & Co., London.
38. Sirica, AE and Pitot, HC (1980) Pharmacol Rev 31, 205-228.
39. Steward, AR, Dannan, GA, Guzelian, PS and Guengerich, FP (1985) Mol Pharmacol 27, 125-132.

CHAPTER 4

CHOLESTEROL 7α-HYDROXYLASE ACTIVITY AND BILE ACID SYNTHESIS IN HEPATOCYTES OF UNWEANED AND WEANED PIGS IN MONOLAYER CULTURE

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SUMMARY

Activity of cholesterol 7α-hydroxylase (EC 1.14.13.17) in freshly isolated hepatocytes from unweaned piglets (2-3 weeks old) was 16 times lower as compared to hepatocytes from weaned piglets (7-8 weeks old). The monolayer culture activity of the enzyme remained low in unweaned piglet hepatocytes. In contrast, in cultured hepatocytes from weaned piglets cholesterol 7α-hydroxylase activity declined during the first day of culture, but was restored during the next two culture days, provided that fetal bovine serum (10%) was added to the culture medium. Addition of dexamethasone (50 nM) and insulin (135 nM) to the medium further enhanced cholesterol 7α-hydroxylase activity to values similar to those in freshly isolated hepatocytes and retarded the decline of enzyme activity after the third culture day. Cultured hepatocytes from weaned and unweaned piglets synthesized similar types of bile acids from [^{14}C]cholesterol, among which hyocholic acid (the most prominent), hyodeoxycholic acid, chenodeoxycholic acid, murocholic acid and lithocholic acid could be identified. Ninetyfive percent of radiolabelled bile acids synthesized was conjugated, mainly with glycine, but also with taurine, sulfate and glucuronic acid. The rate of mass production of bile acids by cultured hepatocytes of weaned piglets (as measured by gas chromatography) parallelled cholesterol 7α-hydroxylase activity, and was low in the absence of serum, but increased in medium containing fetal bovine serum, dexamethasone and insulin to a rate lying in the range of 75% of the in vivo bile acid production during the third culture day. Bile acid production by unweaned piglet hepatocytes was 3 times lower under these conditions.
It is concluded that hepatocytes from young weaned pigs cultured in medium containing 10% fetal bovine serum, offer a suitable in vitro model for the study of bile acid synthesis, in view of the high cholesterol 7α-hydroxylase activities and bile acid production rates.

INTRODUCTION

Bile acid synthesis is the major pathway for elimination of cholesterol from the body. Our knowledge of its regulation is still incomplete. Bile acids are believed to exert feedback inhibition on their own synthesis, at the level of the rate-limiting enzyme cholesterol 7α-hydroxylase (EC 1.14.13.17) (1). There is some evidence for effects of hormones on bile acid synthesis (1,2). Furthermore, *in vivo* studies suggest a link between bile acid synthesis on the one hand and de novo synthesis of cholesterol and lipoprotein degradation by the liver on the other hand (3,4).

In recent years, monolayer cultures of rat hepatocytes have been used to study regulation of bile acid synthesis at the cellular level. Effects of bile acids (5-7), drugs (8,9), enzyme inhibitors (10,11), and also cholesterol supply by lipoproteins (8,12,13) on bile acid synthesis have been examined. Recently our group started to study the regulation of cholesterol 7α-hydroxylase in cultured rat hepatocytes (14) and observed induction of the enzyme by glucocorticoids (15).

With regard to atherosclerosis, the link between bile acid synthesis and lipoprotein metabolism is of special interest. For this purpose hepatocytes of the rat seem, however, not to be the best choice. First, in the rat, in contrast to man, low-density lipoprotein (LDL) is only a minor lipoprotein (16). Second, decrease of plasma LDL after feeding cholestyramine as observed in man (17) has not been found in rat (18). Hepatocytes of the pig might supply a better model. LDL is a major lipoprotein in this animal (16) and interruption of the enterohepatic circulation results in a decrease of plasma LDL (19) as in man. Furthermore, cultured hepatocytes from neonatal pigs have been shown to internalize and degrade LDL through a saturable process (20,21), as cultured human hepatocytes do (22-24).

These considerations led us to investigate the possibility of using pig hepatocytes in monolayer culture for the study of bile acid synthesis. Initial attempts to culture hepatocytes of four months old pigs failed in our laboratory. Since other groups reporting on cultivation of pig hepatocytes (20,21,25) used young piglets, we then decided to examine this possibility. In the present study bile acid synthesis and cholesterol 7α-hydroxylase activity in cultured hepatocytes from unweaned and weaned piglets were characterized and compared, and the influences of serum and hormone additions to the culture medium on these hepatocyte functions were examined.

MATERIALS

Materials used for isolation and culturing of pig hepatocytes, determination of bile acid synthesis from radiolabelled cholesterol and cholesterol 7α-hydroxylase activity

were obtained from sources described previously (14). Suppliers of reference bile acids were given in Refs. 26 and 27. Taurohyocholic acid was synthesized from hyocholic acid and taurine by the method described in Ref. 28. Hexafluoro-2-propanol and trifluoroacetic anhydride were obtained from Serva (Heidelberg, FRG); pronase from Calbiochem (Behring Diagnostics, La Jolla, USA) and mithramycine from Pfizer (Rotterdam, The Netherlands). Heparin was from Leo Pharmaceutical Products B.V., Weesp, The Netherlands. Hexokinase and glucose-6-phosphate dehydrogenase were from Boehringer (FRG).

METHODS

Pig hepatocyte isolation and culture.

Unweaned (2 to 3 weeks old) and weaned (7 to 8 weeks old) female domestic pigs (hybrid of Large Yorkshire and Nederlands Landvarken) were obtained from the Veterinary Department of the State University of Utrecht (Netherlands). All pigs were sacrified between 8.30 and 9.30 a.m. Pigs were administered i.v. 10 mg/kg hypnodyl (anaesthesia) and 10000 U heparin to prevent blood clotting. After opening of the abdomen the vena porta was cannulated, the vena cava cut, and the liver was immediately perfused under hydrostatic pressure with 1 litre of solution (0°C) containing 10 mM Hepes (pH 7.4), 132 mM NaCl, 6.7 mM KCl, 20 mM D-glucose and 0.5 mM EGTA (solution 1) and subsequently with 1 litre of the same buffer without EGTA (solution 2) supplemented with 20 U/ml heparin. During perfusion the liver was excised. After this preperfusion, aimed to prevent blood clotting during transport, the liver was immersed in solution 2 with heparin and transported on ice to the site of hepatocyte isolation within 60 minutes. For cell isolation, a piece of the lobe that was best blanched, was cut off. All solutions used for subsequent perfusion of the liver lobe were oxygenated by bubbling 95% O_2/5% CO_2 and temperature was kept at 37°C. Non-recirculating perfusion with 500 ml solution 1 at a rate of 120 ml/min was started after insertion of four polyethylene catheters (18 gauge) in vascular orifices at the cut surface. Perfusion was continued with respectively 1 litre of solution 2 and 200 ml of a solution containing 100 mM Hepes (pH 7.6), 67 mM NaCl, 6.7 mM KCl and 5 mM $CaCl_2$ (solution 3). Subsequently, the liver lobe was perfused with solution 3 containing 0.5 mg/ml collagenase and 0.05 mg/ml trypsin inhibitor in a recirculating mode for 20 min and with 0.1% collagenase for 30 min, followed by non-recirculating perfusion with 200 ml collagenase-free solution 3.

Liver tissue was dissociated in Williams E medium (0°C) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were filtered through a 250 μm filter, centrifuged for

1 min at 50 xg and washed three times in the above-mentioned medium. Hepatocytes were seeded at a density of of 1.5 x 10^5 viable cells per cm^2 on 28 cm^2 plastic culture dishes or on 10 cm^2 wells in 6 well trays in Williams E medium with the above-mentioned supplements and 135 nM insulin and 50 nM dexamethasone (by analogy with our standard medium for rat and human hepatocyte cultures (9,14,24,26, 27,29)) and maintained at 37°C in a 5% CO_2/95% air atmosphere. The medium was renewed after 4 h, and every 24 h thereafter. In some experiments, hormone or serum supplements were omitted after 4 h, as will be indicated in the Results section.

Assay of cholesterol-7α-hydroxylase in hepatocyte homogenates and liver microsomes. Cholesterol 7α-hydroxylase activities in homogenates of freshly isolated and cultured hepatocytes and in liver microsomes were determined as has been described for rat hepatocytes and microsomes before (9,14). Liver microsomes were prepared essentially as has been described in (9), except that no KF was added to the buffers. Enzyme activities in hepatocyte homogenates were expressed as pmol 7α-hydroxy-cholesterol.h^{-1}.μg DNA^{-1} and in liver microsomes as pmol.h^{-1}.mg $protein^{-1}$. Formation of 7α-hydroxycholesterol by pig hepatocyte homogenates was linear with respect to DNA concentration between 20 and 100 μg per ml assay buffer. Reaction rate was proportional to incubation time up to 60 min after addition of the NADPH-generating system.

Determination and characterization of bile acid synthesis from [^{14}C]cholesterol. Bile acid formation from radiolabelled exogenous cholesterol was determined by measurement of radioactivity in bile acids accumulated in cells and medium during 24 h incubations with 0.2 μCi [^{14}C]cholesterol/ml medium. [^{14}C]Cholesterol was added to the medium after solubilization in fetal bovine serum. After addition of [^{3}H(G)]-taurocholic acid as recovery standard, bile acids were separated from lipids by extraction with chloroform and methanol and further isolated using a C18-cartridge (Sep-Pak C18, Water Associates, Milford, USA) as described before (30). Bile acids were then either directly separated according to conjugation class by thin-layer chromatography (TLC) or first enzymatically deconjugated by incubation with 12 U cholylglycine hydrolase and 3000 U β-glucuronidase (Helix pomatia, Sigma) (31) for 18 h at 37°C in 10 mM sodium acetate, 10 mM EDTA, 0.15% mercaptoethanol (pH 5.6), and solvolyzed in dioxane with 1 mM HCl and 3% water (Princen H.M.G. et al., manuscript in preparation). Separation by TLC of the bile acids according to conjugation class was done using chloroform/methanol/glacial acetic acid/water (15:5:1:1) denoted below as system A (32). R_f values for reference bile acids (visualized by spraying with 10% sulphuric acid and heating) were: taurocholic acid 3α-sulphate, 0.04; taurochenodeoxycholic acid 3α-sulphate, 0.06; taurohyocholic acid,

0.18; glycolithocholic acid 3α-sulphate, 0.23; taurochenodeoxycholic acid, 0.24; deoxycholic acid 3α-glucuronide, 0.26; chenodeoxycholic 3α-sulphate, 0.29; glycocholic acid, 0.34; glycochenodeoxycholic acid, 0.52; hyocholic acid, 0.78; chenodeoxycholic acid, 0.98. Evidently, no complete separation of taurine, sulphate and glucuronide conjugates was achieved in this TLC system. Therefore, silica zones with unconjugated, glycine conjugated and bile acids more polar than glycine conjugates were scraped off, and radioactivity counted.

Deconjugated bile acids were separated by TLC in toluene/dioxane/glacial acetic acid (20:10:2) (33) (system B). Plates were autoradiographed and silica areas containing radioactive bile acids scraped off. ^{3}H and ^{14}C radioactivities were determined by liquid scintillation counting in Picofluor (Packard), using a double-label programme. Identification of bile acids was achieved by comparison of R_f values to those of reference bile acids applied at the edges of TLC plates and after recovery from the silica by their retention times upon gaschromatography (GLC).

R_f values of reference bile acids in system B: deoxycholic acid 3α-glucuronide, 0.00; chenodeoxycholic acid 3α-sulphate, 0.03; lithocholic acid 3α-sulphate, 0.05; hyocholic acid, 0.20; hyodeoxycholic acid, 0.31; chenodeoxycholic acid, 0.38; murocholic acid, 0.38; lithocholic acid, 0.53. Chenodeoxycholic acid and murocholic acid comigrated in this TLC system, but could be separated by double development in trimethylpentane/isopropanol/glacial acetic acid (30:10:1) (33).

The degree of bile acid sulfation was quantitated by processing parts of media and cell samples with and without the solvolysis step after deconjugation with cholylglycine hydrolase and comparing radioactivity in free bile acids. Similarly the degree of glucuronidation was estimated by analyzing parts of samples processed with and without the β-glucuronidase treatment.

Quantification of mass production of bile acids.

Mass production of bile acids was determined by GLC of bile acids accumulated in cells and medium of two 28 cm^2 culture dishes during 24 h periods, essentially as described by Davis et al (8). At the end of a 24 h culture period medium was aspirated and cells were washed twice with Hanks' buffered salt solution (the first wash was added to the medium). Cells were scraped off with a rubber policeman and suspended in water. After addition of 2,66 μg sodium taurodeoxycholate (equivalent with 2 μg deoxycholic acid) as recovery standard, the cell suspensions and media were diluted with an equal volume 0.1 M NaOH, incubated for 15 min at 60°C, cooled and then extracted using a C18 cartridge. Bile acids were deconjugated with cholylglycine hydrolase and β-glucuronidase, again extracted with a C18 cartridge and subjected to solvolysis. The bile acid containing residue was dissolved in alkaline water (pH 9.3), shaken three times with hexane, acidified to pH 2.8 and bile acids were extracted in

dichloromethane. After evaporation of solvent, bile acids were derivatized with trifluoroacetic anhydride and hexafluoroisopropanol for 1 h at 60°C. The solvent was evaporated and the residue dissolved in 10 μl hexane. Samples of 1 μl were subjected to GLC separation using a Chrompack-Packard 438S gaschromatograph equipped with a CPSil-19 (CB) column (25 m x 0.32 mm i.d.) and flame ionisation detector. Hydrogen was used as carrier gas. Samples were splitted with a 1:10 ratio. Injector and detector temperatures were 280°C. Initial oven temperature was 210°C and was raised after 0.1 min by 1°C/min and after 20 min by 20°C/min to 290°C.
Retention time of deoxycholic acid (internal standard) was 10.70 min. Chenodeoxycholic acid, murocholic acid, hyodeoxycholic acid and hyocholic acid had relative retention times of 1.23, 1.28, 1.34 and 1.45 with respect to deoxycholate.
Bile acids were quantified using peak area ratios. Relationships between weight and peak area were similar for all bile acids synthesized by pig hepatocytes. Detection limit was 1 ng. Due to the presence of bile acids in fetal bovine serum, serum-supplemented culture medium contained cholic acid (0.035 μg/ml), chenodeoxycholic acid (0.162 μg/ml), and lithocholic acid (0.023 μg/ml). Net bile acid synthesis was calculated as the amount in medium and cells (corrected for the amount of bile acids in the medium before incubation) minus the amount in cells 24 h earlier.

Miscellaneous methods.
DNA was measured using mithramycine (34) with calf thymus DNA as standard, protein according to Lowry (35) with BSA as standard, cholesterol according to Gamble (36) and LDH activity in media and cells after lysis of cells with 0.2% Triton X-100 (37). ATP was determined in perchloric acid extracts of hepatocytes. Briefly, for extraction of ATP, 0.3 ml ice-cold 14% perchloric acid was added to 1 ml medium, hepatocyte dishes were placed on ice and after 10 min the contents of the dishes were transferred to a centrifuge tube and kept for another 20 min at 0°C to complete ATP extraction. The tubes were centrifuged for 5 min at 6000 xg, 1 ml supernate was transferred to a clean tube, neutralized with approximately 200 μl, 3 M KOH/0.3 M 3-[N-morpholino]propane-sulphonic acid to pH 7.5, frozen in liquid nitrogen and stored at -80°C until ATP determination. At the day of the ATP measurement the samples were thawed, the precipitated $KClO_4$ removed by centrifugation, and ATP determined using the hexokinase/glucose-6-phosphate dehydrogenase method (38).

Statistical analysis.
Statistical differences were assessed using Student's t-test (two-tailed) for paired data, unless otherwise stated. Values of $p < 0.05$ were considered significant.

RESULTS

Hepatocyte culture and viability.

Hepatocytes freshly isolated from livers of unweaned (2 to 3 weeks old) and weaned (7 to 8 weeks old) piglets had a viability, based upon trypan blue exclusion, of 90 ± 5% (n = 10) and 63 ± 16% (n = 9), respectively. This difference is probably due to the extensive stroma development in the pig liver during growth, which troubles hepatocyte isolation.

In the cell preparation, besides solitary cells, also clusters of cells were present. Most viable cells attached within 4 h to the culture dish plastic. Twenty-four h later most hepatocytes had spread, except for cells in the centre of clusters. Flattening of these cells took another 24 h. After two days, monolayers reached confluency.

Cellular ATP content was low in hepatocytes immediately after isolation (Table 1), which has also been described for rat hepatocytes (39), but ATP was restored to a physiological level (38) during the first two culture days, both in hepatocytes from weaned and unweaned piglets.

LDH leakage to the medium (Table 1) was in agreement with values reported for cultured rat hepatocytes (40), except on the first culture day, indicating the presence of non-viable cells that had not attached to the plastic.

Table 1. Cellular ATP and leakage of LDH to medium.

Leakage of lactate dehydrogenase to medium and cellular ATP content during culture of pig hepatocytes in Williams E medium with 10% fetal bovine serum, 50 nM dexamethasone and 135 nM insulin. Values represent means ± S.D. of three culture experiments with duplicate incubations, and were similar for hepatocytes isolated from unweaned and weaned piglets.

Culture age (h)	ATP (pmol.μg DNA^{-1})	LDH (% in medium)
0	65 ± 50	
24	605 ± 185	24.4 ± 6.4
48	810 ± 300	11.7 ± 1.8
72	960 ± 215	7.2 ± 3.4
96	1000 ± 40	
120	945 ± 115	

Cholesterol 7α-hydroxylase activity in hepatocytes and liver microsomes from unweaned and weaned piglets.

Cholesterol 7α-hydroxylase activities in freshly isolated hepatocytes from weaned

piglets appeared to be on the average sixteen times higher as compared to activities in hepatocytes from unweaned piglets (Table 2). This difference in expression of cholesterol 7α-hydroxylase was confirmed by determination of cholesterol 7α-hydroxylase activities in liver microsomes, which were 166 ± 156 (mean ± S.D.) pmol.h^{-1}.mg protein^{-1} for unweaned (n = 4) and 1714 ± 1150 pmol.h^{-1}.mg^{-1} for young weaned piglets (n = 7) ($p < 0.01$ in unpaired t-test). The considerable variations in enzyme activities are due to interindividual differences, which also were observed in the rat (14,41,42).

When hepatocytes of unweaned piglets were cultured, cholesterol 7α-hydroxylase activity remained low (Table 2). In contrast, enzyme activity in hepatocytes of weaned piglets, cultured in the same medium, declined by 90% during the first day of culture, but then increased again, reaching values similar to those in freshly isolated hepatocytes at the third day of culture. These high cholesterol 7α-hydroxylase activities were maintained during the fourth culture day, and activity amounted still to 60% of the initial value after five days.

Table 2. Comparison of cholesterol 7α-hydroxylase activities in hepatocytes from unweaned and weaned piglets.

Cholesterol 7α-hydroxylase activities were determined in homogenates of hepatocytes, either freshly isolated, or cultured in Williams E medium with 10% fetal bovine serum, 50 nM dexamethasone, and 135 nM insulin and harvested at the indicated time points. Values shown are means (± S.D.) of 5 different culture experiments with hepatocytes from unweaned piglets and 4 different culture experiments with hepatocytes isolated from weaned piglets.

Culture age (h)	Cholesterol 7α-hydroxylase activity (pmol.h^{-1}.μg DNA^{-1})	
	unweaned	weaned
0	0.8 ± 0.4	12.6 ± 5.7
24	0.1 ± 0.3	1.3 ± 0.9
48	0.5 ± 0.6	7.8 ± 4.6
72	0.4 ± 0.6	14.0 ± 6.8
96	0.2 ± 0.7	12.5 ± 2.0
120	0.0 ± 0.6	7.1 ± 1.1

Effect of serum and hormones on cholesterol 7α-hydroxylase activity in cultured hepatocytes from weaned piglets.

We then investigated the effects of serum and hormone additions to the medium on cholesterol 7α-hydroxylase activity in hepatocytes from weaned piglets. Fig. 1 shows that the inclusion of fetal bovine serum was essential for the recovery of enzyme

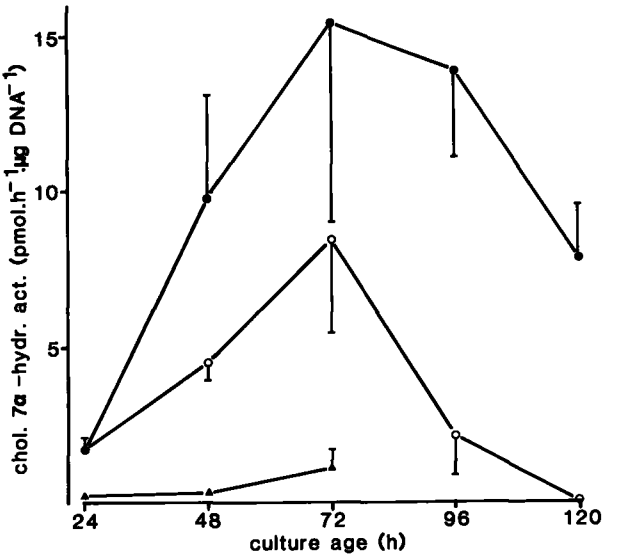

Fig. 1. Influence of fetal bovine serum and hormones on cholesterol 7α-hydroxylase activity in cultured hepatocytes from weaned piglets.
Hepatocytes were plated in Williams E medium (WE) supplemented with 10% fetal bovine serum (FCS) and with or without 50 nM dexamethasone (DEX) and 135 nM insulin (INS). After a 4-h attachment period, medium was renewed with one of the three media indicated below. At the indicated time points cells were harvested and cholesterol-7α-hydroxylase activity was determined. Values represent means ± S.D. of three different culture experiments with duplicate determinations, except for the data at 24 and 48 h in medium without serum, which were obtained in two culture experiments.
●: WE + FCS + DEX + INS; ○: WE + FCS; ▲: WE + DEX + INS (no FCS)

activity between culture day one and three. In the presence of serum, rise of enzyme activity was also observed when dexamethasone and insulin were omitted from the medium but then enzyme activity did not reach the level found in freshly isolated cells. Besides enhancing cholesterol 7α-hydroxylase activity during the recovery period, these hormones partially prevented the fall of enzyme activity during the next two culture days.

Bile acid synthesis by cultured piglet hepatocytes.

a) Formation of labeled bile acids from [^{14}C]cholesterol

Bile acid formation was first characterized by following the conversion of [^{14}C]cholesterol to bile acids during three subsequent days of culture in the presence of 10% fetal bovine serum, 135 nM insulin and 50 nM dexamethasone. There were no significant differences concerning types of formed bile acids and conjugation pattern between hepatocytes isolated from unweaned or weaned piglets.

Separation of formed radiolabelled bile acids by TLC system A (see Materials and Methods) learned that 95% was conjugated, mainly with glycine (38 ± 6% of total bile acids synthesized during the first culture day to 61 ± 5% on the third culture day (n = 3)). Part of the formed bile acids was sulfated, since, upon incubation of hepatocytes with sodium [^{35}S]sulfate, radioactivity was incorporated into bile acids, which were more polar than glycine conjugates upon TLC. [^{35}S]Radioactivity was lost upon solvolysis of bile acids.

Solvolysis of parts of bile acid samples learned that on the average 20% of formed bile acids was sulfated, predominantly metabolites migrating in TLC as monohydroxy bile acids. Additional treatment with β-glucuronidase increased recovery of unconjugated bile acids with 2 to 9% (depending on culture age).

Table 3. Characterization of bile acids formed from exogenous [^{14}C]cholesterol by piglet hepatocytes.

Bile acids synthesized from [^{14}C]cholesterol (0.2 μCi per ml medium) by hepatocytes from unweaned and weaned piglets during three subsequent 24 h culture periods were extracted from cells and medium, deconjugated by incubation with cholylglycine hydroxylase and β-glucuronidase, solvolyzed and separated by TLC in system B (see Materials and Methods section). Ratios of formed bile acids were comparable for hepatocytes from weaned and unweaned piglets, and were combined. Values are expressed as percentage of total radiolabelled bile acids formed during 24-h periods, and are means ± S.D. of 5 independent culture experiments (three with hepatocytes of unweaned and two with hepatocytes of weaned piglets) with duplicate incubations. Mean incorporations were 195 ± 56, 263 ± 127 and 353 ± 132 dpm.24 h^{-1}. μg DNA^{-1} for the three consecutive days, respectively.

culture period (h):	4-28	28-52	52-76
		% of total bile acids	
polar metabolites [a]	19 ± 1	14 ± 2*	13 ± 2+
hyocholic acid	30 ± 5	41 ± 10	50 ± 11+
hyodeoxycholic acid	8 ± 3	9 ± 4	6 ± 3
chenodeoxy- + murocholic acid [b]	9 ± 3	12 ± 6*	10 ± 4
dihydroxy bile acids [c]	14 ± 8	8 ± 4*	8 ± 5
monohydroxy bile acids [d]	20 ± 7	16 ± 6*	14 ± 5+

[a] Radioactive material with Rf value < 0.20 (i.e. Rf of hyocholic acid).
[b] Chenodeoxycholic acid and murocholic acid were not separated in TLC system B, but could be separated in another TLC system (see Materials and Methods section); 90% of radioactivity then appeared as chenodeoxycholic acid.
[c] Two unidentified metabolites, migrating in the area of dihydroxycholanoic acids.
[d] Five metabolites migrating as monohydroxy cholanoic acids, one of these being lithocholic acid.
* denotes significant difference compared to the preceeding time period.
+ denotes significant difference with the first 24-h culture period.

After subjecting media and cell samples to deconjugation with cholyl glycine hydrolase and β-glucuronidase and subsequent solvolysis, most of the radioactivity was found upon TLC in system B in 11 metabolites (Table 3). The most prominent radioactive product was identified as hyocholic acid, accounting for 50% of total radioactivity incorporated in water-soluble metabolites during the third culture day. Another five metabolites migrated in the area of dihydroxy bile acids, among which were hyodeoxycholic acid, chenodeoxycholic acid and murocholic acid. Five metabolites migrated as monohydroxy bile acids. Rates of synthesis of these products declined with culture age. One of these was identified as lithocholic acid. The identity of radiolabelled material which resided on the origin of TLC (designated polar metabolites in Table 3) could not be established. Formation of this material declined with culture age.

b) Mass production of bile acids

Bile acid synthesis by cultured pig hepatocytes was further investigated by mass determinations of formed bile acids by gas-liquid chromatography (GLC). Also in terms of mass, hyocholic acid was by far the major bile acid synthesized. Since in serum-containing medium a considerable amount of chenodeoxycholic acid was present, and this bile acid is converted to hyocholic acid by pig liver cells (43), the amounts of chenodeoxycholic acid and hyocholic acid were added and corrected for chenodeoxycholic acid in serum. Together, these bile acids accounted for more than 85% of total bile acid synthesis. Furthermore hyodeoxycholic acid (comprising on the average 7% of bile acid synthesis) and murocholic acid (no more than 3% of bile acid synthesis) were detected. On the average, 20% of formed bile acids was found inside the cells.

Figure 2 shows mass production of bile acids by cultured hepatocytes obtained from suckling and weaned piglets during three consecutive days. Hepatocytes from weaned piglets synthesized only small amounts of bile acids when cultured in serum-free medium. However, inclusion of 10% fetal bovine serum resulted in a 7 times higher bile acid production. In accordance with cholesterol 7α-hydroxylase activities, bile acid synthesis in the presence of serum and hormones increased during the second and third day of culture. Mean rate of bile acid production by four different weaned piglet hepatocyte preparations during the third culture day amounted to 66 ng.24h^{-1}.μg DNA^{-1}.

Conversely, hepatocytes from unweaned piglets synthesized considerably smaller amounts of bile acids when cultured in the same conditions. Bile acid synthesis rate by these cells was linear from 28-76 h and amounted on the average 21 ng.24 h^{-1}.μg DNA^{-1}.

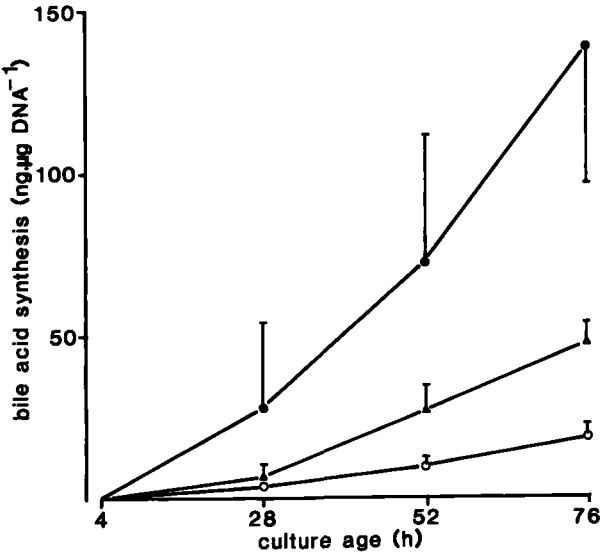

Fig. 2. Mass production of bile acids by hepatocytes of weaned and unweaned piglets in monolayer culture.
Hepatocytes isolated from weaned and unweaned piglets were plated in Williams E medium supplemented with 10% fetal bovine serum, 50 nM dexamethasone and 135 nM insulin. After a 4 h attachment period, medium was replaced by fresh medium with or without serum. At the indicated time points, cells and medium were harvested or cells were supplied with fresh medium. Bile acids in cells and medium of two dishes were enzymatically deconjugated, solvolyzed and determined by GLC. Net synthesis during each 24-h period was calculated. Values are means ± S.D. of three independent culture experiments with duplicate incubations of unweaned piglet hepatocytes, of four culture experiments with weaned piglet hepatocytes in the presence of serum and means (bar indicating the range) of two culture experiments with weaned piglet hepatocytes in absence of serum. Net synthesis rates are plotted cumulatively.
●: Weaned piglet hepatocytes in Williams E medium + 10% fetal bovine serum + 135 nM insulin + 50 nM dexamethasone.
○: Weaned piglet hepatocytes in Williams E medium + 135 nM insulin + 50 nM dexamethasone (no serum).
▲: Unweaned piglet hepatocytes in Williams E medium + 10% fetal bovine serum + 135 nM insulin + 50 nM dexamethasone.

DISCUSSION

Hepatocytes isolated from unweaned piglets have been shown to express in monolayer culture several hepatocyte specific functions, e.g. synthesis and secretion of VLDL, urea and albumin and gluconeogenesis (20,25). In this paper we show that activity of cholesterol 7α-hydroxylase however, was very low both in homogenates of isolated hepatocytes and in liver microsomes of unweaned piglets. In contrast, in microsomes

and hepatocyte-homogenates from weaned piglets, activity of this enzyme was much higher, and comparable to activities in microsomes and isolated hepatocytes of normal, adult rats (14,41). Thus, cholesterol 7α-hydroxylase seems to be induced upon weaning. Data on development of this enzyme in other species are sparse. In the rat, a similar induction has been observed, shortly after weaning (44). Likewise, cholesterol 7α-hydroxylase activity in suckling guinea pigs has been reported to be lower than in adult animals (45). A more extensive study from our laboratory on this interesting phenomenon will be reported in a separate paper.

The difference in expression of cholesterol 7α-hydroxylase activity was maintained in hepatocytes brought into monolayer culture. Enzyme activity in hepatocytes from weaned piglets, cultured in the presence of fetal bovine serum, dexamethasone and insulin was restored, after an initial fall, to the values found in freshly isolated hepatocytes, whereas it remained low in unweaned piglet hepatocytes. In addition, bile acid production was lower in cultured unweaned piglet hepatocytes than in weaned piglet hepatocytes. This difference (3-fold) was, however, considerably smaller than the difference between cholesterol 7α-hydroxylase activities (15 to 35 times at a culture age of 48 and 72 h, respectively). The mean value of cholesterol 7α-hydroxylase activities at 48 h and 72 h in the four different preparations of weaned piglet hepatocytes in which mass production of bile acids was determined, was 9 pmol/h/μg DNA. Extrapolation of this value to bile acid production by the living cell, leads to an expected rate of bile acid synthesis of 86 ng/24 h/μg DNA. This is in reasonable agreement with the actually measured rate (66 ng/24 h/μg DNA). In contrast, the rate of bile acid synthesis in unweaned piglet hepatocytes predicted by their cholesterol 7α-hydroxylase activities (5 ng/24 h/μg DNA) is 4 times lower than the actually measured rate (21 ng/24 h/μg DNA). This suggests that part of the bile acids formed by these cells in culture, may be formed by a route, bypassing cholesterol 7α-hydroxylase. The existence of such a pathway, in which the initial step is 26-hydroxylation of cholesterol has been demonstrated in rat, hamster, rabbit and man (46-48). In this context, it is interesting that homogenates from both weaned and unweaned piglet hepatocytes were well able to convert [^{14}C]cholesterol to 26-hydroxy-cholesterol in the presence of NADPH (Kwekkeboom et al., unpublished results), as homogenates of rat hepatocytes do (14).

Cholesterol 7α-hydroxylase is a cytochrome P-450 enzyme. A well known problem in the use of hepatocytes for the study of cytochrome P-450 enzymes, is the rapid loss of these enzymes in culture (49). This phenomenon was also observed with cholesterol 7α-hydroxylase activity in cultured hepatocytes from weaned piglets, but in the presence of fetal bovine serum, enzyme activity was restored again. We have made the same observation in cultured rat hepatocytes (14). Which factors in fetal bovine serum are responsible for the effect is as yet unknown.

The hormones insulin and dexamethasone were included in our standard medium because of their beneficial effects on cell viability and maintenance of differentiated cell functions (50). We observed that these hormones further induced cholesterol 7α-hydroxylase activity during the second and third culture day to values equal to those in freshly isolated hepatocytes, and partially prevented decline afterwards. Further research is needed to elucidate which of these hormones (or perhaps both) is/are responsible for these effects. Induction of bile acid synthesis by dexamethasone has been observed in rat liver cell hybrids (51) and in cultured rat hepatocytes by our group (15).

The composition of bile acids synthesized by cultured pig hepatocytes agrees well with bile acids synthesized by pig liver in vivo. Hyocholic acid, hyodeoxycholic acid and chenodeoxycholic acid were found to be secreted by bile fistula pigs (43) and to be present in bile of germ-free pigs (52) and are considered to be the primary pig bile acids.

Just like in vivo (53), bile acids synthesized by piglet hepatocytes were mainly conjugated with glycine. Sulfation of bile acids by cultured hepatocytes has already been described for Hep G2 cells (54) and rat hepatocytes (27,55), but to our best knowledge, this is the first report giving evidence for conjugation of bile acids with glucuronic acid by cultured hepatocytes. Bile acid glucuronides have been found in urine, serum and bile (31), and are formed by microsomal UDP-glucuronyltransferase activity (see e.g. Ref. 56).

In table 4 a comparison is made between rates of bile acid synthesis in vivo and in cultured hepatocytes of different species. Published bile acid synthesis rates by rat hepatocytes (cultured in serum-free media) accounted only for 3 to 8% of the in vivo rate in the rat. Comparable low bile acid production was found in this study in pig hepatocytes cultured in serum-free medium. In contrast, rates of bile acid synthesis by weaned piglet hepatocytes in the conditions in which cholesterol 7α-hydroxylase was comparable to the values in freshly isolated hepatocytes (i.e. during the third day of culture in medium with fetal bovine serum, dexamethasone and insulin), were considerably higher. Mean bile acid synthesis rate of 4 preparations of weaned piglet hepatocytes accounted for 75% of the previously reported in vivo rate. Such a high rate of bile acid synthesis has recently also been observed in cultured rabbit hepatocytes (62), surprisingly in serum-free medium.

In conclusion, in view of the high cholesterol 7α-hydroxylase activities and bile acid synthesis rates, hepatocytes from weaned, young pigs cultured in the presence of 10% fetal bovine serum, offer a suitable in vitro system for the study of bile acid synthesis. Recently, we observed that the ability to accumulate bile acids intracellularly, was much longer maintained in cultured pig hepatocytes than in rat and human hepatocytes (29). Moreover, in a preliminary publication (26) we reported that the

Table 4. Comparison of rates of bile acid synthesis in cultured hepatocytes and in vivo.

All rates have been converted to pmol/h/mg cell protein, based on the assumptions that the liver represents 4% of the body weight, 1 g liver contains 180 mg protein, and 1 mol bile acid is equivalent with 400 g. To convert rates of bile acid synthesis obtained in this study, protein/DNA ratio in pig hepatocytes was measured (50 μg protein/μg DNA (n = 3)). Only rates obtained with normal animals or hepatocytes from normal animals are included.

species	experimental system	rate of synthesis (pmol/h/mg protein)	references
rat	in vivo (faecal loss)	300 - 750	57-59
	monolayer culture (serum-free medium)	20 - 23	8,60
pig	in vivo (faecal loss)	180 ± 54[a]	61
	monolayer culture (serum-free)	15 - 24	this study
	monolayer culture (10% fetal bovine serum)	138 (62 - 263)	this study
rabbit	in vivo (faecal loss)	240	62
	monolayer culture (serum-free)	170	62

[a] mean ± S.D.

rate of bile acid synthesis in cultured pig hepatocytes can be suppressed by addition of exogenous bile acids, attesting the suitability of this in vitro model for the study of regulation of bile acid synthesis.

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REFERENCES

1. Björkhem, I (1985) in Sterols and bile acids: New Comprehensive Biochemistry (Danielsson, H and Sjövall, J, eds) Vol 12, pp 231-278, Elsevier, Amsterdam.
2. Bekersky, I and Mosbach, EH (1973) in The bile acids (Nair, PP and Kritchevsky, D, eds) pp 249-258, Plenum Press, New York.
3. Fears, R (1986) in Cholesterol 7α-hydroxylase (7α-monooxygenase) (Fears, R and Sabine, JR, eds) pp 115-132, CRC Press, Boca Raton, Florida.
4. Angelin, B, Björkhem, I and Einarsson, K (1986) in Cholesterol 7α-hydroxylase (7α-monooxygenase) (Fears, R and Sabine, JR, eds) pp 167-177, CRC Press, Boca Raton, Florida.
5. Davis, RA, Highsmith, WE, Malone-McNeal, M, Archambault-Schexnayder, J, Hyde, PM and Kuan, JW (1983) J Biol Chem 258, 4079-4082.
6. Kubaska, WM, Gurley, EC, Hylemon, PB, Guzelian, PS and Vlahcevic, ZR (1985) J Biol Chem 260, 13459-13463.
7. Kubaska, WM, Gurley, EC, Hylemon, PB, Heuman, DM, Mosbach, EH and Vlahcevic, ZR (1987) Biochim Biophys Acta 920, 195-204.
8. Davis, RA, Hyde, PM, Kuan, JW, Malone-McNeal, M and Archambault-Schexnayder, J (1983) J Biol Chem 258, 3661-3667.
9. Princen, HMG, Huijsmans, CMG, Kuipers, F, Vonk, RJ and Kempen, HJM (1986) J Clin Invest 78, 1064-1071.
10. Sampson, WJ, Suffolk, RA, Bowers, P, Houghton, JD, Botham, KM and Suckling, KE (1987) Biochim Biophys Acta 920, 1-8.
11. Sampson, WJ, Houghton, JD, Bowers, P, Suffolk, RA, Botham, KM, Suckling, CJ and Suckling, KE (1988) Biochim Biophys Acta 960, 268-274.
12. Mackinnon, AM, Drevon, CA, Sand, TM and Davis, RA (1987) J Lipid Res 28, 847-855.
13. Ford, RP, Botham, KM, Suckling, KE and Boyd, GS (1985) FEBS Lett 179, 177-180.
14. Princen, HMG, Meijer, P, Kwekkeboom, J and Kempen, HJM (1988) Anal Biochem 171, 158-165.
15. Princen, HMG, Meijer, P and Hofstee, B (1989) Biochem J 262, 341-348.
16. Chapman, MJ (1986) in Methods in Enzymology, (Segrest, JP and Alberts, JJ, eds) Vol 128, pp 70-143, Academic Press Inc., New York.
17. Shepherd, J, Packard, CJ, Bicker, S, Lawrie, TDV and Morgan, HG (1980) N Engl J Med 302, 1219-1222.
18. Spady, DK, Turley, SD and Dietschy, JM (1985) J Lipid Res 26, 465-472.
19. Huff, MW, Telford, DE, Woodcraft, K and Strong, WLP (1985) J Lipid Res 26, 1175-1186.
20. Pangburn, SH, Newton, RS, Chang, C, Weinstein, DB and Steinberg, D (1981) J Biol Chem 256, 3340-3347.
21. Bachorik, PS, Franklin, FA, Virgil, DG and Kwiterovich, PO (1982) Biochemistry 21, 5675-5684.
22. Kosygh, VA, Preohrazhensky, SN, Ivanov, VO, Tsibulsky, VP, Repin, VS and Smirnov, UN (1985) FEBS Lett 183, 17-20.
23. Edge, SB, Hoeg, JM, Triche, T, Schneider, PD and Brewer, HB (1986) J Biol Chem 261, 3800-3806.
24. Havekes, LM, Verboom, H, De Wit, E, Yap, SH and Princen, HMG (1986) Hepatology 6, 1356-1360.
25. Caperna FJ, Failla, ML, Kornegay, ET, Richards, MP and Steele, NC (1985) J Animal Sci 61, 1576-1586.
26. Kwekkeboom, J, Van Voorthuizen, EM, Princen, HMG and Kempen, HJ (1988) Biochim Biophys Res Commun 155, 850-856.
27. Princen, HMG and Meijer, P (1988) Biochem Biophys Res Commun 154, 1114-1121.
28. Tserng, KY, Hackey, DL and Klein, PO (1977) J Lipid Res 18, 404-407.
29. Kwekkeboom, J, Princen, HMG, Van Voorthuizen, EM, Meijer, P and Kempen, HJM (1989) Biochem Biophys Res Commun 162, 619-625.
30. Kempen, HJM, Vos-Van Holstein, MPM and De Lange, J (1982) J Lipid Res 23, 823-830.
31. Stiehl, A, Becker, M, Czygan, P, Frohling, W, Kommerell, B, Rotthauwe, HW and Sehn, M (1980) Eur J Clin Invest 10, 307-316.
32. O'Moore, RRL and Percy-Robb, JW (1973) Clin Chim Acta 43, 39-47.
33. Eneroth, P (1963) J Lipid Res 4, 11-16.
34. Hukkelhoven, MWAC, Vromans, E, Markslag, AMG and Vermorken, AJM (1981) Anticancer Res 1, 341-344.
35. Lowry, OH, Rosebrough, NJ, Farr, AL and Randall, RJ (1951) J Biol Chem 193, 265-275.
36. Gamble, W, Vaughan, M, Kruth, MS and Arigan, J (1978) J Lipid Res 19, 1068-1071.

37. Bergmeyer, HU and Bernt, E (1970) in Methoden der enzymatischen Analyse (Bergmeyer, HU, ed) pp 533-538, Verlag Chemie, Weinheim, FRG.
38. Bergmeyer, HU and Bernt, E (1970) in Methoden der enzymatischen Analyse (Bergmeyer, HU, ed) pp 2020-2035, Verlag Chemie, Weinheim, FRG.
39. Bissell, DM, Hammaker, LE and Meyer, UA (1973) J Cell Biol 59, 722-734.
40. Jauregui, HO, Hayner, NT, Driscoll, JL, Williams-Holland, R, Lipsky, MH and Galletti, PM (1981) In Vitro 17, 1100-1110.
41. Cohen, LH, Griffioen, AM, Wanders, RJA, Van Roermund, CWT, Huijsmans, CMG and Princen, HMG (1986) Biochim Biophys Res Commun 138, 335-341.
42. Hylemon, PB, Gurley, EC, Kubaska, WM, Whitehead, TR, Guzelian, PS and Vlahcevic, ZR (1985) J Biol Chem 260, 1015-1019.
43. Bergström, S, Danielsson, H and Göransson, Å (1959) Act Chem Scand 13, 776-783.
44. Hahn, P and Innis, SM (1984) Biol Neonate 46, 48-52.
45. Li, JR, Subbiah, MTR. and Kottke, BA (1979) Steroids 34, 47-55.
46. Wachtel, N, Emerman, S and Javitt, NB (1968) J Biol Chem 243, 5207-5212.
47. Anderson, KE, Kok, E and Javitt, NB (1972) J Clin Invest 51, 112-117.
48. Ayaki, Y, Kok, E and Javitt, NB (1989) J Biol Chem 264, 3818-3821.
49. Sirica, AE and Pitot, HC (1980) Pharmacol Rev 31, 205-228.
50. Guguen-Guillouzo, C and Guillouzo, A (1983) Mol Cell Biochem 53/54, 35-56.
51. Everson, GT (1987) in Bile acids and the liver (Paumgartner, G, Stiehl, A and Gerok, W, eds) pp 61-64, MTP Press Ltd., Lancaster, UK.
52. Haslewood, GAD (1971) Biochem J 123, 15-18.
53. Haslewood, GAD and Ogan, AU (1957) Biochem J 67, 30 P.
54. Everson, GT and Polokoff, MA (1986) J Biol Chem 261, 2197-2201.
55. Kirkpatrick, RB and Belsaas, RA (1985) J Lipid Res 26, 1431-1437.
56. Radominska-Pyrek, A, Zimniak, P, Golunski, E, Lester, R and Pyrek, J (1986) J Lipid Res 27, 89-101.
57. Myant, NB (1981) The Biology of Cholesterol and Related Steroids, p 247. William Heinemann Medical Books Ltd., London.
58. Kellogg, TF (1973) in The Bile Acids, Chemistry, Physiology and Metabolism, vol. 2 (Nair, PP and Kritchevsky, D, eds) pp 283-304, Plenum Press, New York.
59. Uchida, K, Nomura, Y, Masumi, K, Takase, H, Takano, K and Takeuchi, N (1978) J Lipid Res 19, 544-552.
60. Ford, RP, Botham, KM, Suckling, KE and Boyd, G.S. (1985) Biochim Biophys Acta 830, 185-191.
61. Kim, DN, Rogers, DH, Li, JR, Reiner, JM, Lee, KT and Thomas, WA (1977) Exp Mol Pathol 26, 434-447.
62. Whiting, MJ, Wishart, RA, Growing, MR, McManus, ME and Mackinnon, AM (1989) Biochim Biophys Acta 1001, 176-184.

CHAPTER 5

POSTNATAL DEVELOPMENTAL PROFILE OF 3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE, SQUALENE SYNTHETASE AND CHOLESTEROL 7α-HYDROXYLASE ACTIVITIES IN LIVER OF DOMESTIC SWINE

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SUMMARY

Activities of 3-hydroxy-3-methylglutaryl-CoA reductase, squalene synthetase and cholesterol 7α-hydroxylase, measured in liver microsomal preparations from domestic swine between birth and adolescence, correlated strongly in individual animals. A synchronous increase was observed between 4 and 6 weeks after birth, i.e., immediately after weaning. Rise in activity was highest for HMG-CoA reductase (thirty-fold), and smallest for squalene synthetase (five-fold). In pubertal (16 to 30 weeks old) pigs, activities of these enzymes had the same low values as in suckling piglets. The increase of both HMG-CoA reductase and squalene synthetase activities may be caused by the shift from high-cholesterol milk intake to a chow diet with low-cholesterol content. The rise in cholesterol 7α-hydroxylase activity might be due to other dietary or hormonal factors.

INTRODUCTION

The developmental profile of the rate-limiting enzymes of cholesterol and bile acid synthesis, has been studied only in the rat and fragmentary in the guinea pig. Activity of rat liver HMG-CoA reductase (EC 1.1.1.34), the rate-limiting enzyme in cholesterol biosynthesis, is high in late fetal rats, declines to low values during the suckling period and increases at weaning, overshooting adult levels (1-3). Conversely, no changes in HMG-CoA reductase activity were observed during postnatal development in the guinea pig (4). Activity of cholesterol 7α-hydroxylase (EC 1.14.13.17), the rate-limiting enzyme in bile acid synthesis, was found to be low in fetal and newborn suckling rats, but at weaning the enzyme activity rapidly increased and a circadian rhythm was established (5,6). Data for activity of this enzyme in

developing guinea pigs are contradictory: cholesterol 7α-hydroxylase activity in suckling animals has been reported to be lower (4), equal to (7) or even higher than in adults (8).
Apparently, marked changes in activities of these enzymes occur in the developing rat liver, but it is not clear whether these also take place in other species. This is particularly interesting, because during postnatal ontogeny, pronounced variations in serum cholesterol levels have been observed in all animal species so far examined. Serum cholesterol rapidly rises after birth, and during the suckling period a hypercholesterolemia is developed. After weaning serum cholesterol drops sharply (2,9-11).
In the present study we have examined activities of these enzymes during postnatal development of a third animal species, the domestic swine (Sus domesticus). In addition, the developmental profile of squalene synthetase (EC 2.5.1.21) was studied, since this enzyme has been mentioned as a secondary regulation site in cholesterol biosynthesis, probably playing a role in the metabolic targeting of mevalonate to non-sterol metabolites (12-14).
The pig is considered to be more comparable with man in respect to its cholesterol metabolism, than the rat. First, its plasma cholesterol is transported for a large part in LDL, whereas in the rat HDL is the major lipoprotein class (15). Second, its hepatocytes express active receptor mediated LDL uptake and degradation (16). Moreover, the pig is an omnivorous and diurnal animal.

METHODS

Piglets (F_1-hybrids of Large Yorkshire and Nederlands Landvarken) were bred at the pig farm of the Veterinary Department, State University of Utrecht, The Netherlands. From the third week after birth, piglets had ad libitum access to piglet chow (Brokking B.V., Utrecht) and water. They were removed from their sows at 4 weeks after birth.
Enzyme activities were determined in liver microsomes from 21 female swine, varying in age from 2 to 30 weeks. Livers were excised at 9 a.m. after anaesthesia with Stressnil and Hypnodil (Janssen Pharmaceuticals, Beerse, Belgium), and immediately cooled on ice. In most cases, microsomes were prepared from fresh liver tissue. In five cases, pieces of liver were frozen in liquid nitrogen and stored at -80°C prior to microsome preparation.
Liver tissue was put through a stainless steel press yielding pulp which was free of connective tissue (17) and homogenized in 100 mM sucrose, 100 mM potassium phosphate (pH = 7.4), 2 mM EDTA and 5 mM DTT in a Potter-Elvehjem

homogenizer with motor-driven pestle. Microsomes were prepared as previously described (18), resuspended in the buffer mentioned above, frozen in liquid nitrogen and stored at -80°C. Microsomal protein and cholesterol were determined by the methods of Lowry (19) and Gamble (20), respectively.

The assays for HMG-CoA reductase squalene synthetase and cholesterol 7α-hydroxylase activities, as previously described for rat liver microsomes (13,18,21) were adapted to determination of enzyme activities in pig liver microsomes.

Pilot experiments with the HMG-CoA reductase assay showed that formation of [^{14}C]mevalonic acid by pig liver microsomes was linear with incubation time up to 50 min and with microsomal protein concentration up to at least 2.5 mg/ml at a substrate concentration of 0.5 mM. Therefore, the assay was performed for 40 min with 1.5 mg microsomal protein per ml.

Squalene synthetase activity in pig liver microsomes was saturated at a substrate concentration of 40 μM, while slight substrate inhibition occurred at substrate concentrations above 90 μM as was previously reported by Krishna et al. (22). Reaction rates were linear with microsomal protein up to 0.4 mg per ml and with incubation times up to 20 min. Squalene synthetase activities were assayed as described in ref. (13) with 80 μM [^{14}C]farnesylpyrophosphate, and 0.3 mg microsomal protein per ml during 15 minutes incubations.

Cholesterol 7α-hydroxylase activities were measured with 0.5 mg microsomal protein in one ml assay mixture and an incubation time of 30 min. Formation of 7α-hydroxy-cholesterol by pig liver microsomes was linear with respect to microsomal protein up to 0.6 mg/ml, and proportional to incubation time up to 60 min. For calculation of enzyme activity (in terms of pmoles 7α-hydroxy-cholesterol formed), it was assumed that 70% of the endogenous microsomal unesterified cholesterol had access to the enzyme (23).

The effect of freezing of liver tissue prior to microsome preparation on enzyme activities was investigated by comparison of enzyme activities in microsomes prepared from three different livers prior to, or after, storage of whole liver tissue at -80°C. Cholesterol 7α-hydroxylase and squalene synthetase activities were not significantly different in both sets of microsome preparations, but HMG-CoA reductase activities were 78 ± 12% (n = 3) lower in the microsomes prepared from frozen liver tissue compared with activities in the microsomes prepared from the same livers before freezing. Therefore, only the values of the HMG-CoA reductase activities obtained with microsomes prepared from fresh liver are presented.

RESULTS AND DISCUSSION

Figure 1 shows that activities of HMG-CoA reductase, squalene synthetase and cholesterol 7α-hydroxylase were markedly elevated in most piglets which were 6 to 8 weeks old, i.e. immediately after weaning. In pubertal (16 to 30 weeks old) swine, activities were decreased again.

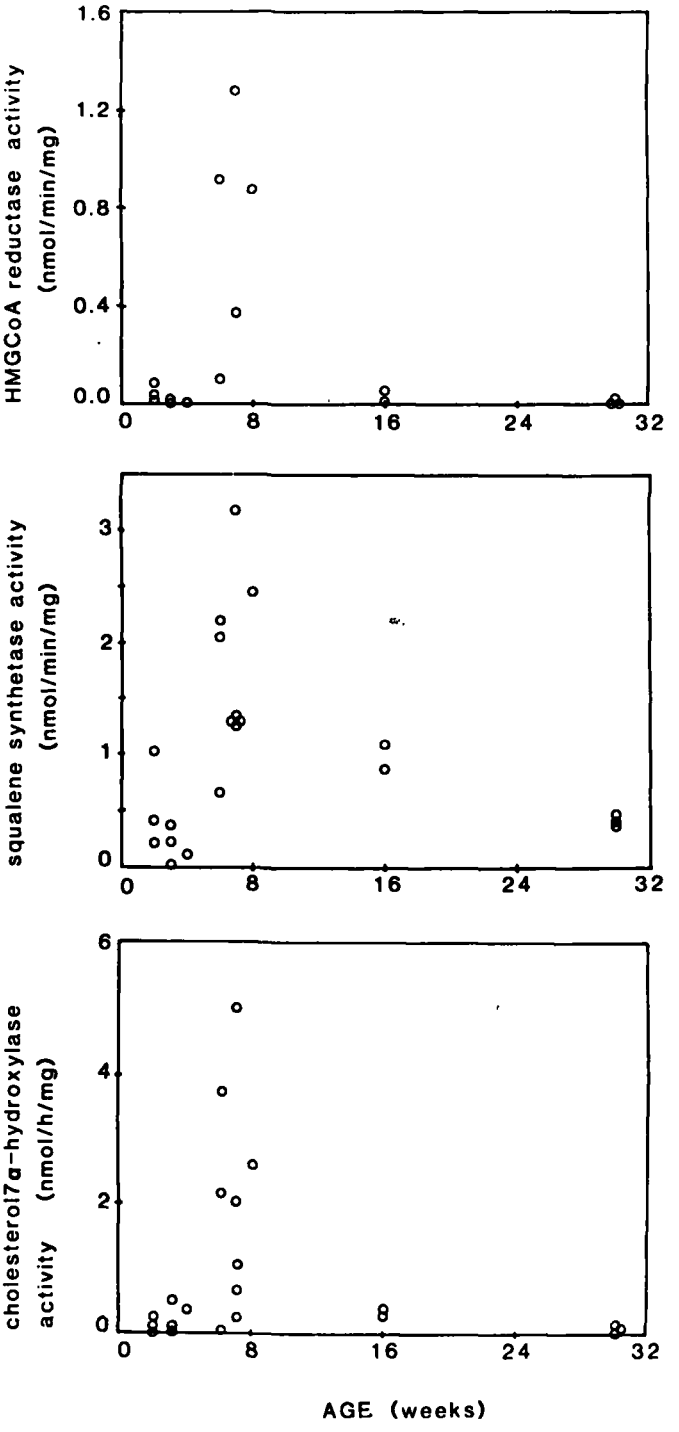
According to this variation, three age-groups were formed: suckling animals (2 to 4 weeks old) weaned animals (6 to 8 weeks old) and pubertal animals (16 and 30 weeks old). One-way analysis of variance showed that differences in activities between these age-groups were significant for each enzyme (Table 1). The increase in activity at weaning was significant for each enzyme and was most prominent for HMG-CoA reductase (thirty-fold). Cholesterol 7α-hydroxylase activity was elevated twelve-fold and squalene synthetase five-fold. In pubertal swine activities of these enzymes were similar to those in suckling animals.

Table 1. Comparison of HMG-CoA reductase, squalene synthetase and cholesterol 7α-hydroxylase activities in suckling (2 to 4 weeks old), weaned (6 to 8 weeks old) and pubertal (16 and 30 weeks old) swine.

From the data depicted in Fig. 1, means ± S.E.M. for each age-group of n animals were calculated. Data were analysed according to this classification with one-way analysis of variance (ANOVA). F-values are presented in the last column. Significancy of differences between the age-groups was tested for each enzyme with Fischer's LSD test. Values with different superscripts (a,b) are significantly different ($p < 0.05$).

	age groups			ANOVA
	suckling	weaned	pubertal	F
HMG-CoA reductase (pmol/min/mg)	23 ± 13[a] (n = 6)	708 ± 210[b] (n = 5)	17 ± 10[a] (n = 5)	11.91 ($p < 0.005$)
Squalene synthetase (pmol/min/mg)	347 ± 125[a] (n = 7)	1759 ± 257[b] (n = 9)	652 ± 143[a] (n = 5)	13.51 ($p < 0.005$)
Cholesterol 7α-hydroxylase (pmol/h/mg)	168 ± 72[a] (n = 7)	1941 ± 554[b] (n = 9)	177 ± 54[a] (n = 5)	6.49 ($p < 0.01$)

Fig. 1 (next page). HMG-CoA reductase, squalene synthetase and cholesterol 7α-hydroxylase activities during postnatal development of swine.
Activities of HMG-CoA reductase, squalene synthetase and cholesterol 7α-hydroxylase were determined in liver microsomes from swine varying in age from 2 to 30 weeks. Each point represents the mean of duplicate determinations of the enzyme activity in microsomes of one animal. Since loss of HMG-CoA reductase occurred during freezing and thawing of liver tissue prior to microsome preparation, merely the values for this enzyme obtained with microsomes prepared from fresh liver are presented.


HMGCoA reductase activity (nmol/min/mg)
1.6
1.2
0.8
0.4
0.0
0
8
16
24
32
squalene synthetase activity (nmol/min/mg)
3
2
1
0
0
8
16
24
32
cholesterol7α-hydroxylase activity (nmol/h/mg)
6
4
2
0
0
8
16
24
32
AGE (weeks)

The postnatal developmental patterns of HMG-CoA reductase and cholesterol 7α-hydroxylase in domestic swine liver as reported here, are similar to those in rat liver (1-3,5,6). The increase of squalene synthetase activity at weaning has not been previously reported, and is a new example of covariation of this enzyme and HMG-CoA reductase. Just like the induction of these two enzymes by cholestyramine treatment (13), the variation in HMGCoA-reductase activity is much higher than that in squalene synthetase activity. A marked rise of squalene synthetase activity during the middle of the nursing period, as suggested for rats (24), was not observed in swine. These changes classify these enzymes, with regard to their ontogeny, to the late suckling cluster, as defined by Greengard (25).

Table 2 shows that the activities of HMG-CoA reductase, squalene synthetase and cholesterol 7α-hydroxylase were correlated significantly in the individual animals. Together with the parallel changes during the postnatal period, this suggests that these enzymes may be regulated by (a) common factor(s) or that changes in one of them are causal for changes in the others. Suckling animals, including pigs, have high serum cholesterol levels (2,9-11), mainly due to the high cholesterol content of the milk (10). After weaning to a low cholesterol diet, serum cholesterol decreases sharply. Therefore the low HMG-CoA reductase activities in suckling rats, and the increase in activity of this enzyme after weaning have been attributed to the opposite variations in serum cholesterol (2,9). This may also be the case in swine. It has been reported that in swine supplementation of the diet with cholesterol results in an increase of serum cholesterol and a decrease of liver HMG-CoA reductase activity

Table 2. Correlations of the activities of HMG-CoA reductase, squalene synthetase and cholesterol 7α-hydroxylase and correlations between enzyme activities and microsomal unesterified cholesterol in n individual animals.

Correlation statistics on the values depicted in Fig. 1 and the assessory microsomal free cholesterol values were performed with the Spearman's rank correlation test.

Correlated parameters	Spearman's rank correlation coefficient
HMG-CoA reductase - squalene synthetase	0.89 (n = 16)*
HMG-CoA reductase - cholesterol 7α-hydroxylase	0.52 (n = 16)*
squalene synthetase - cholesterol 7α-hydroxylase	0.78 (n = 21)*
HMG-CoA reductase - microsomal cholesterol	0.19 (n = 16)
squalene synthetase - microsomal cholesterol	-0.14 (n = 21)
cholesterol 7α-hydroxylase - microsomal cholesterol	-0.04 (n = 21)

*denotes significant correlation ($p < 0.05$)

(26). Since the pig chows used in this study contained, as measured by gas-liquid chromatography, merely 0.016% (wt) cholesterol, while sow's milk contains 0.22% on solid basis (11), it is conceivable that the observed increase in HMG-CoA reductase activity upon weaning was caused by a decreased delivery of cholesterol to the liver. In this up-regulation of HMG-CoA reductase an intracellular sterol pool is probably involved.

Strikingly, there was virtually no correlation between the activities of the three enzymes and microsomal unesterified cholesterol content (Table 2). However, the regulatory sterol pool may not correspond with the microsomal cholesterol.

Changes in serum cholesterol could also lead to the observed variations in squalene synthetase activity, since in vitro studies with Hep G2 cells showed that activity of this enzyme is inhibited by sterols (14).

On the other hand, cholesterol seems not to be the regulating factor in the postnatal variation of cholesterol 7α-hydroxylase activity. In the pig, cholesterol feeding has no effect on activity of this enzyme, notwithstanding increase of serum cholesterol concentration (26,27). In the rat, supplementation of the diet with cholesterol even stimulates cholesterol 7α-hydroxylase activity (28). Possibly, the pig chow exerts a cholestyramine-like effect on the intestinal absorption of bile acids, resulting in a reduced feedback inhibition of cholesterol 7α-hydroxylase. Such an effect has been ascribed to dietary fibers, but experimental data are not quite consistent (29). Alternatively, the changes of cholesterol 7α-hydroxylase activity in the postnatal period may be connected to the ontogeny of the activity of the hypothalamo-pituitary-adrenal axis (5). Recently we showed that glucocorticoids induce this enzyme in cultured rat hepatocytes (30). Possibly, the hypercholesterolemia in newborns is enhanced by the relative underdevelopment of bile acid synthesis in this life period.

It is remarkable that activities of all three enzymes are decreased again in the pubertal period. Decline of HMGCoA-reductase and cholesterol 7α-hydroxylase activities during the first year of life has also been observed in the rat (2,5,31,32), but in this species enzyme activities did not return to the low suckling values. Whether gonadal hormones play any role in this phenomenon is unknown. Further research is needed to establish whether the factors suggested above are actually responsible for the observed changes in the neonatal period.

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REFERENCES

1. McNamara, DJ, Quackenbush, FW and Rodwell, VW (1972) J Biol Chem 247, 5805-5810.
2. Ness, GC, Miller, JP, Moffler, MH and Woods, LS (1979) Lipids 14, 447-450.
3. Hahn, P and Walker, BL (1979) Can J Biochem 57, 1216-1219.
4. Li, JR, Subbiah, R and Kottke, BA (1979) Steroids 34, 47-55.
5. Van Cantfort, MJ (1972) C R Acad Sci Paris 274, 2593-2595.
6. Hahn, P and Innis, SM (1984) Biol Neonate 46, 48-52.
7. Subbiah, MTR, Yunker, RL, Menkhaus, A and Poe, B (1985) Am J Physiol 249, E251-E256.
8. Yunker, RL and Subbiah, MTR (1985) Biochem Biophys Res Commun 132, 702-707.
9. Hahn, P (1982) in Annual Review of Nutrition (Darby, WJ, Broquist, HP and Olson, RE, eds) Vol 2, pp 91-112, Palo Alto, California.
10. Roberts, DCK, Huff, MW and Carroll, KK (1979) Nutr Metab 23, 476-486.
11. Carroll, KK and Hamilton, RMG (1973) Lipids 8, 635-640.
12. Brown, MS and Goldstein, JL (1980) J Lipid Res 21, 505-517.
13. Cohen, LH, Griffioen, AM, Wanders, RJA, Van Roermund, CWT, Huysmans, CMG and Princen, HMG (1986) Biochem Biophys Res Commun 138, 335-341.
14. Cohen, LH, Van Miert, E and Griffioen, M (1989) Biochim Biophys Acta 1002, 69-73.
15. Chapman, MJ (1986) in Methods in Enzymology (Segrest, JP and Alberts, JJ, eds) Vol 128, pp 70-143, Academic Press Inc, New York.
16. Pangburn, SH, Newton, RS, Chang, C, Weinstein, DB and Steinberg, D (1981) J Biol Chem 256, 3340-3347.
17. Popjak, G (1969) in Methods in Enzymology (Clayton, RB, ed.) Vol 15, pp 393-454, Academic Press Inc, New York.
18. Princen, HMG, Huijsmans, CMG, Kuipers, F, Vonk, RJ and Kempen, HJM (1986) J Clin Invest 78, 1064-1071.
19. Lowry, OH, Rosebrough, NJ, Farr, AL and Randall, RJ (1951) J Biol Chem 193, 265-275.
20. Gamble, W, Vaughan, M, Kruth, MS and Arigan, J (1978) J Lipid Res 19, 1068-1071.
21. Cohen, LH and Griffioen, M (1984) Biochem J 255, 61-67.
22. Krishna, G, Whitlock, JW, Feldbrugge, DH and Porter, JW (1966) Arch Biochem Biophys 114, 200-215.
23. Van Cantfort, J and Gielen, J (1975) Eur J Biochem 55, 33-40.
24. Bruenger, E and Rilling, HC (1986) Biochim Biophys Acta 876, 500-506.
25. Greengard, O (1970) in Biochemical Actions of Hormones (Litwack, G, ed) Vol 1, pp 53-87, Academic Press, New York.
26. Kim, DN, Rogers, DH, Li, JR, Reiner, JM, Lee, KT and Thomas, WA (1977) Exp Mol Pathol 26, 434-447.
27. Li, JR, Kim, DN, Lee, KT, Reiner, JM and Thomas, WA (1980) Exp Mol Pathol 32, 52-60.
28. Björkhem, I (1985) in Sterols and Bile Acids (Danielsson, H and Sjövall, J, eds) pp 231-278, Elsevier, Amsterdam.
29. Ginter, E (1986) in Cholesterol 7α-hydroxylase (Fears, R and Sabine, JR, eds) pp 103-114, CRC Press, Boca Raton, Florida.
30. Princen, HMG, Meijer, P and Hofstee, B (1989) Biochem J 262, 341-348.
31. Choi, YS, Ide, T and Sugano, M (1987) Exp Gerontol 22, 339-349.
32. Story, JA and Kritschevski, O (1974) Experientia 30, 242-243.

CHAPTER 6

FEEDBACK INHIBITION OF BILE ACID SYNTHESIS IN CULTURED PIG HEPATOCYTES

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SUMMARY

Bile acid synthesis by cultured pig hepatocytes, as measured by conversion of [^{14}C]cholesterol to bile acids, increased during the second and third day of culture. This rise was inhibited after addition of various conjugated and unconjugated bile acids in a concentration of 100 μM. It could be completely prevented by cycloheximide, indicating that de novo protein synthesis is required for the increase in bile acid formation. No effect of exogenous bile salts on LDH release to the medium or on cellular ATP content was observed, demonstrating that hepatocyte viability was not affected. During the period in which bile acid synthesis was inhibited, pig hepatocytes were able to accumulate taurocholic acid (100 μM) up to 7-18 nmol per mg cell protein (decreasing during culture time). It is concluded that feedback regulation of bile acid synthesis is exerted by direct action of bile acids on the hepatocyte.

INTRODUCTION

The catabolism of cholesterol to bile acids is the predominant pathway through which cholesterol is eliminated from the body. An important regulatory mechanism of this process is thought to be exerted by the bile acids returning to the liver via the enterohepatic circulation. This concept of negative feedback control is based on in vivo studies in which increased bile acid synthesis following biliary diversion and restoration of synthesis rates to normal after intraduodenal infusion of bile acids were observed (1).
However, in recent years in experiments with cultured rat hepatocytes no inhibition of bile acid synthesis by its end products could be demonstrated (2-4). This casted doubt upon the theory that bile acids act directly on the liver to repress bile acid synthesis.

In this paper we describe experiments with cultured pig hepatocytes, and show that in these cells bile acid synthesis can directly be inhibited by incubation of these cells with bile acids.

MATERIALS AND METHODS

Materials.

Williams E culture medium and fetal bovine serum were purchased from Flow Laboratories, Irvine, Scotland. [4-^{14}C]Cholesterol, [^{3}H(G)]taurocholic acid and [24-^{14}C]taurocholic acid were obtained from New England Nuclear, Dreieich, FRG. Taurocholic acid was obtained from Sigma Chemicals, St. Louis, MO, USA; glycohyodeoxycholic, hyocholic and hyodeoxycholic acid were from Steraloids Inc., Wilton, NH, USA; chenodeoxycholic acid from Serva, Heidelberg, FRG and glycochenodeoxycholic acid from Supelco, Bellefonte, PA, USA. Taurohyocholic acid was synthesized from hyocholic acid and taurine as described in reference 5.
All bile acids used were pure (> 99%), as verified by gas-liquid chromatography, except chenodeoxycholic and glycochenodeoxycholic acid which contained respectively 6% and 7% impurities, consisting of other bile acids.

Methods.

Pig hepatocyte isolation and culture. Hepatocytes were isolated from livers of seven weeks old female pigs (hybrid of Large Yorkshire and Nederlands Landvarken) by subsequent perfusion with a Ca^{2+}-free buffer supplemented with EGTA and with collagenase solution.
Hepatocytes were seeded at a density of 1.5 10^5 viable cells/cm^2 on 60 mm diameter plastic culture dishes (Costar, Cambridge, MA) in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 135 nM insulin, 50 nM dexamethasone, 100 IU/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in a 5% CO_2/95% air atmosphere. The medium (2.5 ml/dish) was renewed after 4 h, and every 24 h thereafter. Non-conjugated bile acids used for regulation studies were administered in ethanol: 0.9% NaCl (1:1) to the culture medium. Conjugated bile acids in a mixture of ethanol and 0.9% NaCl (1:4). Ethanol concentration never exceeded 0.1%, which had no influence on bile acid synthesis from [^{14}C]cholesterol.

Conversion of labeled cholesterol into bile acids. Bile acid formation from [4-^{14}C]cholesterol was determined by adding 0.5 μCi [4-^{14}C]cholesterol solubilized in

fetal bovine serum to each culture dish, and harvesting cells and medium 24 hours later (3,6).
After addition of 0.01 μCi [^{3}H(G)]taurocholic acid as recovery standard, bile acids were separated from lipids by extraction with chloroform and methanol (7) and further isolated using a Sep-pak C18 cartridge as described before (8). The bile acids were recovered from the cartridge with methanol. ^{3}H and ^{14}C radioactivity in part of the methanol eluate were determined by liquid scintillation counting in Picofluor^R (Packard) using a double label program with external standardization for quench correction.
Added bile acids in the concentrations used in these experiments did not influence cellular uptake of labeled cholesterol, as determined by counting the radioactivity in the chloroform phase after extraction of the cells with chloroform/methanol.

Bile acid uptake. Uptake of [^{14}C]taurocholic acid was studied in culture dishes of 35 mm diameter with 1.5.10^6 hepatocytes and 1 ml culture medium essentially as described in (9). At 24, 48 and 72 h culture time 20 μl of a solution of 2.5 mM [^{14}C]taurocholic acid (0.1 μCi) in Williams E medium was added to each dish. Hepatocytes were incubated at 37°C in 5% CO_2/95% air. At the indicated time points dishes were placed on ice, the medium was aspirated and cells were washed five times with ice-cold Hank's balanced salt solution. Cells were then scraped off with a rubber policeman and suspended in water. An aliquot was taken for protein determination and radioactivity in part of the sample was determined by liquid scintillation counting in Picofluor^R. Uptake was expressed as nmol taurocholic acid/mg cell protein. Non-specific binding was measured at 0°C and was substracted from values obtained at 37°C.

Protein and DNA determination. Protein was determined by the method of Lowry (10) and DNA using mithramycin (11) with calf thymus DNA as a standard.

LDH and ATP determination. LDH activity was determined in media and cells, after lysis in 0.2% Triton X-100, as described (12). ATP was extracted with perchloric acid and determined using the hexokinase/glucose-6-phosphate dehydrogenase method (13).

RESULTS

Effect of added bile acids on bile acid synthesis.
Bile acid synthesis was determined by measurement of conversion of [4-^{14}C]chole-

sterol to methanol/ water soluble materials during 24-h periods (6,25), consisting of conjugated bile acids. After enzymatic deconjugation with cholylglycine hydrolase, hyocholic acid (the main bile acid), hyodeoxycholic acid, chenodeoxycholic acid and lithocholic acid could be identified by thin layer and gas-liquid chromatography as will be reported extensively elsewhere (J. Kwekkeboom, H.M.G. Princen, E.M. van Voorthuizen, H.J.M. Kempen, submitted). Since in incubations without exogenous bile acids, on the average 25% of bile acids was detected inside the cells, radioactivity in medium and cells was added. Fig. 1 shows that conversion of [4-^{14}C]cholesterol to bile acids increased during three subsequent 24-h culture periods. This rise was completely abolished by the addition of 5 μM cycloheximide during the second and third 24-h culture period, indicating that de novo protein synthesis is required for the elevation of bile acid formation. However, this rise was also inhibited by 100 μM taurocholic acid present in the culture medium from 28-76 h. This concentration approximates portal bile acid concentration in the pig (on the average 84 μM with maximal concentrations of 134 μM postprandially (14). To find out whether this inhibitory effect was also elicited by other bile acids, we incubated hepatocytes with several other conjugated and un-conjugated bile acids in a concentration of 100 μM. All inhibited bile acid synthesis during the third 24-h culture period, suppression ranging from 26 to 59% (Table 1).

Uptake of taurocholic acid.

Since in experiments with rat hepatocytes in which no feedback inhibition was observed, cellular uptake of added bile acids was poor (3,4), the capacity of pig hepatocytes in culture to accumulate added bile acids was assessed. Fig. 2 shows the time course of uptake of 50 μM [^{14}C]taurocholic acid by hepatocytes cultured for 24, 52 and 76 h. Taurocholic acid concentration reached a plateau after 2 h. Hepatocytes cultured for 24 h were able to concentrate 50 μM taurocholic acid up to 13 nmol.mg protein^{-1}. Assuming that the cellular volume of pig hepatocytes is the same as for cultured rat hepatocytes (2.5 μl/mg protein) (15,16), this is equivalent to an apparent intracellular taurocholic acid concentration of 5.2 mM, which is in the same order of magnitude as values reported for freshly isolated (17,18) and 27 h cultured (9) rat hepatocytes incubated with taurocholic acid. Three hour incubations with 100 μM taurocholic acid resulted in an intracellular concentration of 18 nmol.mg protein, 14 nmol.mg protein^{-1} and 7 nmol.mg protein^{-1} at 24, 48 and 72 h, respectively. Therefore, although taurocholic acid concentrating capacity declined during culture time, pig hepatocytes remained able to concentrate taurocholic acid intracellularly during the time in which feedback studies were performed.

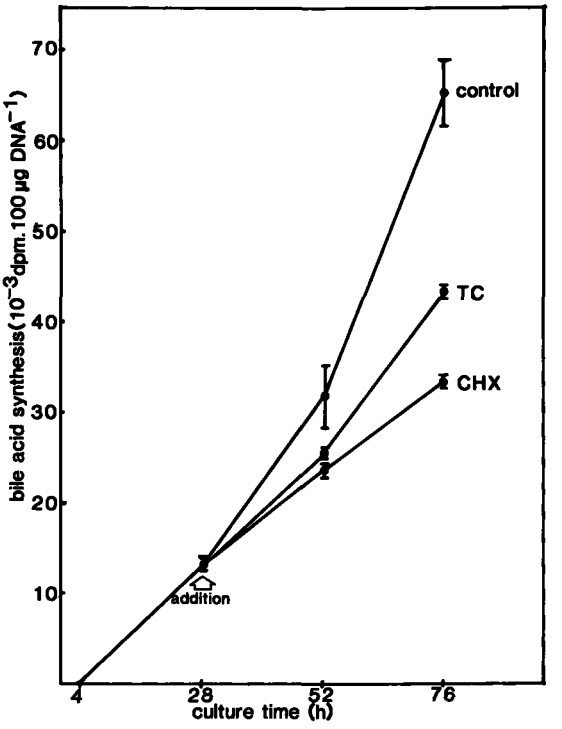

Fig. 1. Influence of taurocholic acid and cycloheximide on the time course of bile acid synthesis from [^{14}C]cholesterol by cultured pig hepatocytes. Four, 28 and 52 h after plating medium was replaced by fresh medium containing [^{14}C]cholesterol and 24 h later media and cells were harvested and ^{14}C-labeled bile acids determined. Values represent the sum of intracellular and extracellular bile acids, and figures of subsequent 24-h periods were plotted cumulatively. Cycloheximide (CHX; 5 μM) and taurocholic acid (TC; 100 μM) were added from 28 to 76 h culture time. Values are expressed as means of triplicate determinations ± S.D.

Effect of bile acids on cell viability.

Table 2 shows that LDH release to the medium was not enhanced in incubations with bile acids in the concentration used in feedback experiments and that ATP concentration remained the same as in the control, in good agreement with the physiological level (19).

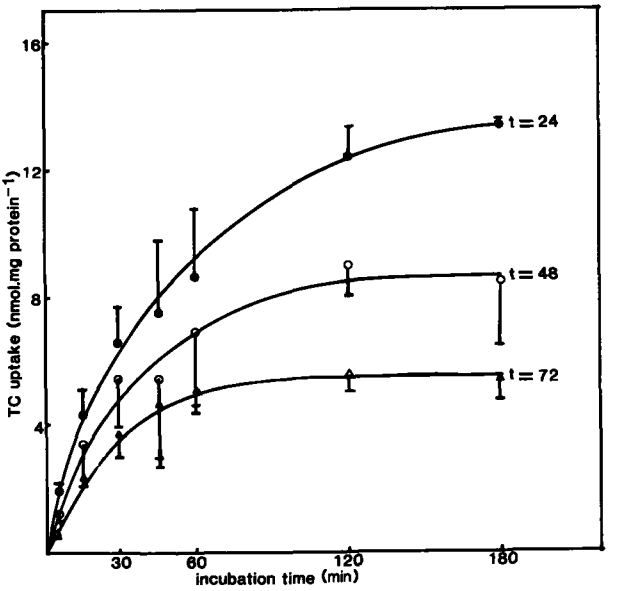

Fig. 2. Intracellular accumulation of taurocholic acid by pig hepatocytes. Hepatocytes were at three different culture times incubated with 50 μM [^{14}C]taurocholic acid at 37°C. At the indicated time points taurocholic acid uptake was determined and expressed as nmol.mg cell protein^{-1}. Plots represent means ± S.D. of three different culture experiments with duplicate incubations.

Table 1. Effect of added bile acids on bile acid synthesis.

Indicated bile acids were added to the medium at 28 h and along with tracer at 52 h culture time. Cells and media were harvested at 76 h. The data are expressed as percent of bile acid synthesis by control hepatocytes (41244 ± 9925 dpm. 100 μg DNA^{-1}. 24 h^{-1} in 3 different experiments with duplicate incubations).

addition (100 μM)	n*	bile acid synthesis (% of control)
taurocholic acid	3	51 ± 16
glycohyodeoxycholic acid	2	62 ± 3
glycochenodeoxycholic acid	2	50 ± 15
taurohyocholic acid	1	71
hyocholic acid	1	74
chenodeoxycholic acid	1	53
hyodeoxycholic acid	1	41

*n = number of different culture experiments with duplicate incubations.

Table 2. Effect of bile acids on leakage of LDH to medium and cellular ATP content.

Hepatocytes were incubated from 28 to 76 h with 100 μM of the indicated bile acids. Medium was renewed at 28 and 52 h. LDH activity in cells and media and cellular ATP content were determined as described under Materials and Methods. Data represent means of duplicate determinations in two different cultures ± S.D.

additions	percent LDH in medium	ATP (nmol.mg cell protein^{-1})
control (0.1% ethanol)	6.0 ± 3.7	21.0 ± 2.3
taurocholic acid	5.7 ± 2.3	22.1 ± 2.9
glycohyodeoxycholic acid	3.1 ± 2.0	n.d.*
glycochenodeoxycholic acid	5.4 ± 1.9	n.d.
hyocholic acid	3.8 ± 1.8	25.8 ± 4.5
chenodeoxycholic acid	5.0 ± 0.5	24.0 ± 1.5
hyodeoxycholic acid	4.1 ± 1.6	n.d.

*n.d. = not done.

DISCUSSION

Since there is a time lag between the interruption of the enterohepatic circulation and the increase in bile acid synthesis and cholesterol-7α-hydroxylase activity (20-22), and since this increase is prevented by treatment of the animals with actinomycin D (23) it has been concluded that feedback inhibition of bile acid synthesis occurs at the level of protein synthesis (1). Clearly, in our pig hepatocyte cultures, bile acid synthesis was partly dependent on protein synthesis, as can be concluded from the abolishment of the increase in bile acid formation by cycloheximide. It seems likely therefore, that synthesis of enzyme, which is thought to be suppressed by bile acids in feedback regulation, takes place in the conditions of our cell culture.

We have shown here, that this increase of bile acid synthesis was inhibited by a number of different conjugated and unconjugated bile acids.

The reasons for the lack of feedback inhibition of bile acid synthesis in cultured rat hepatocytes reported by other groups (2-4), may be of two kinds. First, their data show constant, instead of increasing rates of bile acid synthesis (2,24,25) and decreasing cholesterol-7α-hydroxylase activities (25) during the time they studied the effect of added bile acids. Therefore it is questionable whether de novo synthesis of bile acid forming enzymes took place in these hepatocytes. Second, in their experiments cellular bile acid uptake was probably seriously impaired. Kubaska et al. reported that cholic acid (100 μM) was concentrated by hepatocytes to only 0.21

nmol.mg protein^{-1} (3), ursocholic acid (100 μM) to 0.25 nmol.mg protein^{-1} and ursodeoxycholic acid (100 μM) to 0.4 nmol.mg protein^{-1} (4) at 48 h culture time. These figures are 35 or more times lower than those reported in this paper for cultured pig hepatocytes. Loss of bile acid uptake during hepatocyte culture has been reported by several authors (26-28). Stabilization of this cell function could be achieved by coculture with liver epithelial cells (26) or by addition of dexamethasone and tocopherol to the medium (27). The presence of both these agents in our culture medium may be the reason for the relative stability of this process in our pig hepatocyte culture system.

In conclusion, we have demonstrated that bile acids inhibit an increase in the rate of bile acid synthesis in cultured pig hepatocytes by direct action on the hepatocyte. It is necessary to establish whether this inhibition is exerted by a direct effect on expression of cholesterol-7α-hydroxylase as suggested before. Therefore further research will concentrate on the effect of bile acids on cholesterol-7α-hydroxylase activity in cultured hepatocytes, using the sensitive assay for activity of this enzyme in cultured hepatocytes we recently described (29).

ACKNOWLEDGEMENTS

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REFERENCES

1. Björkhem I (1985) in Sterols and Bile Acids (Danielsson H and Sjövall J, eds.). Elsevier, Amsterdam, pp. 231-278.
2. Davis RA, Highsmith WE, Malone McNeal M, Archambault Schexnayder J, and Kuan JCW (1983) J Biol Chem 258, 4079-4082.
3. Kubaska WM, Gurley EC, Hylemon PB, Guzelian PS, and Vlahcevic ZR (1985) J Biol Chem 260, 13459-13463.
4. Kubaska WM, Gurley EC, Hylemon PB, Heuman DM, Mosbach EH, and Vlahcevic ZR (1987) Biochim Biophys Acta 920, 195-204.
5. Tserng KY, Hachey DL, and Klein PO (1977) J Lipid Res 18, 404-407.
6. Princen HMG, Huijsmans CMG, Kuipers F, Vonk RJ, and Kempen HJM (1986) J Clin Invest 78, 1064-1071.
7. Bligh EG, and Dyer JW (1959) Can J Biochem Biophys 37, 911-917.
8. Kempen HJM, Vos-van Holstein MPM, and De Lange J (1982) J Lipid Res 23, 823-830.
9. Schwarz LR, and Barth CA (1979) Hoppe Seyler's Z physiol Chem 360, 1117-1120.
10. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) J Biol Chem 193, 265-275.
11. Hukkelhoven MWAC, Vromans E, Markslag AMG, and Vermorken AJM (1981) Anticancer Res 1, 341-344.

12. Bergmeijer HU, and Bernt E (1970) in Methoden der enzymatischen Analyse (Bergmeijer HU, ed). Verlag Chemie, pp. 533-538.
13. Ibidem, pp. 2020-2035.
14. Legrand-Defretin V, Juste C, Corring T, and Rerat A (1986) Am J Physiol 250, G295-G301.
15. Kletzien RF, Pariza MW, Becker JE, and Potter VR (1975) Anal Biochem 68, 537-544.
16. Scharschmidt BF, and Stephens JE (1981) Proc Natl Acad Sci USA 78, 986-990.
17. Schwarz LR, Burr R, Schwenk M, Pfaff E, and Greim H (1975) Eur J Biochem 55, 617-623.
18. Gewirtz DA, Randolph JK, and Goldman ID (1984) Hepatology 4, 205-212.
19. Dickson AJ, and Pogson CI (1977) FEBS Lett 83, 27-32.
20. Danielsson H, Einarsson K, and Johansson G (1967) Eur J Biochem 2, 44-49.
21. Shefer S, Hauser S, Bekersky I, and Mosbach EH (1970) J Lipid Res 11, 404-411.
22. Botham KM, Lawson ME, Beckett GJ, Percy-Robb IW, and Boyd GS (1981) Biochim Biophys Acta 665, 81-87.
23. Einarsson K, and Johansson G (1968) FEBS Lett 1, 219-222.
24. Davis RA, Hyde PM, Kuan JCW, Malone-McNeal M, and Archambault-Schexnayder J (1983) J Biol Chem 258, 3661-3667.
25. Hylemon PB, Gurley EC, Kubaska WM, Whitehead TR, Guzelian PS, and Vlahcevic ZR (1985) J Biol Chem 260, 1015-1019.
26. Foliot A, Glaise D, Erlinger S, and Guguen-Guillouzo C (1985) Hepatology 5, 215-219.
27. Galivan J (1982) Arch Biochem Biophys 214, 850-852.
28. Petzinger E, and Frimmer M (1988) Biochim Biophys Acta 937, 135-144.
29. Princen HMG, Meijer P, Kwekkeboom J, and Kempen HJM (1988) Anal Biochem 171, 158-165.

CHAPTER 7

BILE ACIDS EXERT NEGATIVE FEEDBACK CONTROL ON BILE ACID SYNTHESIS IN CULTURED PIG HEPATOCYTES BY SUPPRESSION OF CHOLESTEROL 7α-HYDROXYLASE ACTIVITY

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SUMMARY

Feedback regulation of bile acid synthesis by its end products was studied in cultured hepatocytes of young weaned pigs. We previously showed that conversion of exogenous [^{14}C] cholesterol into bile acids was suppressed by addition of bile acids to the culture medium (Kwekkeboom et al., Biochem Biophys Res Commun 155, 850-856). In the present study, the effects of bile acids on bile acid mass production and cholesterol 7α-hydroxylase activity were examined. Mass production of bile acids was strongly inhibited by addition of taurocholic acid (50 and 100 μM) to the culture medium. The inhibitory action was exerted specifically on activity of cholesterol 7α-hydroxylase, since conversion of [^{14}C] 7α-hydroxycholesterol to bile acids by pig hepatocytes was not affected. Suppression of cholesterol 7α-hydroxylase activity after incubation of the hepatocytes with taurocholic acid was concentration- and time-dependent. Maximum suppression (-80%) was achieved after a 20 to 30-h incubation of hepatocytes with 100 μM of this bile acid. Decline of enzyme activity caused by 100 μM taurocholic acid followed first order kinetics with a half-life of 11 h. Taurocholic acid had no direct effect on cholesterol 7α-hydroxylase activity in homogenates of hepatocytes, as assessed by addition of the bile acid to the assay mixture.
The effects of several other bile acids in a concentration of 100 μM on cholesterol 7α-hydroxylase activity, were examined in 48 h incubations. Glycochenodeoxycholic and glycohyodeoxycholic acids, which are the major bile acids in pig bile, their unconjugated forms and also deoxycholic and cholic acid pronouncedly inhibited activity of the enzyme. In contrast, hyocholic acid failed to inhibit, while ursodeoxycholic acid was a weak inhibitor.

It is concluded that bile acids, in physiological concentrations (i.e. as observed in portal blood), inhibit bile acid synthesis in cultured pig hepatocytes, by suppression of cholesterol 7α-hydroxylase activity through a direct effect on the hepatocyte.

INTRODUCTION

Hepatic conversion of cholesterol to bile acids is a major pathway for removal of cholesterol from the body. The most important form of regulation of this process is thought to be exerted via the enterohepatic circulation. Interruption of it, either by biliary drainage or feeding of bile acid binding resins, leads to a several-fold increase of bile acid synthesis (1). The major site of regulation was found to be the 7α-hydroxylation of cholesterol (1). Since intraduodenal and intravenous infusions of taurocholate and cholate into bile diverted rats were observed to inhibit bile acid synthesis (2,3), it was concluded that the bile acids returning via the portal vein to the liver are responsible for the inhibition found with intact enterohepatic circulation. However, recent attempts to demonstrate an inhibitory effect of infused taurocholate yielded not only positive (4,5), but also negative results (6-8). Furthermore, it was reported that bile acids in concentrations even exceeding those in portal blood, failed to inhibit bile acid synthesis in suspension or monolayer cultures of rat (9-11) and rabbit (12) hepatocytes. These findings challenged the concept of negative feedback regulation of bile acid synthesis by its end products. Alternatively, it was shown that not taurocholic acid, but intravenously infused deoxycholic and taurodeoxycholic (13), or lithocholic and 3β-hydroxy-5-cholenoic acids (14) inhibited bile acid synthesis in rat and rabbit respectively. This suggests that not the primary, but the secondary bile acids would regulate bile acid synthesis.

Recently, we reported that taurocholate and several other conjugated and unconjugated bile acids, in a concentration comparable to that found in portal blood, depressed formation of bile acids from [^{14}C]cholesterol in cultured hepatocytes of young weaned pigs (15). None of these bile acids affected hepatocyte viability, as assessed by cellular ATP content and release of lactate dehydrogenase into the culture medium, in the concentration used in that study. Thus, in cultured pig hepatocytes, bile acid synthesis, at least from exogenous cholesterol, is regulated by its end products.

Further characterization of this model learned that these pig hepatocytes in monolayer culture expressed, after a recovery period of two days, cholesterol 7α-hydroxylase activities comparable with the values found in freshly isolated hepatocytes, provided that fetal bovine serum and the hormones insulin and dexamethasone were included in the culture medium (16). In these conditions, rate of bile acid

synthesis was in the range of the in vivo bile acid production in the pig. Furthermore, we found that cultured pig hepatocytes retained their capacity to concentrate bile acids intracellularly for at least three days. In contrast, rat and human hepatocytes in monolayer culture lost this ability already during the second culture day (17).
Thus, monolayer cultures of pig hepatocytes offer a suitable in vitro system for the study of feedback regulation of bile acid synthesis. In this study, we examined the effects of exogenous bile acids on bile acid mass production and cholesterol 7α-hydroxylase activity in cultured pig hepatocytes.

MATERIALS

Materials used for isolation and culturing of pig hepatocytes and determination of cholesterol 7α-hydroxylase activity were obtained from sources described previously (18). Hexafluoro-2-propanol, trifluoroacetic anhydride and deoxycholic acid were obtained from Serva, Heidelberg, FRG; pronase from Calbiochem (Behring Diagnostics, La Jolla, USA); mithramycine from Pfizer, Rotterdam, The Netherlands. Heparin was from Leo Pharmaceutical Products B.V., Weesp, The Netherlands and Sep-pak C18 cartridges were from Water Associates, Milford, USA.
Taurocholic acid sodium salt, glycochenodeoxycholic acid sodium salt, hyocholic acid, chenodeoxycholic acid and hyodeoxycholic acid were purchased from Sigma, St. Louis, MO, USA. Ursodeoxycholic acid, glycohyodeoxycholic acid and 7α-hydroxycholesterol were from Steraloids Inc., Wilton, NH, USA. All bile acids used were pure (> 98%), as verified by gas-liquid chromatography, except chenodeoxycholic acid which contained 7% impurities, consisting of other bile acids. [^{3}H(G)]taurocholic acid was obtained from New England Nuclear, Dreieich, FRG. [4-^{14}C] 7α-hydroxycholesterol was prepared from [4-^{14}C]7-ketocholesterol, which is the most abundant impurity in commercially available [4-^{14}C]cholesterol, by reduction with sodium borohydride (19).

METHODS

Pig hepatocyte isolation and culture

Female domestic pigs (7 to 8 weeks old, hybrid of Large Yorkshire and Nederlands Landvarken) were obtained from the Veterinary Department of the State University of Utrecht (Netherlands). All pigs were sacrified between 8.30 and 9.30 a.m. Pigs were administered i.v. 10 mg/kg hypnodyl (anaesthesia) and 10000 U heparin to prevent blood clotting. After opening of the abdomen the vena porta was cannulated, the vena cava cut, and the liver was immediately perfused under hydrostatic pressure

with 1 litre solution (0°C) containing 10 mM Hepes (pH 7.4), 132 mM NaCl, 6.7 mM KCl, 20 mM D-glucose and 0.5 mM EGTA (solution 1) and subsequently with 1 litre of the same buffer without EGTA (solution 2) supplemented with 20 U/ml heparin. All solutions were sterilized by passage through 0.2 μ filters. During perfusion the liver was excised. After this preperfusion, aimed to prevent blood clotting during transport, the liver was immersed in solution 2 with heparin and transported on ice to the site of hepatocyte isolation within 60 minutes. For cell isolation, a piece of the lobe that was best blanched, was cut off. All solutions used for subsequent perfusion of the liver lobe were oxygenated by bubbling 95% O_2/5% CO_2 and temperature was kept at 37°C. Non-recirculating perfusion with 500 ml solution 1 at a rate of 120 ml/min was started after insertion of four polyethylene catheters (18 gauge) in vascular orifices at the cut surface. Perfusion was continued with respectively 1 litre of solution 2 and 200 ml of a solution containing 100 mM Hepes (pH 7.6), 67 mM NaCl, 6.7 mM KCl and 5 mM $CaCl_2$ (solution 3). Subsequently, the liver lobe was perfused with solution 3 containing 0.5 mg/ml collagenase and 0.05 mg/ml trypsin inhibitor in a recirculating mode for 20 min and with 0.1% collagenase for 30 min, followed by non-recirculating perfusion with 200 ml collagenase-free solution 3.

Liver tissue was dissociated in Williams E medium (0°C) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were filtered through a 250 μm filter, centrifuged for 1 min at 50 xg and washed three times in the above-mentioned medium.

Hepatocytes were seeded at a density of of 1.5 x 10^5 viable cells/cm^2 on 28 cm^2 plastic culture dishes or on 10 cm^2 wells in 6 well trays in Williams E medium with the above-mentioned supplements and 135 nM insulin and 50 nM dexamethasone and maintained at 37°C in a 5% CO_2/95% air atmosphere. The medium was renewed after 4 h, and every 24 h thereafter. In some experiments, the hormone supplements were omitted after 4 h, as is indicated in the Results section.

For regulation studies, bile acids were dissolved to the required concentration in culture medium and added to the hepatocytes with medium change. Alternatively, bile acids were added from 100 times concentrated stocks in culture medium.

Quantification of mass production of bile acids

Bile acids, accumulated in cells and medium of two 28 cm^2 culture dishes during 24 h periods, were determined by gas-liquid chromatography (GLC), essentially as described by Davis et al. (20). At the end of a 24 h culture period medium was aspirated and cells were washed twice with Hanks' buffered salt solution (both washes were added to the medium). Cells were scraped off with a rubber policeman, suspended in water and a sample was taken for DNA determination. After addition

of 2.66 μg sodium taurodeoxycholate (equivalent with 2 μg deoxycholic acid) as recovery standard, the cell suspensions and media were diluted with an equal volume of 0.1 M NaOH, incubated for 15 min at 60°C, cooled and then extracted using a C18 silica cartridge. Bile acids were deconjugated with cholylglycine hydrolase, extracted again with a C18 silica cartridge and subjected to solvolysis. The bile acid containing residue was dissolved in alkaline water (pH 9.3), shaken three times with hexane, acidified to pH 2.8 and bile acids were extracted in dichloromethane. After evaporation of solvent, bile acids were derivatized with trifluoroacetic anhydride and hexafluoroisopropanol for 1 h at 60°C. The solvent was evaporated and the residue dissolved in 10 to 20 μl hexane. Samples of 1 μl were subjected to GLC separation using a Chrompack-Packard 438S gaschromatograph equipped with a CPSil-5 (CB) column (25 m x 0.22 mm i.d.) and flame ionisation detector. Hydrogen was used as carrier gas. Samples were splitted with a 1:10 ratio. Injector and detector temperatures were 280°C. Initial oven temperature was 230°C and was raised after 0.1 min by 1°C/min and after 20 min by 20°C/min to 290°C.
Retention time of deoxycholic acid (internal standard) was 10.30 min. Cholic acid, hyocholic acid, chenodeoxycholic acid and hyodeoxycholic acid had relative retention times of 1.02, 1.07, 1.13 and 1.22 with respect to deoxycholate.
Bile acids were quantified using peak area ratios. Relationships between weight and peak area were similar for all bile acids synthesized by pig hepatocytes. Detection limit was 1 ng. Due to the presence of bile acids in fetal bovine serum, culture medium contained cholic acid (0.035 μg/ml), chenodeoxycholic acid (0.162 μg/ml), and lithocholic acid (0.023 μg/ml) resulting in a total bile acid concentration of 0.5 μM. Net bile acid synthesis was calculated as the amount in medium and cells (corrected for the amount of bile acids in the medium before incubation) minus the amount in cells 24 h earlier.

Determination of bile acid synthesis from [^{14}C]7α-hydroxycholesterol

Bile acid formation from radiolabelled 7α-hydroxycholesterol was determined as described (21) by measurement of radioactivity in bile acids accumulated in cells and medium during 1 to 8 h incubations with 3 nCi [^{14}C] 7α-hydroxycholesterol/ml medium supplemented with non-radioactive 7α-hydroxycholesterol to 1 μM. [^{14}C] 7α-Hydroxycholesterol was added to the medium after solubilization in fetal bovine serum. After addition of [^{3}H(G)]-taurocholic acid as recovery standard, bile acids were separated from lipids by extraction with chloroform and methanol and further isolated using a C18-cartridge as described before (22). The bile acids were recovered from the cartridge with methanol. ^{3}H and ^{14}C radioactivity in a sample of the methanol eluate were determined by liquid scintillation counting in Picofluor (Packard) using a double label program and external standardization for quench correction.

Assay of cholesterol-7α-hydroxylase in hepatocyte homogenates

Cholesterol 7α-hydroxylase activities in homogenates of cultured pig hepatocytes were determined as has been described before for rat hepatocytes (18). Enzyme activities in hepatocyte homogenates were expressed as pmol 7α-hydroxycholesterol.h^{-1}. μg DNA^{-1}. Formation of 7α-hydroxycholesterol by pig hepatocyte homogenates was linear with respect to DNA concentration between 20 and 100 μg per ml assay buffer and with incubation time up to at least 60 min after addition of the NADPH-generating system. Therefore, the assay was performed with 20-100 μg DNA per ml assay mixture and an incubation time of 30 min.

Miscellaneous methods

DNA was measured using mithramycine (23) with calf thymus DNA as standard, and cholesterol according to Gamble (24).

Statistical analysis

Statistical differences were assessed using Student's t-test (two-tailed) for paired data.

RESULTS

Effect of taurocholic acid on mass production of bile acids by cultured pig hepatocytes

Pig hepatocytes in monolayer culture synthesize mainly hyocholic and chenodeoxycholic acids (> 85% of total bile acid synthesis) and furthermore minor amounts of hyodeoxycholic and murocholic acids, but not cholic acid (16). Thus, addition of taurocholic acid to the culture medium does not interfere with determination of total mass production of bile acids by these cells. Taurocholic acid was added to the culture medium during the second and third day of culture in concentrations of 50 and 100 μM. These concentrations are within the range of portal bile acid concentrations in the pig (25) and do not have adverse effects on cell viability as reported before (15). Figure 1 shows that mass production of bile acids in control hepatocytes increased during the second and third culture day. This is in accordance with the rise of cholesterol 7α-hydroxylase activities (16). Addition of 50 μM taurocholic acid inhibited bile acid synthesis, repression amounting to 65% during the second and even to 85% during the third culture day. Administration of 100 μM taurocholate did not result in further suppression.

Effect of taurocholic acid on formation of labelled bile acids from [^{14} C]7α-hydroxycholesterol

In order to define the site of inhibition, hepatocytes were preincubated for 40 h with 100 μM taurocholic acid, and conversion of [^{14}C] 7α-hydroxycholesterol to bile acids by these hepatocytes and control hepatocytes was compared during a subsequent 8-h period. As shown in Fig. 2, taurocholate did not affect formation of bile acids from 7α-hydroxycholesterol. In contrast, as we showed previously (15), conversion of [^{14}C] cholesterol to bile acids by pig hepatocytes is pronouncedly suppressed by taurocholic acid and several other bile acids. This suggests that the inhibition of bile acid synthesis in pig hepatocytes, like in vivo, takes place at the 7α-hydroxylation of cholesterol.

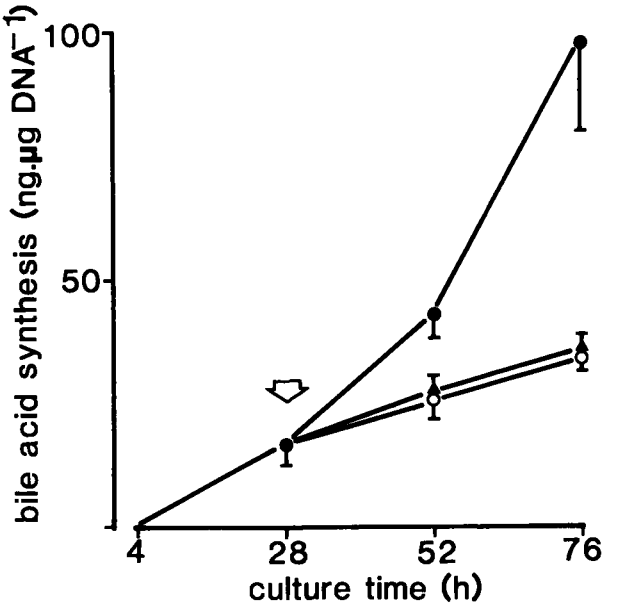

Fig. 1. The effect of taurocholic acid on mass production of bile acids by cultured pig hepatocytes. Taurocholic acid (50 or 100 μM) was added to the culture medium during the second and third culture day. At each time point, cells and media were harvested, or cells were supplied with fresh medium. Bile acids in cells and media were enzymatically deconjugated, solvolyzed and determined by GLC. Net synthesis rates during subsequent 24 h periods were calculated, and cumulatively plotted. Values are the means of duplicate incubations ± range.
●: control; ○: 50 μM taurocholic acid; ▲: 100 μM taurocholic acid.

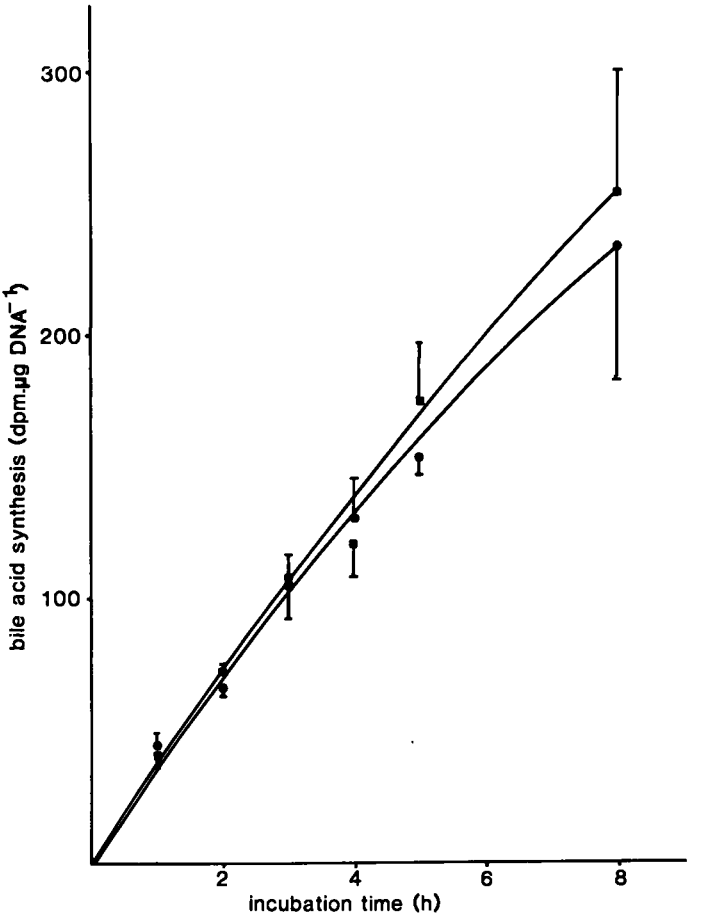

Fig. 2. Effect of taurocholic acid on conversion of [^{14}C]7α-hydroxycholesterol into bile acids by cultured pig hepatocytes.
Pig hepatocytes were cultured from 24 to 64 h in the presence or absence of 100 µM taurocholate. At 64 h medium was aspirated, fresh medium with 3 nCi [4-^{14}C]7α-hydroxycholesterol per ml, supplemented with non-radioactive 7α-hydroxycholesterol to a concentration of 1 µM, was supplied to the cells, and 100 µM taurocholic acid was added to the appropriate dishes. At the indicated time points, cells and media were harvested and radiolabelled bile acids were determined as described in the Methods section. Values are depicted as means ± range of two culture experiments with duplicate incubations. After 8 h, 83% of the added [^{14}C]7α-hydroxycholesterol was converted to bile acids.
●: control; ■: 100 µM taurocholic acid.

Effect of taurocholic acid on cholesterol 7α-hydroxylase activity in cultured pig hepatocytes

Addition of 100 µM taurocholic acid to the culture medium from 24 to 72 h resulted in five separate culture experiments in an 80% ± 9% ($p < 0.01$) inhibition of

cholesterol 7α-hydroxylase activity at 72 h (2.2 ± 1.3 (mean ± S.D.) pmol.h^{-1}. μg DNA^{-1} vs. 10.6 ± 3.2 pmol.h^{-1}. μg DNA^{-1} in control hepatocytes).

Figure 3 shows that the inhibition of cholesterol 7α-hydroxylase by exogenous taurocholic acid was concentration-dependent. Suppression was already observed at an extracellular concentration of 10 μM. Maximum inhibition was achieved at 50 to 100 μM exogenous taurocholate. The time course of inhibition at 100 μM taurocholate (Fig. 4) shows that maximum inhibition was reached after an incubation period of 20 to 30 h. The relationship between the logarithm of enzyme activity and incubation time was linear, indicating that the decline of enzyme activity followed first order kinetics with a half-life of 11 h.

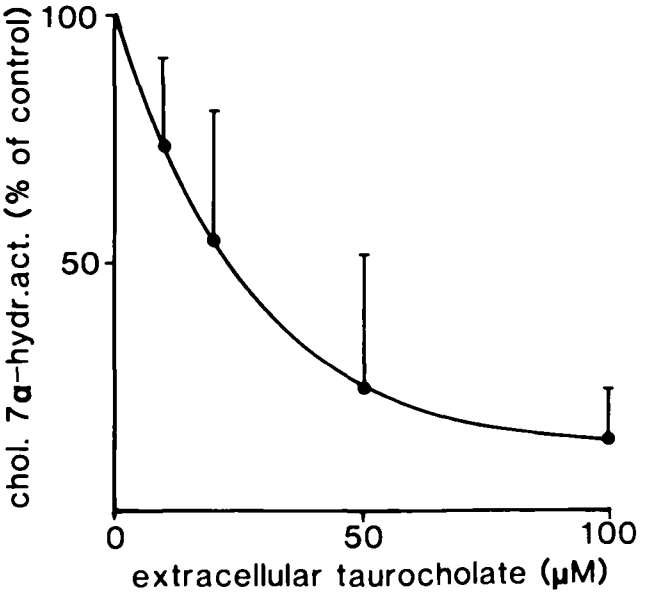

Fig. 3. Concentration dependency of suppression of cholesterol 7α-hydroxylase activity in cultured pig hepatocytes by taurocholic acid.
Taurocholic acid (10 to 100 μM) was added to the culture medium during the second and third culture day. At the end of this period cells were harvested and cholesterol 7α-hydroxylase activity determined. The data are expressed as percentage of the enzyme activity of controls (no bile acid added), and are means ± S.D. of three different culture experiments. Cholesterol 7α-hydroxylase activities in control hepatocytes amounted to 10.5 ± 4.5 pmol.h^{-1}. μg DNA^{-1} (mean ± S.D.).

In all these experiments, hepatocytes were cultured in the presence of dexamethasone and insulin, in order to achieve cholesterol 7α-hydroxylase activities in the cultured hepatocytes comparable to those in freshly isolated hepatocytes (16). To exclude the possibility that the observed inhibition of the enzyme represented only a repression

of induction of cholesterol 7α-hydroxylase by (one of these) hormones, the effects of exogenous bile acids in hepatocytes cultured in hormone-free medium were examined in an additional set of experiments. Table 1 shows that omission of the hormones resulted in a 30% lowered enzyme activity and that taurocholic and glycochenodeoxycholic acid suppressed cholesterol 7α-hydroxylase activity also under these culture conditions.

Table 1. Effect of bile acids on cholesterol 7α-hydroxylase activity in pig hepatocytes cultured in presence or absence of hormones.

After an attachment period of 4 h, pig hepatocytes were cultured in Williams E (WE) medium containing 10% fetal bovine serum (FCS) and with or without 50 nM dexamethasone (DEX) and 135 nM insulin (INS). Bile acids were added to the culture medium during the second and third culture day and cholesterol 7α-hydroxylase activity was measured at the end of the third day. Values represent means ± range of two different culture experiments.

culture medium + additions	cholesterol 7α-hydroxylase activity (pmol.h^{-1}.μg DNA^{-1})	percent inhibition by bile acids
WE + FCS + DEX + INS	16.0 ± 3.5	
WE + FCS + DEX + INS + taurocholate (100 μM)	2.8 ± 0.4	83
WE + FCS + DEX + INS + glycochenodeoxycholate (100 μM)	7.0 ± 1.4	56
WE + FCS	10.8 ± 0.4	
WE + FCS + taurocholate (100 μM)	5.9 ± 0.8	45
WE + FCS + glycochenodeoxycholate (100 μM)	4.7 ± 0.4	56

To determine whether taurocholic acid exerted its effect by direct inhibition of cholesterol 7α-hydroxylase, the bile acid was added to the cholesterol 7α-hydroxylase assay mixture. Cultured pig hepatocytes are able to accumulate 100 μM exogenous taurocholate intracellularly up to 17 nmol per mg cell protein (17). The maximum amount of cell protein put into the assay mixture is 5 mg per ml (equivalent with 100 μg DNA per ml). Thus, when cholesterol 7α-hydroxylase activity is determined in homogenates of hepatocytes cultured in the presence of 100 μM taurocholate, bile acid concentrations up to 85 μM can be estimated to occur in the assay mixture. Therefore, the direct effect of taurocholate in concentrations up to 100 μM on cholesterol 7α-hydroxylase activities in homogenates of freshly isolated hepatocytes was examined. No significant inhibition of enzyme activities was observed (data not shown).

Effects of different bile acids on cholesterol 7α-hydroxylase activity in cultured pig hepatocytes

In order to find out whether feedback inhibition of cholesterol 7α-hydroxylase was specific with respect to bile acid structure, pig hepatocytes were incubated for 48 h with several conjugated and unconjugated bile acids, and cholesterol 7α-hydroxylase

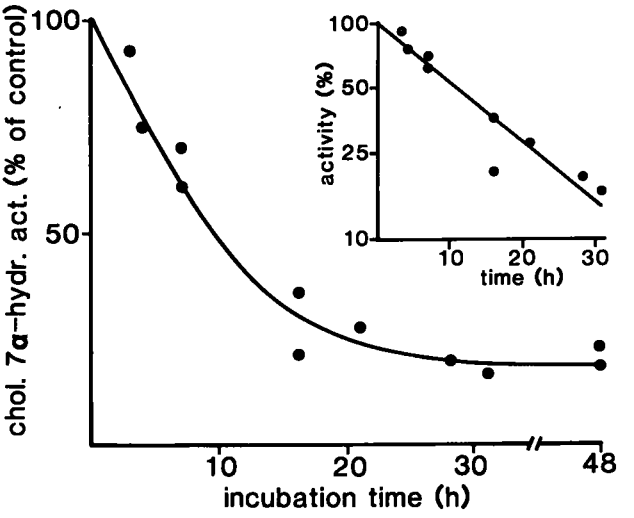

Fig. 4. Time course of suppression of cholesterol 7α-hydroxylase activity in cultured pig hepatocytes by taurocholate.
Taurocholate (100 μM) was added to the culture medium between 24 h and 72 h of culture age so that cells exposed for different times to the bile acid were harvested simultaneously. Values were obtained in two different culture experiments and are depicted as percentage of the enzyme activity of controls (no bile acid added). Insert: same data plotted semi-logarithmically. The line was fitted to the points by the methods of least squares (r = -0.95).

activities were determined at the end of these incubations (Table 2). Glycohyodeoxycholic and glycochenodeoxycholic acids, the most prominent bile acids found in pig bile (26), their unconjugated forms and also deoxycholic and cholic acid were found to be strong inhibitors. In contrast, hyocholic acid almost failed to inhibit the enzyme. Ursodeoxycholic acid elicited only weak suppression.

DISCUSSION

This study shows that bile acid synthesis in cultured pig hepatocytes is inhibited by a direct effect of bile acids on the hepatocyte. Like in vivo (1,27-29), the suppressive

action was exerted specifically on cholesterol 7α-hydroxylase and not on enzymes catalyzing subsequent steps in the pathway from cholesterol to bile acids. The time course of inhibition of cholesterol 7α-hydroxylase is in agreement with the time course of inhibition of bile acid synthesis observed upon bile acid infusion into bile diverted rats (5,27). Maximal inhibition was observed over the whole range of the physiological portal bile acid concentrations in the pig over the day (60-130 μM) (25). This does not imply that under physiological conditions cholesterol 7α-hydroxylase is maximally suppressed in all hepatocytes. At normal bile acid load, only periportal hepatocytes are involved in bile acid uptake (30,31), while perivenous hepatocytes are exposed to only low bile acid concentrations. As a consequence, cholesterol 7α-hydroxylase activity is suppressed in periportal, but not in perivenous hepatocytes, at least in the rat (32).

Table 2. Effect of different bile acids on cholesterol 7α-hydroxylase activity in pig hepatocytes.

Cultured pig hepatocytes were incubated with different bile acids (100 μM) during the second and third culture day. Cells were harvested at 72 h and cholesterol 7α-hydroxylase activities were determined. Data are expressed as percent enzyme activity as compared to control (no bile acid added), and are means ± S.D. or range (for n = 2) of experiments with n different hepatocyte preparations.

added bile acid (100 μM)	n	cholesterol 7α-hydroxylase activity (% of control)
taurocholic acid	5	21 ± 9
cholic acid	2	25 ± 15
glycochenodeoxycholic acid	3	44 ± 22
chenodeoxycholic acid	3	39 ± 8
glycohyodeoxycholic acid	2	34 ± 17
hyodeoxycholic acid	3	21 ± 12
deoxycholic acid	2	41 ± 10
hyocholic acid	3	92 ± 3
ursodeoxycholic acid	3	70 ± 14

Considerable differences between bile acid species with respect to their inhibitory potential were observed in this study. Hyocholic acid and ursodeoxycholic acid were weak inhibitors of cholesterol 7α-hydroxylase in cultured pig hepatocytes. This agrees with other studies in which ursodeoxycholic acid feeding and infusion did not appreciably inhibit bile acid synthesis and cholesterol 7α-hydroxylase activity in rat and man (4,5,33-36), and hyocholic acid feeding failed to lower cholesterol 7α-hydroxylase activity in the rat (33). On the other hand, chenodeoxycholic and deoxycholic acids were found to be potent inhibitors both in cultured pig hepatocytes and in vivo in rat and man (13,33-37). Concerning the effect of taurocholic acid on bile acid synthesis, there is disagreement in the literature. Although feeding of this bile

acid consistently was found to inhibit bile acid synthesis and cholesterol 7α-hydroxylase (8,33,37,38), inhibition upon intraduodenal or intravenous infusion was not always observed (see Introduction). Recently Stange et al. (13) suggested that the disagreement on the effect of taurocholate might be due to variable formation of secondary bile acids. In our study, a pronounced inhibitory effect of taurocholate on bile acid synthesis in pig hepatocytes was observed. However, it can as yet not be fully excluded that the inhibitory effects were exerted by metabolites of added bile acids, since cultured hepatocytes, at least of the rat, are able to metabolize bile acids to some extent (39).

Our results on the bile acid structure-inhibition relationship generally agree with those found by Heuman et al. (33) in the rat, except for hyodeoxycholic acid, which did not inhibit cholesterol 7α-hydroxylase in that study. They proposed that the capacity of bile acids to inhibit cholesterol 7α-hydroxylase activity is related to their hydrophobicity.

In contrast with the results obtained in the present study, two research groups have reported lack of inhibition of bile acid synthesis by exogenous bile acids in cultured rat hepatocytes (10,11). The reasons for the discrepancy between their and our results might be of two kinds:

1. Rat hepatocytes in monolayer culture rapidly lose the capacity to take up (40) and accumulate bile acids intracellularly (16,41,42), while cultured pig hepatocytes retain this function for at least three days (15,17).
2. The rate of bile acid synthesis by cultured hepatocytes from untreated rats, as reported by Davis et al. (10,20), amounting to 20 pmol/h/mg cell protein, represents only 3 to 8% of the in vivo rate in the rat (300 to 750 pmol/h/mg (43-45)). Vlahcevic's group performed their experiments with hepatocytes isolated from cholestyramine treated rats, which synthesized bile acids at a 7 times higher rate (46). However, cholesterol 7α-hydroxylase activities in these hepatocytes were still 50 times lower as compared to activity of the enzyme in livers of cholestyramine treated rats, measured with the same assay method (47). In contrast, cholesterol 7α-hydroxylase activities in pig hepatocytes, cultured in the medium used in this study, equalled, after a recovery period of two days, the values found in freshly isolated hepatocytes (16). Moreover, after this recovery period, rate of bile acid production (55 ng/24 h/μg DNA, Fig. 1, which is equivalent with 115 pmol/h/mg cell protein) was in the range of the in vivo value as assessed by measurement of daily faecal loss (180 ± 54 pmol/h/mg protein, mean ± S.D. (48)).

The mechanism of repression of cholesterol 7α-hydroxylase by bile acids is unknown. In accordance with others (11,49,50) we found that bile acids do not directly inhibit activity of the enzyme. Since activity of the enzyme was measured with exogenous substrate, under saturating conditions, the observed effects cannot be explained by

changes in substrate availability. Probably, bile acids regulate cholesterol 7α-hydroxylase activity by modulation of synthesis or degradation of the enzyme. This hypothesis is supported by the observation that the rise in cholesterol 7α-hydroxylase activity in rats after bile canulation is prevented by actinomycin D (51).
The in vitro model presented in this paper provides an excellent means to investigate the molecular basis of this regulation process. Such a study must await the availability of antibodies against cholesterol 7α-hydroxylase.

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REFERENCES

1. Björkhem I (1985) In: Danielsson H, Sjövall J, eds. Sterols and Bile Acids. Amsterdam, Elsevier, 231-278.
2. Shefer S, Hauser S, Bekersky I, Mosbach EH (1969) J Lipid Res 10, 646-655.
3. Pries JM, Gustafson A, Wiegand D, Duane WC (1983) J Lipid Res 24, 141-146.
4. Spady DK, Dietschy JM (1987) In: Paumgartner G, Stiehl A, Gerok W, eds. Bile Acids and the Liver. Lancaster, MTP Press, pp 15-24.
5. Heuman DM, Hernandez CA, Hylemon PB, Kubaska WM, Hartman C, Vlahcevic ZR (1988) Hepatology 8, 358-365.
6. Davis RA, Musso CA, Malone-McNeal M, Lattier GR, Hyde PM, Archambault-Schexnayder J, Straka M (1988) J Lipid Res 29, 202-211.
7. Duane WC, McHale AP, Hamilton JN (1988) J Lipid Res 29, 212-214.
8. Stange EF, Scheibner J, Lutz C, Ditschuneit H (1988) Hepatology 8, 879-886.
9. Botham KM, Lawson ME, Beckett GJ, Percy-Robb IW, Boyd GS (1981) Biochim Biophys Acta 666, 238-245.
10. Davis RA, Highsmith WE, Malone-McNeal M, Archambault-Schexnayder J, Hyde PM, Kuan JW (1983) J Biol Chem 258, 4079-4082.
11. Kubaska WM, Gurley EC, Hylemon PB, Guzelian PS, Vlahcevic ZR (1985) J Biol Chem 260, 13459-13463.
12. Whiting MJ, Wishart RA, Growing MR, McManus ME, Mackinnon AM (1989) Biochim Biophys Acta 1001, 176-184.
13. Stange EF, Scheibner J, Ditschuneit H (1989) J Clin Invest 84, 173-180.
14. Hall R, Kok E, Javitt NB (1988) FASEB J 2, 152-156.
15. Kwekkeboom J, Van Voorthuizen EM, Princen HMG, Kempen HJM (1988) Biochem Biophys Res Commun 155, 850-856.
16. Kwekkeboom J, Princen HMG, Van Voorthuizen EM, Kempen HJM (1989) Biochim Biophys Acta (in press).
17. Kwekkeboom J, Princen HMG, Van Voorthuizen EM, Meijer P, Kempen HJM (1989) Biochem Biophys Res Commun 162, 619-625.
18. Princen HMG, Meijer P, Kwekkeboom J, Kempen HJM (1988) Anal Biochem 171, 158-165.
19. Nimmannit S, Porter JW (1980) Arch Biochem Biophys 201, 533-543.

20. Davis RA, Hyde PM, Kuan JW, Malone-McNeal M, Archambault-Schexnayder, J (1983) J Biol Chem 258, 3661-3667.
21. Princen HMG, Huijsmans CMG, Kuipers F, Vonk RJ, Kempen HJM (1986) J Clin Invest 78, 1064-1071.
22. Kempen HJM, Vos-Van Holstein MPM, De Lange J (1982) J Lipid Res 23, 823-830.
23. Hukkelhoven MWAC, Vromans E, Markslag AMG, Vermorken AJM (1981) Anticancer Res 1, 341-344.
24. Gamble W, Vaughan M, Kruth MS, Avigan J (1978) J Lipid Res 19, 1068-1071.
25. Legrand-Defretin V, Juste C, Corring T, Rerat A (1986) Am J Physiol 250, G295-G301.
26. Haslewood GAD (1971) Biochem J 123, 15-18.
27. Shefer S, Hauser S, Bekersky I, Mosbach EH (1970) J Lipid Res 11, 404-411.
28. Johansson G (1970) Eur J Biochem 17, 292-295.
29. Mosbach EH, Rothschild MA, Bekersky I, Oratz M, Mongelli J (1971) J Clin Invest 50, 1720-1730.
30. Jones AL, Hradek GT, Renston RH, Wong KY, Karlaganis G, Paumgartner G (1980) Am J Physiol 238, 9233-9237.
31. Groothuis GMM, Hardonk MJ, Keulemans KPT, Nieuwenhuis P, Meijer DKF (1982) Am J Physiol 243, 9455-9462.
32. Ugele B, Kempen HJM, Gebhardt R, Meijer P, Burger HJ, Princen HMG (1989) In: Paumgartner G, Stiehl A, Gerok W, eds. Trends in Bile Acid Research. Lancaster, Kluwer Academic Publishers, pp 53-62.
33. Heuman DM, Vlahcevic ZR, Bailey ML, Hylemon PB (1988) Hepatology 8, 892-897.
34. Bertolotti M, Carulli N, Menozzi D, Zironi D, Digrisolo A, Pinetti A, Baldini MG (1986) J Lipid Res 27, 1278-1286.
35. Nilsell K, Angelin B, Leyd B, Einarsson K (1983) Gastroenterology 85, 1248-1256.
36. Pooler PA, Duane WC (1988) Hepatology 8, 1140-1146.
37. Shefer S, Hauser S, Lapar V, Mosbach EH (1973) J Lipid Res 14, 573-580.
38. Danielsson H (1973) Steroids 22, 667-676.
39. Princen HMG, Meijer P (1988) Biochem Biophys Res Commun 154, 1114-1121.
40. Foliot A, Glaise D, Erlinger S, Guguen-Guillouzo C (1985) Hepatology 5, 215-219.
41. Petzinger E, Frimmer M (1988) Biochim Biophys Acta 937, 135-144.
42. Galivan J (1982) Arch Biochem Biophys 214, 850-852.
43. Myant NB (1981) The Biology of Cholesterol and Related Steroids. London: William Heinemann Medical Books Ltd., p 247.
44. Kellogg TF (1973) In: Nair PP, Kritchevsky D, eds. The Bile Acids, Chemistry, Physiology and Metabolism, Vol. 2. New York, Plenum Press, pp 283-304.
45. Uchida K, Nomura Y, Masumi K, Takase H, Takano K, Takeuchi N (1978) J Lipid Res 19, 544-552.
46. Hylemon PB, Gurley EC, Kubaska WM, Whitehead TR, Guzelian PS, Vlahcevic ZR (1985) J Biol Chem 260, 1015-1019.
47. Shefer S, Hauser S, Mosbach EH (1968) J Lipid Res 9, 328-333.
48. Kim DN, Rogers DH, Li JR, Reiner JM, Lee KT, Thomas WA (1977) Exp Mol Pathol 26, 434-447.
49. Schwartz MA, Margolis S (1983) J Lipid Res 24, 28-33.
50. Van Cantfort J, Gielen J (1975) Eur J Biochem 55, 33-40.
51. Einarsson K, Johansson G (1968) FEBS Lett 1, 219-222.

CHAPTER 8

COMPARISON OF TAUROCHOLATE ACCUMULATION IN CULTURED HEPATOCYTES OF PIG, RAT AND MAN

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SUMMARY

Intracellular accumulation at 37°C of 50 μM [^{14}C] taurocholic acid by hepatocytes of pig and rat, cultured for 24 hours, and by human hepatocytes, cultured for 12 hours, reached equilibrium after an incubation time of 1 to 2 hours. Maximum capacity to accumulate taurocholate intracellularly was assessed in 3-hour incubations with increasing extracellular taurocholate concentrations. Accumulation capacity of pig and rat hepatocytes was saturated at 100 μM, while uptake by human hepatocytes slightly increased even further above this concentration. At extracellular concentrations of 100 to 500 μM, hepatocytes of these three species concentrated taurocholic acid intracellularly to between 13 and 17 nmol per mg cell protein, corresponding to an intracellular concentration which was 10-70 times higher than the added extracellular concentration. With proceeding culture age, accumulation capacity of rat and human hepatocytes declined steeply (-80% and -60%, respectively between the first and second culture day). In contrast, in cultured pig hepatocytes, this capacity was only 40% lower on the third day compared to the first day of culture.
It is concluded that in cultured pig hepatocytes, the capacity to accumulate bile acids is retained for a longer time than in cultured rat and human hepatocytes.

INTRODUCTION

Bile acids are efficiently extracted from plasma by the liver. Previous studies using isolated perfused liver, freshly isolated and cultured rat hepatocytes have shown that hepatic bile acid uptake is a saturable, sodium-dependent and concentrative process (1-7). The maximum capacity of hepatocytes for bile acid accumulation (at saturating extracellular bile acid concentration) has been determined only in rat hepatocytes cultured for two days (7,8). However, rat hepatocytes, maintained in monolayer culture under usual conditions, loose their capacity to accumulate bile acids during

the first (9) or first few culture days (10,11). In connection with our study on feedback regulation of bile acid synthesis in cultured hepatocytes of the pig, we have shown that hepatocytes of this animal species concentrated taurocholic acid intracellularly even after three days in monolayer culture (12). In the present study, we compared the capacities of cultured pig, rat and human hepatocytes to accumulate taurocholic acid as a function of extracellular taurocholate concentration and of culture age.

MATERIALS AND METHODS

Materials used for hepatocyte isolation and culture were obtained from sources described in (13) and (14). Sodium [24-^{14}C]taurocholate (56 mCi/mmol) and sodium [24-^{14}C]cholate (56 mCi/mmol) were obtained from Amersham, England. Unlabeled sodium taurocholate, sodium cholate and DL-α-tocopherol were from Sigma, St. Louis, U.S.A.

Hepatocytes were isolated by perfusion with Ca^{2+}-free buffer and with collagenase solution from the livers of seven weeks old female pigs (hybrid of Large Yorkshire and Nederlands Landvarken) (12), male Wistar rats (250-350 g) (13) and human donor liver (14).

Human liver tissue was obtained through the Auxiliary Partial Liver Transplantation Programme carried out at the Department of Surgery of the University Hospital Dijkzigt in Rotterdam, The Netherlands. Consent to use the remaining non-transplantated part of the liver for scientific research was given by the Medical Ethical Committee of the University Hospital Dijkzigt, Rotterdam.

Hepatocytes were seeded at a density of 1 to 1.5 x 10^5 viable cells/cm^2 on 10 cm^2 wells in 6-wells trays (Costar, Cambridge, U.S.A.) or on 10 cm^2 plastic culture dishes (Greiner, Nürtingen, FRG). Cells were cultured in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 135 nM insulin and 50 nM dexamethasone and incubated at 37°C in a 5% CO_2/95% air atmosphere (12-14). The medium of pig and rat hepatocytes (1 ml per dish) was renewed after 4 hours; that of human hepatocytes after 12 hours, and every 24 hours thereafter.

Intracellular accumulation of [24-^{14}C]taurocholate or [24-^{14}C]cholate was determined essentially as described (6). Twenty μl culture medium with 0.1 μCi [^{14}C]taurocholate or 0.1 μCi [^{14}C]cholate, supplemented with unlabeled taurocholate or cholate to 50 nmol, was added to each dish. Alternatively, to investigate concentration dependency of bile acid uptake, 0.1 μCi[^{14}C]taurocholic acid and varying amounts of unlabeled taurocholate were added to the dishes. At the indicated time points, dishes were placed on ice, the medium was aspirated and cells were washed five times with 0.5

ml Hanks' balanced salt solution (0°C). Cells were then scraped off with a rubber policeman and suspended in water. An aliquot of cell suspension was taken for protein determination (by the Lowry method (15)) and radioactivity in part of the sample was determined by liquid scintillation counting, with automatic correction for quenching by the external standard ratio method. Intracellular accumulation was expressed as nmoles bile acid/mg cell protein. Adsorption of label at 0°C was considered to represent non-specific binding, and was subtracted from the values obtained at 37°C.

RESULTS

Capacity of cultured hepatocytes of pig, rat and man to accumulate bile acids intracellularly

Intracellular bile acid accumulation was first studied with pig and rat hepatocytes cultured for 24 hours and with human hepatocytes cultured for 12 hours. The fact

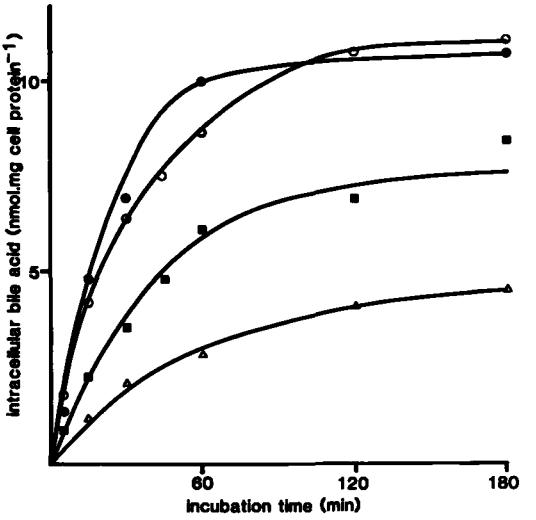

Fig. 1. Time course of uptake of 50 μM [^{14}C]taurocholate by cultured pig, rat and human hepatocytes and of 50 μM [^{14}C]cholate by cultured pig hepatocytes.
Pig and rat hepatocytes cultured for 24 hours, and human hepatocytes cultured for 12 hours, were incubated with 50 μM [^{14}C]taurocholate or 50 μM [^{14}C]cholate at 37°C. At the indicated time points cellular bile acid accumulation was determined. Values are expressed as nmol bile acid.mg cell protein^{-1}, and represent means of experiments with three (pig and human hepatocytes) or five (rat hepatocytes) different hepatocyte preparations with duplicate incubations.
○: taurocholate accumulation by pig hepatocytes.
●: taurocholate accumulation by rat hepatocytes.
■: taurocholate accumulation by human hepatocytes.
△: cholate accumulation by pig hepatocytes.

that a different time point was used for human hepatocytes was due to the time of human hepatocyte isolation, which was always at night (in connection with auxiliary liver transplantation).

Figure 1 shows that accumulation at 37°C of 50 μM [^{14}C]taurocholate by cultured rat hepatocytes reached steady-state after 1 hour and by pig and human hepatocytes after 2 hours. [^{14}C]cholate (50 μM) was accumulated by pig hepatocytes to a two-fold lower level than its conjugated counterpart. Therefore, taurocholate was used in all subsequent experiments. A further reason for its use is that in portal blood, the greater part of bile acids is in the conjugated form (16).

Steady-state intracellular taurocholate concentrations were determined after 3-hour incubations. The capacity of pig and rat hepatocytes to accumulate the bile acid was saturated at an extracellular taurocholate concentration of 100 μM (Fig. 2). Accumulation in human hepatocytes still exhibited a small increase between 100 μM and 300 μM extracellular taurocholate concentration. Maximum steady-state intracellular taurocholate content (at extracellular concentrations of 100 to 500 μM) amounted to 13 to 17 nmol/mg cell protein in hepatocytes of these three species.

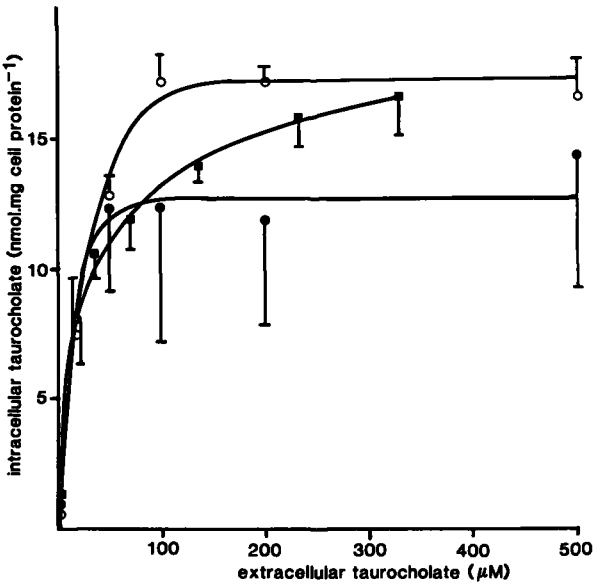

Fig. 2. Accumulation of taurocholate by cultured hepatocytes of pig, rat and man as a function of extracellular taurocholate concentration.
Hepatocytes were maintained in monolayer culture for 24 hours (pig and rat) or 12 hours (human) and steady-state intracellular taurocholate concentrations were determined after 3-hour incubations with 0.1 μCi [^{14}C]taurocholate and varying amounts of unlabeled taurocholate at 37°C. Values represent means ± S.D. of two (pig and human) or three (rat) different culture experiments with duplicate incubations. ○: pig hepatocytes; ●: rat hepatocytes; ■: human hepatocytes.

The possibility that intracellular taurocholic acid accumulation was due to impaired capability to secrete this bile acid was investigated for pig hepatocytes.
Figure 3 shows that, upon change to taurocholate-free medium, these hepatocytes secreted intracellular taurocholate almost completely within 60 minutes.

Effect of culture age on taurocholate accumulation

Table 1 shows that the capacity of rat and human hepatocytes to accumulate taurochocholate declined steeply with proceeding culture age (-80% and -60%, respectively, between the first and second day of culture). At the third culture day, rat hepatocytes were no longer able to concentrate taurocholate intracellularly. In

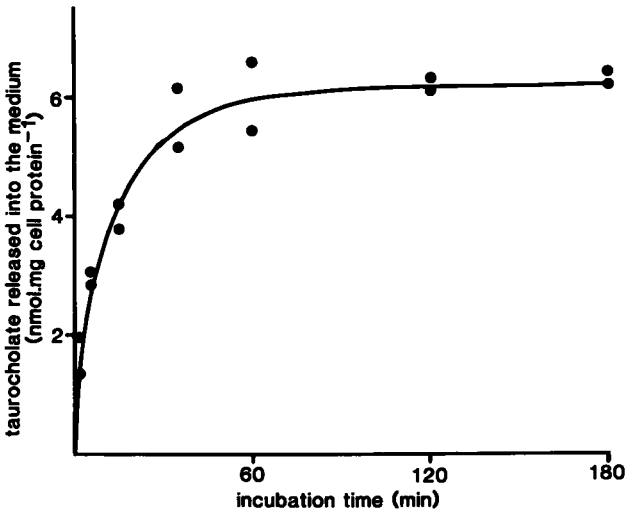

Fig. 3. Pig hepatocytes, cultured for 24 h, were loaded with 50 μM [^{14}C]taurocholate in 1 ml culture medium for 3 hours at 37°C. During this incubation, hepatocytes of this individual animal accumulated the bile acid up to 7 nmol per mg cell protein. Then [^{14}C]taurocholate-containing medium was aspirated, cells were washed rapidly twice with 1 ml culture medium (20°C) and incubated with 1.5 ml taurocholate-free culture medium. Appearance of ^{14}C-radioactivity in medium was followed by taking aliquots of medium at the indicated time points. The figure shows nmol taurocholate released to the medium per mg cell protein. Each point represents one determination in one culture dish. After 180 minutes the medium was aspirated. Residual [^{14}C]taurocholate in cells, which was determined after washing the cells five times with Hanks' buffered salt solution, amounted to merely 0.67 nmol per mg cell protein.

contrast to rat and human hepatocytes, accumulation by pig hepatocytes decreased by only 30% between the first and second culture day, and not significantly further during the third day.
Gallivan (10) reported that loss of cholic acid accumulation by cultured rat hepatocytes could be partially prevented by addition of dexamethasone together with

DL-α-tocopherol to the culture medium, with maximal effect at 10 nM dexamethasone and 10 μM tocopherol. However, we observed no effect of inclusion of 10 μM DL-α-tocopherol in our culture medium (which contained 50 nM dexamethasone) on taurocholate accumulation during culture age (data not shown).

Table 1. Accumulation of 50 μM [^{14}C]taurocholate by cultured pig, rat and human hepatocytes during culture age.

Steady-state intracellular [^{14}C]taurocholate levels were determined after 3-hour incubations with 50 μM [^{14}C]taurocholate at 37°C. Values are means ± S.D. of n different culture experiments with duplicate incubations.

Culture age (h)	pig hepatocytes n = 3	rat hepatocytes n = 7	human hepatocytes n = 3
		(nmol taurocholate.mg cell protein^{-1})	
12			8.61 ± 2.52
24	11.01 ± 3.48	10.77 ± 3.74	
36			3.23 ± 1.72
48	7.69 ± 2.55	2.32 ± 0.67	
60			1.22 ± 0.24
72	6.21 ± 0.81	0.22 ± 0.13[a]	

[a] Equivalent with an intracellular concentration of approximately 80 μM.

DISCUSSION

In this paper we have shown that hepatocytes of pig, rat and man cultured for a short time in monolayer were equally able to accumulate high amounts of taurocholate. To the best of our knowledge, this is the first study in which bile acid uptake by human hepatocytes has been examined. Steady-state intracellular taurocholate concentrations observed in this study at extracellular concentrations up to 50 μM were comparable to those reported for freshly isolated (3,17) and 27 hours cultured rat hepatocytes (6). These authors did not, however, determine bile acid accumulation at higher extracellular concentrations. Significantly lower taurocholate accumulation was observed in rat hepatocytes which had been maintained in culture for two days, probably due to loss of uptake capacity during culture (7,8). Assuming that the cellular volumes per mg cell protein of cultured pig and human hepatocytes are in the same order as that of cultured rat hepatocytes (2.5 μl/mg protein (7,18)), maximum apparent intracellular concentrations of 5 to 7 mM were achieved. This means that hepatocytes were able to concentrate taurocholate at an extracellular concentration

of e.g. 100 μM 50 to 70 times. At lower extracellular concentrations, intracellular to extracellular concentration gradients were even higher. In hepatocytes, bile acids are bound to proteins or stored in vesicles (19-21). However, we cannot exclude the possibility that part of the accumulated bile acids are in fact present in the bile canaliculi. Although bile acid concentrations in rat portal serum (30 to 200 μM (16,22,23) are comparable to medium concentrations to which hepatocytes were exposed in this study, amounts of bile acids found in rat liver in vivo are much lower (ranging from 0.8 to 1.7 nmol/mg protein (22,24-27)), than those accumulating in cultured hepatocytes. Similar low bile acid concentrations have been measured in human liver (27,28). The reason for this discrepancy may be that, at normal bile acid load, only periportal hepatocytes are involved in bile acid uptake from the plasma (29,30), although centrilobular hepatocytes have a similar intrinsic ability to take up bile acids (30,31). Thus, under physiological conditions, the liver has an overcapacity for extraction of bile acids from the portal blood.

Since cultured pig hepatocytes retain their capacity to accumulate taurocholate for at least three days, these cells may offer a better *in vitro* model than cultured rat or human hepatocytes for the study of bile acid uptake. Furthermore, if intracellular bile acids constitute the signal for feedback inhibition of bile acid synthesis, these hepatocytes may be preferable to rat and human hepatocytes for the study of this form of regulation. Indeed, we have recently found that bile acid synthesis in pig hepatocytes is suppressed by addition of bile acids to the culture medium (12).

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REFERENCES

1. Dietmaier, A, Gasser, R, Graf, J and Peterlik, M (1976) Biochim Biophys Acta 443, 81-91.
2. Reichen, J and Paumgartner, G (1976) Am J Physiol 231, 734-742.
3. Schwarz, LR, Burr, R, Schwenk, M, Pfaff, E and Greim, H (1975) Eur J Biochem 55, 617-623.
4. Anwer, MS and Hegner, D (1978) Hoppe-Seyler's Z physiol Chem 359, 181-192.
5. Blitzer, BL, Ratoosh, SL, Donovan, CB and Boyer, JL (1982) Am J Physiol 243, G48-G53.
6. Schwarz, LR and Barth, A (1979) Hoppe-Seyler's Z physiol Chem 360, 1117-1120.
7. Scharschmidt, BF and Stephens, JE (1981) Proc Natl Acad Sci USA 78, 986-990.
8. Van Dijke, RW, Stephen, JE and Scharschmidt, BF (1982) Am J Physiol 243, G484-G492.

9. Petzinger, E and Frimmer, M (1988) Biochim Biophys Acta 937, 135-144.
10. Gallivan, J (1982) Arch Biochem Biophys 214, 850-852.
11. Foliot, A, Glaise, D, Erlinger, S and Guguen-Guillouzo, C (1985) Hepatology 5, 215-219.
12. Kwekkeboom, J, Van Voorthuizen, EM, Princen, HMG and Kempen, HJM (1988) Biochem Biophys Res Commun 155, 850-856.
13. Princen, HMG, Meijer P, Kwekkeboom, J and Kempen, HJM (1988) Anal Biochem 171, 158-165.
14. Princen, HMG, Huijsmans, CMG, Kuipers, F, Vonk, RJ and Kempen, HJM (1986) J Clin Invest 78, 1064-1071.
15. Lowry, OH, Rosebrough, NJ, Farr, AL and Randall, RJ (1951) J Biol Chem 193, 265-275.
16. Cronholm, T and Sjövall, J (1967) Eur J Biochem 2, 375-383.
17. Gewirtz, DA, Randolph, JK and Goldman, ID (1984) Hepatology 4, 205-212.
18. Kletzien, RJ, Pariza, MW, Becker, JE and Potter, VR (1975) Anal Biochem 68, 537-544.
19. Frimmer, M and Ziegler, K (1988) Biochim Biophys Acta 947, 75-99.
20. Sugiyama, J, Yamada, T and Kaplowitz, N (1983) J Biol Chem 258, 3602-3607.
21. Lamri, Y, Roda, A, Dumont, M, Feldmann, G and Erlinger, S (1988) J Clin Invest 82, 1173-1182.
22. Okishio, T and Nair, PP (1966) Biochemistry 5, 3662-2668.
23. Botham, KM, Lawson, ME, Beckett, GJ, Percy-Robb, IW and Boyd, GS (1981) Biochim Biophys Acta 665, 81-87.
24. Strange, RC, Beckett, GJ and Percy-Robb, IW (1979) Biochem J 158, 71-78.
25. Strange, RC, Chapman, BT, Johnston, JD, Nimmo, IA and Percy-Robb, IW (1979) Biochim Biophys Acta 573, 535-545.
26. Simion, FA, Fleischer, B and Fleischer, S (1984) Biochemistry 23, 6459-6466.
27. Greim, H, Trülzsch, D, Czygan, P, Hutterer, F, Schaffner, F and Popper, H (1975) Ann NY Acad Sci 212, 139-147.
28. Greim, H, Czygan, P, Schaffner, F and Popper, H (1973) Biochem Med 8, 280-286.
29. Jones, AL, Hradek, GT, Renston, RH, Wong, KY, Karlaganis, G and Paumgartner, P (1980) Am J Phsyiol 238, 9233-9237.
30. Groothuis, GMM, Hardonk, MJ, Keulemans, KPT, Nieuwenhuis, P and Meijer, DKF (1982) Am J Physiol 243, 9455-9462.
31. Ugele, B, Locher, M, Burger, HJ and Gebhart, R (1987) In Bile Acids and the Liver (G. Paumgartner, A. Stiehl, W. Gerok, Eds.), pp. 153-160. MTP Press, Lancaster.

SUMMARY

In this thesis, the development of an in vitro system of pig liver parenchymal cells in primary monolayer culture, suitable for the study of the regulation of bile acid synthesis at the cellular level, is described. By the use of this system, it was demonstrated for the first time at the cellular level, that feedback inhibition of bile acid synthesis is achieved by a direct effect of bile acids on the liver parenchymal cell. The study was performed with hepatocytes of the pig in stead of the rat, since with regard to lipoprotein metabolism, the pig is more comparable to the human than the rat is.

In chapter 1, the motivation of this study, and the current knowledge of bile acid synthesis and its regulation are reviewed. It is emphasized that there is a lot of confusion with regard to the question which factors are regulating bile acid synthesis directly. In chapter 2, the results of the study are discussed. The complete experimental work is described in the chapters 3 to 8.

In chapter 3, a method to determine activity of cholesterol 7α-hydroxylase in relatively small amounts of hepatocytes (1-2 million) is described. For this purpose, the activity was determined in total cell homogenates, avoiding subcellular fractionation. The method facilitates the study of the regulation of this enzyme in cultured hepatocytes.

Chapter 4 deals with cholesterol 7α-hydroxylase activity and bile acid synthesis in monolayer cultures of piglet hepatocytes. A marked difference in cholesterol 7α-hydroxylase activities was observed between unweaned (two to three weeks old) and newly weaned (seven to eight weeks old) piglets. Activity of the enzyme was very low in hepatocytes isolated from unweaned animals and sixteen times higher in hepatocytes from newly weaned piglets. This difference was confirmed by measurements of cholesterol 7α-hydroxylase activity in liver microsomal preparations (chapter 5). However, not only the activity of cholesterol 7α-hydroxylase, but also the activities of 3-hydroxy-3-methylglutaryl-CoA reductase and squalene synthetase were strongly increased after weaning.

In monolayer cultures of hepatocytes from unweaned piglets, cholesterol 7α-hydroxylase activity was very low (chapter 4). When hepatocytes of newly weaned piglets were brought into culture, the initially high cholesterol 7α-hydroxylase activity declined by 90% during the first culture day. In the presence of fetal bovine serum, the activity of this enzyme was restored during two subsequent culture days. In serum-free medium, both cholesterol 7α-hydroxylase activity and bile acid production were low. When, in addition to the fetal bovine serum, also the hormones insulin and dexamethasone were included into the culture medium, cholesterol 7α-hydroxylase activity reached at the third culture day values similar to those found in the freshly

isolated hepatocytes. Rate of bile acid production by the hepatocytes under these culture conditions, was comparable to the rate in the pig in vivo.

In chapters 6 and 7 it is demonstrated, by the use of the in vitro system of hepatocytes from newly weaned piglets, cultured in medium supplemented with fetal bovine serum and the hormones dexamethasone and insulin, that bile acid synthesis in pig hepatocytes is susceptible to feedback inhibition. Both bile acid synthesis from radiolabelled cholesterol and mass production of bile acids were found to be suppressed upon the addition of bile acids to the culture medium. In the concentration used in this study (100 μM), these bile acids had no adverse effects on cell viability. The inhibitory effect was exerted specifically on the activity of cholesterol 7α-hydroxylase. Conversion of radiolabelled 7α-hydroxycholesterol into bile acids was not inhibited. Suppression of cholesterol 7α-hydroxylase activity upon the addition of taurocholic acid to the culture medium was concentration-dependent (a maximal inhibition of 80% was achieved by an exogenous bile acid concentration of 50 to 100 μM) and followed first-order kinetics, with a half-life of 11 hours. Bile acids did not inhibit the activity of the enzyme directly, but probably exert their effect by regulation of the amount of enzyme protein. Marked differences were found in the relative effectiveness of different bile acids in the inhibition of cholesterol 7α-hydroxylase activity.

Chapter 8 shows that hepatocytes of rat, pig and man during the first culture day accumulated taurocholic acid intracellularly to the same extent. Hepatocytes of rat and human lost this capacity for the greater part during the second culture day, while pig hepatocytes even at the third culture day had retained 60% of their original capacity to accumulate the bile acid. This difference may be one possible explanation for the fact that feedback inhibition of bile acid synthesis could be demonstrated in this study with pig hepatocytes, while other research groups reported that bile acid synthesis in cultured rat hepatocytes was not susceptible to inhibition by bile acids.

SAMENVATTING

In dit proefschrift wordt de ontwikkeling beschreven van een in vitro systeem van varkens leverparenchymcellen in primaire monolaagcultuur, dat geschikt is voor de bestudering van de regulatie van galzuursynthese op cellulair niveau. Met behulp van dit in model werd voor het eerst op cellulair niveau aangetoond dat feedback remming van galzuursynthese tot stand komt door een direkt effekt van galzuren op de leverparenchymcel.

In hoofstuk 1 wordt de motivatie van het in dit proefschrift beschreven onderzoek, en tevens de huidige kennis betreffende galzuursynthese en de regulatie ervan, besproken. Galzuren worden gesynthetiseerd door de leverparenchymcellen (hepatocyten), via een reeks metabole reakties, vanuit cholesterol. Ze worden door de hepatocyten uitgescheiden in de gal en komen zo terecht in de darm, waar ze een essentiële rol spelen bij de absorptie van vetten. Uitscheiding van galzuren via de gal is, tezamen met de aan galzuursecretie gekoppelde uitscheiding in de gal van cholesterol zelf, de kwantitatief belangrijkste wijze waarop verwijdering van cholesterol uit het lichaam kan plaatsvinden. Middelen die de synthese van galzuren stimuleren, zullen daarom mogelijk een serum cholesterol verlagende werking hebben, en bruikbaar zijn voor de behandeling van hypercholesterolemie, één van de belangrijkste risico faktoren voor coronaire en perifere vaatziekten. De bestaande middelen die galzuursynthese stimuleren, zijn harsen, die in de darm galzuren adsorberen. Ze veroorzaken inderdaad een verlaging van het serum cholesterol, maar worden slecht verdragen. Het doel van het in dit proefschrift beschreven onderzoek, was het verkrijgen van meer gedetailleerde kennis omtrent de regulatie van galzuursynthese, teneinde nieuwe mogelijkheden te vinden voor het ontwikkelen van galzuursynthese-stimulerende farmaca.
De huidige kennis van de wijze waarop het lichaam de synthese van galzuren reguleert, is zeer beperkt. Van de galzuren die via de gal naar de darm worden uitgescheiden, wordt 95% weer door de darm geresorbeerd en door het portale bloed naar de lever terug getransporteerd. Het is bekend, dat onderbreking van deze zogenaamde enterohepatische circulatie resulteert in een verhoogde galzuursynthese. Dit effekt is voornamelijk toe te schrijven aan een verhoging van de aktiviteit van het enzym dat de eerste stap in de omzetting van cholesterol naar galzuren katalyseert, namelijk het cholesterol 7α-hydroxylase. Tot voor enkele jaren werd daarom algemeen verondersteld, dat galzuursynthese wordt gereguleerd door een feedback remming door haar eindprodukten. Experimenten waaruit bleek dat galzuursynthese in rat lever parenchymcellen in monolaagcultuur niet geremd wordt door toevoeging van galzuren

aan het kweekmedium, brachten echter twijfels omtrent deze theorie teweeg. Bovendien werd door verschillende onderzoekers gerapporteerd dat bij ratten intraduodenaal of intraveneus toegediende galzuren geen effekt op de galzuursynthese hadden. Anderen vonden overigens wel remming van de galzuursynthese in dergelijke experimenten. Verder zijn er aanwijzingen dat galzuursynthese ook kan worden gereguleerd door hormonen (o.a. glucocorticoïden) en de beschikbaarheid van cholesterol in de parenchymcellen.

Om te kunnen bestuderen welke van de genoemde faktoren direkt, zonder tussenkomst van andere faktoren, de galzuursynthese reguleren, werden in deze studie monolaag cultures van varkens leverparenchymcellen opgezet. Bovendien werden methoden opgezet om de produktie van galzuren en de aktiviteit van het enzym cholesterol 7α-hydroxylase in deze cultures te kunnen bepalen. De keus voor lever cellen van het varken i.p.v. de rat werd ingegeven door het feit dat het varken qua lipoproteinen metabolisme meer op de mens gelijkt dan de rat.

In hoofdstuk 2 worden de resultaten van het experimentele werk besproken. Details van het onderzoek en een meer uitgebreide bespreking van de resultaten kunnen worden gevonden in de hoofdstukken 3 tot en met 8.

In hoofdstuk 3 wordt de ontwikkeling beschreven van een methode, die het mogelijk maakt de aktiviteit van cholesterol 7α-hydroxylase te bepalen in betrekkelijk kleine hoeveelheden geïsoleerde hepatocyten (1-2 millioen). Hiertoe werd de aktiviteit van dit enzym gemeten in totale celhomogenaten, zonder voorafgaande subcellulaire fraktionering. Met deze methode werden de mogelijkheden tot bestudering van de regulatie van dit enzym in gekweekte hepatocyten in belangrijke mate uitgebreid. De methode werd in eerste instantie opgezet voor rat hepatocyten, maar in het vervolg van deze studie gebruikt voor varkens levercellen.

In hoofdstuk 4 wordt een beschrijving gegeven van de galzuursynthese en cholesterol 7α-hydroxylase aktiviteiten in cultures van varkenshepatocyten onder verschillende kweekomstandigheden. Omdat pogingen tot het in kweek brengen van hepatocyten van oudere varkens mislukten, vanwege de slechte hechting van deze cellen aan het plastic van de kweekschaaltjes, werden in deze studie 2 tot 7 weken oude biggen als donor voor lever parenchymcellen gebruikt. Er bleek een groot verschil te zijn in cholesterol 7α-hydroxylase aktiviteit tussen niet gespeende (2 tot 3 weken oude) en pas gespeende (7 tot 8 weken oude) biggen. De aktiviteit van het enzym was zeer laag in hepatocyten geïsoleerd van niet gespeende dieren, maar 16 maal hoger in lever parenchymcellen van pas gespeende biggen. Dit verschil werd bevestigd door metingen van de cholesterol 7α-hydroxylase aktiviteit in lever mikrosoom preparaten (hoofstuk 5). Tegelijkertijd wordt in hoofdstuk 5 aangetoond dat niet alleen de aktiviteit van het cholesterol 7α-hydroxylase, maar ook die van 3-hydroxy-3-methylglutaryl-CoA reduktase en squaleen synthetase (twee mikrosomale enzymen

betrokken bij de cholesterol synthese) sterk verhoogd waren na spenen.
In monolaag cultures van hepatocyten van niet gespeende biggen was de cholesterol 7α-hydroxylase aktiviteit dan ook zeer laag (hoofdstuk 4). Wanneer hepatocyten van pas gespeende biggen in kweek werden gebracht, daalde de initiëel hoge cholesterol 7α-hydroxylase aktiviteit met 90% gedurende de eerste kweekdag. Gedurende de volgende twee dagen herstelde de aktiviteit zich. Voorwaarde voor dit herstel was de toevoeging van foetaal kalfs serum aan het kweek medium. In serum-vrij medium bleef zowel de cholesterol 7α-hydroxylase aktiviteit als de galzuurproduktie, die werd gemeten met een gevoelige methode, gebruikmakend van capillaire gas-chromatografie, laag. Wanneer naast foetaal kalfs serum bovendien de hormonen insuline en dexamethason werden toegevoegd, bereikte de cholesterol 7α-hydroxylase aktiviteit op de derde kweekdag waarden die gelijk waren aan die in de vers geïsoleerde hepatocyten. De galzuurproduktie door de gekweekte hepatocyten was onder deze omstandigheden vergelijkbaar met die in het varken in vivo.
De gekweekte varkens hepatocyten synthetiseerden uit exogeen radioaktief gemerkt cholesterol 11 verschillende water-oplosbare produkten, waarvan 95% geconjugeerd bleek te zijn. Het kwantitatief belangrijkste produkt werd geïdentificeerd als hyocholzuur, het belangrijkste primaire galzuur bij het varken. Vier andere metabolieten werden geïdentificeerd als hyodeoxycholzuur, chenodeoxycholzuur, murocholzuur en lithocholzuur.
In de hoofdstukken 6 en 7 wordt aangetoond dat de galzuursynthese in hepatocyten van pas gespeende biggen, gekweekt in medium met foetaal kalfs serum en de hormonen dexamethason en insuline, gereguleerd kan worden door feedback remming door galzuren. In hoofdstuk 6 wordt beschreven dat verschillende typen galzuren bij toegevoeging aan het kweekmedium, de vorming van galzuren door de levercellen uit radioactief gemerkt cholesterol onderdrukken. In de gebruikte concentratie (100 μM) hadden deze galzuren geen nadelige invloed op de plasma membraan (vastgesteld aan de hand van afgifte van lactaat dehydrogenase aan het kweekmedium) of op het cellulaire ATP niveau. In hoofdstuk 7 wordt deze vorm van regulatie nader onderzocht. Toevoeging van het galzuur taurocholzuur aan het kweek medium bleek ook de massaproduktie van galzuren door de gekweekte hepatocyten te remmen. Vastgesteld kon worden dat deze inhibitie specifiek op het cholesterol 7α-hydroxylase plaatsvond en niet op vervolgstappen in de metabole route van cholesterol naar galzuren: de omzetting van radioaktief 7α-hydroxycholesterol naar galzuren door de gekweekte hepatocyten werd niet geremd, maar de aktiviteit van het cholesterol 7α-hydroxylase wel. Remming van de aktiviteit van dit enzym door toevoeging van taurocholzuur aan het kweekmedium was concentratie-afhankelijk (maximale remming van 80% werd bereikt bij 50 tot 100 μM exogeen taurocholzuur) en volgde eerste-orde kinetiek, met een halfwaarde tijd van 11 uur. De onderdrukking van de

cholesterol 7α-hydroxylase aktiviteit vindt niet plaats door een direkte inhibitie van de enzym aktiviteit, maar waarschijnlijk door een regulatie van de hoeveelheid enzym eiwit.
Nagegaan werd of er verschillen zijn in de relatieve effektiviteiten van verschillende galzuren m.b.t. remming van het cholesterol 7α-hydroxylase. Evenals taurocholzuur remden ook glycochenodeoxycholzuur, glycohyodeoxycholzuur, cholzuur, chenodexoycholzuur, hyodeoxycholzuur en deoxycholzuur de aktiviteit van het cholesterol 7α-hydroxylase. Hyocholzuur remde de aktiviteit van het enzym echter niet en ursodeoxycholzuur slechts zwak.
In hoofdstuk 8 wordt aangetoond dat hepatocyten van zowel de rat, het varken als de mens tijdens de eerste kweekdag in gelijke mate in staat waren taurocholzuur intracellulair te accumuleren. Hepatocyten van de rat en de mens verloren deze capaciteit echter grotendeels gedurende de tweede kweekdag, terwijl varkens levercellen zelfs op de derde kweekdag nog 60% van de oorspronkelijke capaciteit tot accumulatie van het galzuur bezaten. Dit verschil is mogelijk één van de redenen waarom feedback regulatie van galzuursynthese wel kon worden aangetoond in deze studie met varkenshepatocyten, terwijl andere onderzoeksgroepen die met monolaag cultures van rat hepatocyten werkten, daartoe niet in staat waren.
Door deze studie is een goed in vitro systeem beschikbaar gekomen om het moleculaire mechanisme van de remming van galzuursynthese door galzuren te bestuderen. Inzicht hierin kan mogelijkheden openen om antagonisten van deze remming te ontwikkelen. Van dergelijke verbindigen kan worden verwacht dat ze een serum cholesterol verlagende werking hebben.

ABBREVIATIONS

BHT	butylated hydroxytoluene
cDNA	copy DNA
DTT	dithiothreitol
FCS	fetal calf serum
GLC	gas-liquid chromatography
GLC-MS	gas-liquid chromatography - mass spectrometry
HDL	high density lipoproteins
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA reductase	3-hydroxy-3-methylglutaryl-CoA reductase
LDH	lactate dehydrogenase
LDL	low density lipoproteins
Rf	retardation factor
TLC	thin-layer chromatography
VLDL	very low density lipoproteins

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 9 mei 1959 te Aagtekerke. Hij volgde het VWO aan de Chr. Scholengemeenschap Walcheren te Middelburg en behaalde het diploma in 1977. Hij studeerde vervolgens Biologie aan de Rijksuniversiteit te Utrecht. In 1980 legde hij het kandidaatsexamen Medische Biologie af. Zijn doktoraal studie bestond uit: hoofdvak Vergelijkende Endocrinologie, bijvakken Experimentele Pathologie en Fysiologische Chemie en nevenrichting Didaktiek van de biologie. Het doktoraalexamen werd afgelegd in 1984. Van januari 1984 tot juni 1985 was hij als docent biologie verbonden aan de Chr. Scholengemeenschap De Driestar te Gouda. Vanaf juni 1985 tot november 1989 was hij werkzaam op het Gaubius Instituut TNO te Leiden (direkteur Prof. dr. P. Brakman). Hier verrichte hij, o.l.v. Dr. H.J.M. Kempen en Dr. H.M.G. Princen, onderzoek naar de regulatie van galzuursynthese in levercellen in kweek. De resultaten van dit werk zijn in dit proefschrift beschreven. Na zijn promotieonderzoek is hij benoemd als universitair docent aan het E.C. Slater Instituut voor Biochemisch Onderzoek van de Universiteit van Amsterdam.

SLOTWOORD

Bij het voltooien van dit proefschrift wil ik mijn erkentelijkheid uitspreken voor de uiterst plezierige samenwerking met de collega's van het Gaubius Instituut TNO in de afgelopen jaren. Een aantal personen wil ik speciaal noemen:
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