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Processing of cholesteryl ester from low-density lipoproteins in the rat

Hepatic metabolism and biliary secretion after uptake by different hepatic cell types

Folkert KUIPERS,*‡ J. Fred NAGELKERKE,† Hille BAKKEREN,† Rick HAVINGA,*
Theo J. C. VAN BERKEL† and Roel J. VONK*

*Department of Pediatrics, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, and †Center for Bio-Pharmaceutical Sciences, Division of Bio-Pharmaceutics, Sylvius Laboratories, P.O. Box 9503, 2300 RA Leiden, The Netherlands

Biliary secretion of the cholesteryl ester moiety of (modified) low-density lipoprotein (LDL) was examined under various experimental conditions in the rat. Human LDL or acetylated LDL (acetyl-LDL), radiolabelled with [^{3}H]cholesteryl oleate, was administered intravenously to unanaesthetized rats equipped with permanent catheters in the bile duct, duodenum and heart. LDL was cleared relatively slowly from plasma, mainly by Kupffer cells. At 3 h after injection, only 0.9% of the radioactivity was found in bile; after 12 h this value was 4.5%. Uptake of LDL by hepatocytes was stimulated by treatment of the rats with 17α-ethinyloestradiol (EE; 5 mg/kg for 3 successive days); this resulted in a more rapid secretion of radioactivity into bile, 3.9% and 12.4% after 3 h and 12 h respectively. The extremely rapid uptake of acetyl-LDL via the scavenger pathway, mainly by endothelial cells, resulted in the secretion of only 2.1% of its ^{3}H label into bile within 3 h, and 9.5% within 12 h. Radioactivity in bile was predominantly in the form of bile acids; only a small part was secreted as free cholesterol. However, the specific radioactivity of biliary cholesterol was higher than that of bile acids in all three experimental conditions. EE-treated animals did not form cholic acid from [^{3}H]cholesteryl oleate, which was a major product of the cholesteryl oleate from LDL and acetyl-LDL in untreated rats, but formed predominantly very polar bile acids, i.e. muricholic acids. It is concluded that uptake of human LDL or acetyl-LDL by the liver of untreated rats is not efficiently coupled to biliary secretion of cholesterol (bile acids). This might be due to the anatomical localization of their principal uptake sites, the Kupffer cells and the endothelial cells respectively. Induction of LDL uptake by hepatocytes by EE treatment warrants a more efficient disposition of cholesterol from the body via bile.

INTRODUCTION

The importance of the liver for uptake and degradation of circulating lipoproteins has been well established (Attie *et al.*, 1982; Mahley & Innerarity, 1983). Most hepatic cell types, i.e. hepatocytes, endothelial cells and Kupffer cells, are involved in these processes under various experimental conditions in the rat (van Berkel *et al.*, 1982; Harkes & van Berkel, 1983, 1984*a*,*b*). Recognition and uptake of lipoproteins by liver cells is mainly mediated by specific receptors (Mahley & Innerarity, 1983), although receptor-independent uptake can also be of quantitative importance (Spady *et al.*, 1984). Relatively little attention has been paid to the fate of the cholesterol moiety of lipoproteins after their hepatic uptake. In particular, few data exist on the coupling of hepatic uptake of lipoproteins to biliary secretion of cholesterol (bile acids), although it is well known that the hepatobiliary pathway is the main route for removal of cholesterol from the body (Dietschy, 1984; Packard & Shepherd, 1982).

In a previous paper (Nagelkerke *et al.*, 1986) we reported on the hepatic uptake of [^{3}H]cholesteryl oleate incorporated into human low-density lipoproteins (LDL) in control rats and the rats treated with 17α-ethinyloestradiol (EE). In control rats, LDL cholesteryl ester was mainly taken up by Kupffer cells. Uptake was followed by rapid hydrolysis and [^{3}H]cholesterol was transferred to other sites in the body. EE treatment, which selectively induces LDL (apolipoprotein B,E) receptors on rat hepatocytes (Harkes & van Berkel, 1983, 1984*b*), strongly increased uptake of LDL cholesteryl ester by this cell type.

In the present study, we investigated the biliary secretion of cholesterol and bile acids derived from LDL cholesteryl oleate in EE-treated and untreated rats. For comparison, we also studied hepatic processing and biliary secretion of cholesteryl oleate associated with acetylated LDL (acetyl-LDL). These chemically modified lipoproteins are cleared to a large extent by liver endothelial cells via the scavenger pathway (van Berkel *et al.*, 1982; Nagelkerke *et al.*, 1983; Nagelkerke & van Berkel, 1986). The experimental set-up of the present studies (Kuipers *et al.*, 1985) allowed us to correlate the cellular sites of hepatic lipoprotein uptake, i.e. predominantly Kupffer cells (LDL), hepatocytes (LDL in EE-treated rats), or endothelial cells (acetyl-LDL), with the rate of biliary secretion of metabolic products derived from the cholesteryl ester moieties.

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Abbreviations used: LDL, low-density lipoprotein; EE, 17α-ethinyloestradiol.

‡ To whom reprint requests should be addressed.

the American Association for the Study of Liver Diseases, Chicago, IL, U.S.A., in November 1986 (Kuipers *et al.*, 1986*a*).

METHODS AND MATERIALS

Animals

Male Wistar rats (275–325 g) were housed in individual Plexiglass chambers in a light- and temperature-controlled room (light 06:00–18:00 h; 20 °C). The rats were equipped with permanent catheters in the bile duct, the duodenum and the heart as described previously (Kuipers *et al.*, 1985). The bile-duct and duodenum catheters were immediately connected to each other, in order to maintain an intact enterohepatic circulation of bile acids. The heart catheter allowed blood sampling and intracardial administration of compounds under normal feeding conditions (Steffens, 1969). The rats were allowed to recover from the operation during 1 week.

Six rats were injected subcutaneously with 5 mg of EE/kg body wt. for 3 successive days before the experiments. Control rats received the solvent (propylene glycol) in equivalent amounts.

Lipoprotein isolation

LDL was isolated from plasma of healthy volunteers and labelled with [1,2,6,7-^{3}H]cholesteryl oleate (82.7 Ci/mmol; Amersham International, Amersham, Bucks., U.K.), as described in detail previously (Nagelkerke *et al.*, 1986). The labelling procedure did not alter plasma decay and cellular uptake sites of the LDL particles after intravenous administration to rats (Nagelkerke *et al.*, 1986). Radiolabelled LDL was acetylated as described by Basu *et al.* (1976).

Experimental procedures

The lipoproteins (100 μg of protein/100 g body wt.) dissolved in 0.01 M-phosphate-buffered saline (pH 7.4) were introduced via the heart catheter at noon. The bile-duct catheter was then connected to a fraction collector, and bile was collected continuously into preweighed test tubes in 1 h fractions for 12 h, followed by ten 6 h fractions. After weighing, to determine bile flow, the samples were stored at −20 °C. Blood was sampled from the heart catheter at different time intervals after injection, transferred to heparinized test tubes and immediately centrifuged (~ 7500 *g*, 10 min).

Analyses

Radioactivity was measured in plasma and bile in 50 μl samples, and also in the chloroform phase after lipid extraction of the bile (Bligh & Dyer, 1959) to determine the radioactivity in the cholesterol fraction. Bile was decolorized with an equal volume of H_2O_2 before addition of the scintillation fluid. Radioactivity was determined in an Isocap scintillation counter, programmed to correct for quenching.

The biliary bile acid concentration was determined by an enzymic assay (Sterognost-Flu; Nyegaard and Co., Oslo, Norway). Cholesterol in bile was measured after lipid extraction as described by Gamble *et al.* (1978).

Radiolabelled bile acids synthesized in the liver from [^{3}H]cholesteryl ester were analysed by h.p.l.c. as described by Kuipers *et al.* (1986*b*).

Statistics

Data are presented as means ± S.E.M. When appropriate, differences between means were tested for significance by using Student's *t* test.

RESULTS

LDL or acetyl-LDL, radiolabelled with [^{3}H]-cholesteryl oleate, was intracardially injected into EE-treated or untreated rats via a permanent heart catheter. Collection of bile was started immediately after administration of the lipoproteins. Under all circumstances, bile acid secretion decreased to about 5–7% of its initial value within 3 h, owing to exhaustion of the endogenous bile acid pool (Kuipers *et al.*, 1985). After this period, bile acid secretion directly reflects their hepatic synthesis rate. Bile acid synthesis tended to be lower in EE-treated rats than in untreated rats, but the difference was not statistically significant (results not shown). In contrast, bile flow was significantly lower during the course of the experiment in EE-treated animals, as compared with the untreated animals. This was largely due to a 40% decrease in the so-called bile acid-independent fraction of bile flow (BAIF); regression analysis of the relation between flow and bile acid output (Berthelot *et al.*, 1970) revealed a BAIF of 3.2 μl/min per 100 g body wt. in control rats and 2.2 μl/min per 100 g body wt. in EE-treated animals (values from the initial 6 h bile samples after interruption of the enterohepatic circulation were used for this analysis).

Fig. 1 shows the plasma decay of radioactivity after administration of the [^{3}H]cholesteryl oleate-labelled lipoproteins. EE treatment caused a marked acceleration of the clearance of LDL from plasma. At 3 h after injection, 75% of the administered amount of LDL was still present in plasma of control rats, compared with only 11% in EE-treated animals. After 24 h these values were 20 and 1.2% respectively. Acetyl-LDL was cleared

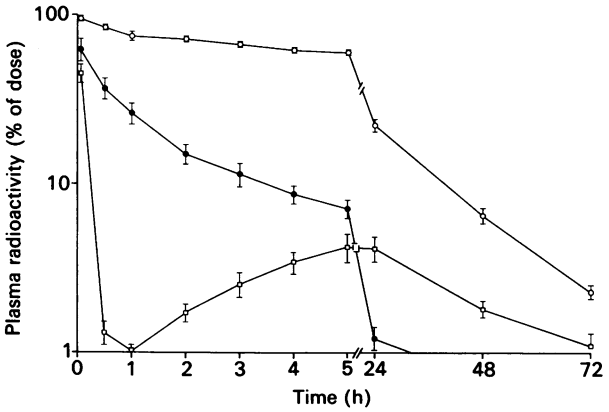

Fig. 1. Plasma radioactivity during 72 h after injection of [^{3}H]cholesteryl oleate-labelled LDL in control (○) and oestrogen-treated rats (●), or of [^{3}H]cholesteryl oleate-labelled acetyl-LDL (□)

Values represent the percentage of the administered amount of radioactivity present in the plasma compartment. The 100% value was calculated from the administered dose and the rat's plasma volume; plasma volume was determined as described by Bijsterbosch *et al.* (1981). Results are means ± S.E.M. ($n = 6$).

very rapidly from the circulation. At 1 h after injection almost no radioactivity was left in the plasma compartment. Thereafter, plasma radioactivity increased again, and remained at a value of about 5% for up to 24 h. Density-gradient centrifugation of plasma obtained at 5 h after injection of acetyl-LDL revealed that, at this time point, radioactivity was no longer associated with acetyl-LDL, but was mainly recovered in the high-density-lipoprotein range.

The biliary secretion of radioactivity under the three experimental conditions is illustrated in Fig. 2. Biliary secretion showed an initial peak in the second hour after administration of LDL. At the longer time intervals a clear circadian variation was noticed, with maximal secretion during the dark periods. The initial peak in biliary secretion in EE-treated rats was 5 times higher than in control rats receiving LDL, and secretion remained significantly higher during 48 h after injection. The secretion of acetyl-LDL-derived radioactivity was initially intermediate between that of LDL in control and EE-treated rats, but increased to values similar to those in the latter group between 6 and 9 h after injection. Radioactivity in bile was mainly in the form of bile acids under all three conditions studied, the remainder being free cholesterol (Fig. 3). The ratio [^{3}H]cholesterol to [^{3}H]-bile acids (Fig. 3, 'Ratio') was markedly increased in the EE-treated rats compared with controls receiving LDL. This ratio was lower for acetyl-LDL than for LDL in control rats.

The specific radioactivity of secreted bile acids was initially very low, owing to the secretion of 'preformed' bile acids from the intestinal bile acid pool, but rapidly increased after the exhaustion of this pool. The specific radioactivity of the biliary cholesterol fraction was 40–60 times that of the bile acid fraction during the first 1 h after injection of LDL, in both controls and EE-treated rats (Fig. 4). For acetyl-LDL, this ratio was initially only

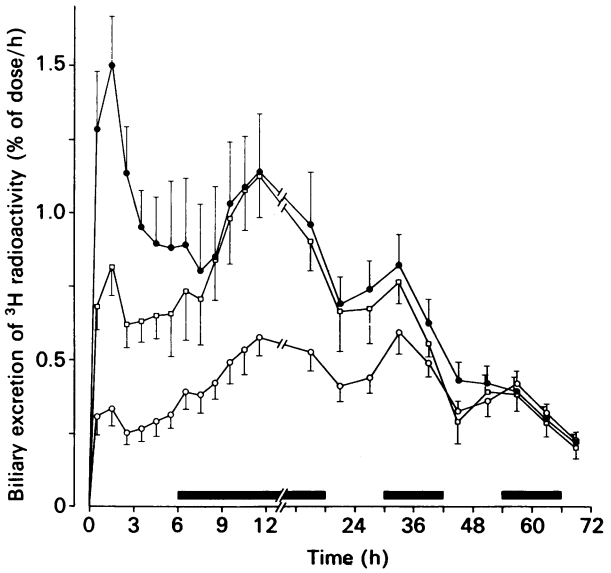

Fig. 2. Biliary excretion of ^{3}H radioactivity during 72 h after injection of [^{3}H]cholesteryl oleate-labelled LDL in control (○) and oestrogen-treated rats (●), or of [^{3}H]cholesteryl oleate-labelled acetyl-LDL (□)

Results are means ± S.E.M. ($n = 6$). Black horizontal bars indicate the dark periods.

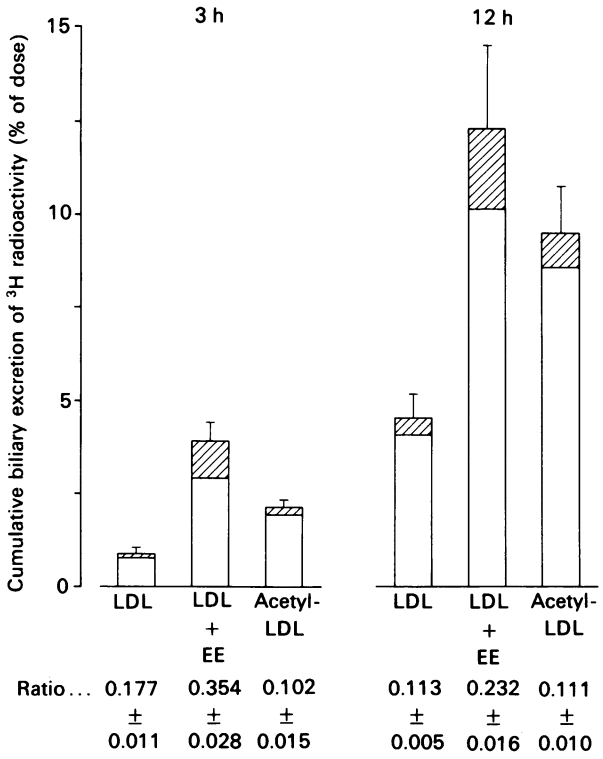

Fig. 3. Total amount of radioactivity excreted into bile at 3 h and 12 h after injection of [^{3}H]cholesteryl oleate-labelled lipoproteins, and amount of radioactivity in the cholesterol (▨) and bile acid (□) fractions

Results are means ± S.E.M. ($n = 6$). The 'Ratio' is [^{3}H]-cholesterol/[^{3}H]bile acids.

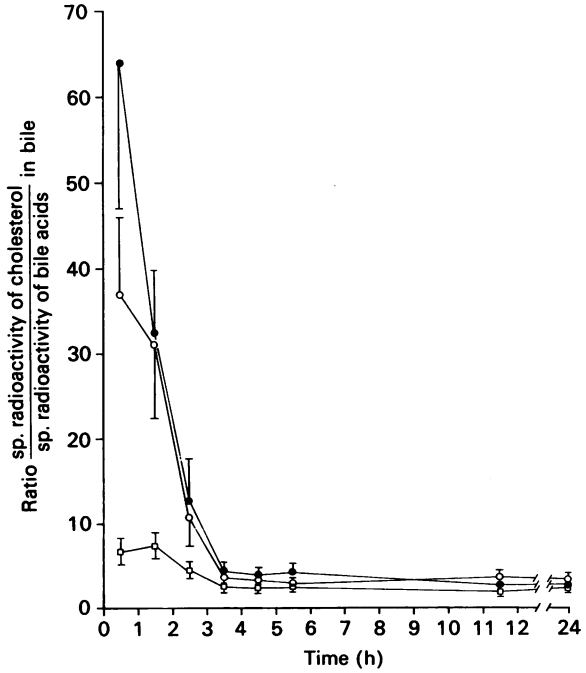

Fig. 4. Ratio between specific radioactivities of cholesterol and the bile acids in bile during 24 h after injection of [^{3}H]cholesteryl oleate-labelled LDL in control (○) and oestrogen-treated rats (●), or of [^{3}H]cholesteryl oleate-labelled acetyl-LDL (□)

Results are means ± S.E.M. ($n = 6$).

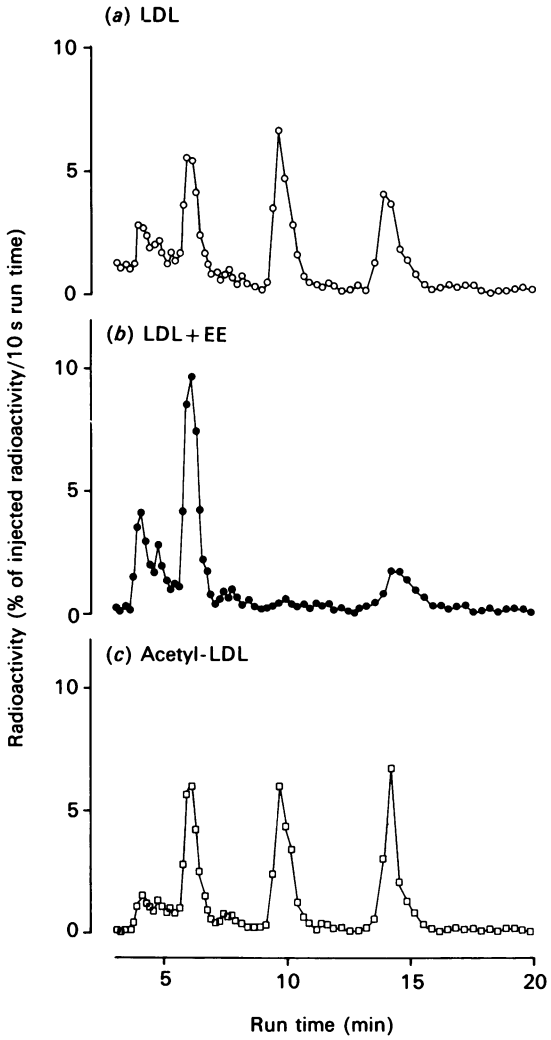

Fig. 5. H.p.l.c. elution profiles of ^{3}H-labelled bile acids in bile, obtained from representative experiments at 12 h after intravenous administration of [^{3}H]cholesteryl oleate-labelled LDL to control (○) or oestrogen-treated rats (●), or of [^{3}H]cholesteryl oleate-labelled acetyl-LDL (□)

Retention times of bile acid standards are: taurocholic acid 9.9 min, taurochenodeoxycholic acid 14.5 min. Conjugates of α- and β-muricholic acids are eluted between 5.5 and 7 min in this system (Kuipers *et al.*, 1986*b*).

about 6; in all three experimental conditions the ratio stabilized at 2–3 after the exhaustion of endogenous bile acid pool.

Radiolabelled bile acids synthesized from [^{3}H]-cholesteryl oleate in the various experiments were analysed by h.p.l.c. Fig. 5 shows the h.p.l.c. elution profiles of [^{3}H]bile acids in bile samples collected during the 12th hour after lipoprotein administration in representative experiments [the h.p.l.c. procedure does not allow separation of the conjugates of α- and β-muricholic acid (Kuipers *et al.*, 1986*b*)]. The pattern of bile acids formed from [^{3}H]cholesteryl oleate carried by LDL and by acetyl-LDL was very similar in untreated rats. In both cases, taurocholic acid was a main product. In contrast, almost no ^{3}H-labelled cholic acid conjugates were found in bile of EE-treated rats injected with LDL. In this case, radioactivity was co-eluted with the muricholic acid fraction and, to a lesser extent, with taurochenodeoxycholic acid.

DISCUSSION

In the present study, the delivery of lipoprotein-associated cholesteryl ester from blood to bile was investigated by comparing three different routes for hepatic uptake of the lipoprotein. Well-characterized lipoprotein particles were used, of which the receptor-mediated uptake in various hepatic cell types was established in previous studies (van Berkel *et al.*, 1982; Harkes & van Berkel, 1983, 1984*a*,*b*; Nagelkerke *et al.*, 1983, 1986). [^{3}H]Cholesteryl oleate was incorporated into the particles by biological procedures, in order not to alter their behaviour *in vivo* (Nagelkerke *et al.*, 1986). Because rat plasma lacks a cholesteryl ester transfer protein (Barter & Lally, 1978), the label remained associated with the particles until they were removed from the circulation (Nagelkerke *et al.*, 1986).

Our results indicate that the introduction of LDL cholesteryl oleate into hepatocytes, after induction of LDL (apo B,E) receptors on these cells by EE (Chao *et al.*, 1979), leads to an accelerated appearance of radioactivity in bile. Already at 1 h after injection of LDL most of the radioactivity in the bile was present in the form of bile acid. This indicates that uptake, hydrolysis and conversion into bile acids of LDL cholesteryl oleate occurs within a 1 h time course. Although the amounts of biliary radioactivity were smaller with acetyl-LDL and LDL injected in control rats, such a rapid metabolic conversion into bile acids was also noticed in these experiments. Although the initial uptake of acetyl-LDL into hepatocytes (37% of injected dose at 10 min after injection; Harkes & van Berkel, 1984*a*; Nagelkerke *et al.*, 1986) is quantitatively comparable with that of LDL in EE-treated rats (30% of injected dose at 30 min after injection; Nagelkerke *et al.*, 1983), similar biliary secretion rates of radioactivity were reached only after 6 h. This suggests that the EE-induced LDL receptor is more tightly coupled to bile secretion than is the scavenger receptor from parenchymal cells which is involved in the acetyl-LDL recognition process (van Berkel *et al.*, 1982). Initial uptake of LDL by Kupffer cells, as occurs in control rats (Harkes & van Berkel, 1983, 1984*a*), was only secondarily coupled to bile secretion (see Nagelkerke *et al.*, 1986), probably owing to the anatomical localization of the Kupffer cells in the liver. Apparently, relatively slow secondary transport processes are involved in transfer of cholesterol from Kupffer cells to parenchymal cells, eventually leading to its biliary secretion. The observed increase in plasma radioactivity in acetyl-LDL-injected rats and the association of this radioactivity with lipoproteins in the high-density range suggests processing of these particles by endothelial cells and subsequent release of the cholesterol moiety to plasma lipoproteins, which may eventually transport it to parenchymal cells. Such an 'intrahepatic reverse cholesterol transport' may also be operative in transport of cholesterol from Kupffer cells to hepatocytes (Kuipers *et al.*, 1986*b*).

Biliary secretion of radiolabelled bile acids showed a clear circadian rhythm, with maximal secretion in the dark periods. This pattern corresponds well to that described for the activity of 7α-hydroxylase (Mitropoulos

et al., 1973), the rate-limiting enzyme in bile acid synthesis, and for biliary bile acid output in the bile-fistula rat (Ho & Drummond, 1975; Vonk *et al.*, 1978). Only a small proportion of biliary radioactivity was in the form of free cholesterol. The relative amount of [^{3}H]-cholesterol was larger in bile of EE-treated rats than in that of untreated rats after injection of LDL. However, the ratio of the specific radioactivities of biliary cholesterol to that of bile acids was similar in both groups. During the period with high bile acid secretion immediately after interruption of the enterohepatic circulation, predominantly caused by 'preformed' bile acids from the intestinal pool, this ratio was 40–60. The ratio was 7–10 times lower for acetyl-LDL-derived radioactivity; this again may be the result of different metabolic behaviour after uptake by another receptor mechanism (i.e. the scavenger receptor). In all experiments, however, this ratio stabilized at values in the range 3–6, indicating that lipoprotein cholesteryl ester enters a pool from which it can be recruited for bile acid synthesis but is preferentially used for direct secretion into bile.

Besides its effect on hepatic lipoprotein uptake, EE exerts a number of additional effects on hepatic cholesterol metabolism, and also on the process of bile formation. Oestrogen treatment increases the activity of the enzyme acyl-CoA:cholesteryl acyltransferase in hepatocytes, resulting in storage of increased amounts of cholesteryl esters in the liver (Nagelkerke *et al.*, 1986; Davis *et al.*, 1978). Furthermore, it decreases the activity of 7α-hydroxylase (Bonorris *et al.*, 1977; Davis *et al.*, 1986) and the maximal secretory rate of bile acids and the magnitude of the BAIF (Gumucio & Valdivieso, 1971). We found a 40% decrease in the BAIF, which is in good agreement with studies in which similar doses of the oestrogen have been applied in rats (Gumucio & Valdivieso, 1971).

A very striking observation was the complete absence of radiolabelled cholic acid conjugates from bile of the treated animals, and the relatively increased formation of very polar bile acids, including the muricholic acids, from [^{3}H]cholesteryl oleate. Kern *et al.* (1977) reported a decrease in biliary cholic acid content and a simultaneous increase in 6-hydroxylated bile acids after administration of 1 mg of EE/day for 5 days to male Sprague–Dawley rats. However, the relative amount of cholic acid was still 33% in their study. Since those authors studied bile acid composition in bile samples collected over a period of 3–4 h immediately after creation of a bile fistula, this cholic acid may have been maintained in the intestinal bile acid pool during the treatment period. The observed changes in synthesis of the various bile acids from [^{3}H]cholesteryl oleate may have been a response to the induced cholestasis, analogous to the increase in β-muricholic acid formation seen after bile-duct ligation in the rat (Kinugasa *et al.*, 1981). On the other hand, the effect may have been more specific. For instance, EE may alter the activity of the microsomal enzymes 12α-hydroxylase and/or 26-hydroxylase, which have been claimed to be of regulatory importance for the ratio between newly synthetized cholic acid and chenodeoxycholic acid in rats (Björkhem *et al.*, 1973).

The present results show that LDL cholesteryl ester comprises only a small fraction for biliary steroid secretion in the rat, which is in accordance with findings by Bhattacharya *et al.* (1986). This, and also the relatively slow secretion of cholesteryl esters from acetyl-LDL, can probably be explained by initial uptake of these lipoproteins by hepatic sinusoidal cells, followed by their degradation and re-entry of cholesterol in the blood compartment, and the eventual secondary delivery of the cholesterol moiety into a pool available for bile acid synthesis in the hepatocytes. Uptake of LDL and of modified (acetyl) LDL by hepatic sinusoidal cells may provide the body with a protective mechanism which converts these atherogenic cholesterol vehicles into a less harmful species, i.e. high-density lipoprotein. Such a mechanism may especially be of importance in situations with low LDL-receptor activity on hepatocytes, as observed in untreated rats, or after diet-induced down-regulation of hepatocyte LDL receptors in other animals, including man.

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