Developmental expression of elements of hepatic cholesterol metabolism in the rat

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Abstract The expression of several key enzymes and receptors of rat hepatic cholesterol metabolism was studied during development. Among major findings were: acyl coenzyme A:cholesterol acyltransferase, the cholesteryl ester hydrolases, cholesterol-7α-hydroxylase and the α2-macroglobulin receptor (LRP) were very low in fetal livers, but all were induced shortly before birth, suggesting that these elements are important for extrauterine life. Although the other elements continued to increase, by day 6 of postnatal life, cholesterol-7α-hydroxylase had reached undetectable levels. It reappeared by day 12 of suckling, placing it in the group of late-appearing activities necessary for the fully mature hepatic phenotype. Changes in acyl coenzyme A:cholesterol acyltransferase activity appeared due predominantly to changes in amount of active protein. The cholesteryl ester hydrolase (CEH) activities all showed different developmental patterns, suggesting that each was a unique activity. The bile salt-dependent CEH activity was much higher in the suckling period than in the adult where it was almost undetectable, suggesting that this CEH may have its major importance in the suckling period of development. Low density lipoprotein receptors exhibited a pattern very different from that of the α2-macroglobulin receptors and did not show consistent correlation with any other elements. At some developmental time points, the relationships amongst the elements differed significantly from the adult pattern. These studies provide for the first time an integrated picture of developmental expression of key elements of hepatic cholesterol metabolism and set the stage for further studies on their modes of regulation.—**Smith, J. L., S. R. Lear, and S. K. Erickson.** Developmental expression of elements of hepatic cholesterol metabolism in the rat. *J. Lipid Res.* 1995. **36:** 641–652.

Supplementary key words acyl coenzyme A:cholesterol acyltransferase • cholesteryl ester hydrolase • cholesterol-7α-hydroxylase • 3-hydroxy-3-methylglutaryl coenzyme A reductase • low density lipoprotein receptor • α2-macroglobulin receptor (LRP)

The liver plays an important role in maintaining cholesterol homeostasis in the body. In the adult this is achieved by integrated regulation of a group of hepatic enzymes, receptors, and other proteins key for cholesterol, lipoprotein, and biliary metabolism. However, to date the interactions and regulation of these pathways to maintain cholesterol homeostasis during development have not been systematically studied in an integrated fashion.

The developmental pattern of activity (1–5) and specific mRNA content (6) for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme for mevalonate and isopentenoid synthesis, including cholesterol, has been reported in rat liver during development. Limited data are available on the response of hepatic acyl coenzyme A:cholesterol acyltransferase (ACAT) activity (7, 8) as are limited data on expression of rat β-very low density lipoprotein (VLDL) binding sites (recognizing both apolipoprotein (apo) E and apoB) during development (9). The mRNA level for the hepatic low density lipoprotein (LDL) receptor has been examined at some postnatal developmental points in rats (10), but no data have been reported on the expression of LDL receptor protein during development. No data have been reported on ontogeny of the LDL receptor-related protein (LRP, α2-macroglobulin receptor), proposed to be involved in clearance of apoE-containing lipoproteins (11–13). The patterns of rat liver cholesteryl ester hydrolase (CEH) activities during development also have not been reported. The developmental expression of mRNA for a variety of apolipoproteins and lecithin:cholesterol acyltransferase (LCAT) has been determined in rat liver (14–19), and changes in plasma lipoprotein patterns (9, 20–23) and plasma LCAT (9) during some stages of development have been described. Data also have been published on developmental expression of elements of bile acid synthesis, secretion, and metabolism (24–31).

We hypothesized that changes in the relationships or expression of these elements occur at key points in development, for example, when the animal as a whole is known to undergo dietary and hormonal changes, e.g., at

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CEH, cholesteryl ester hydrolase; ACAT, acyl coenzyme A:cholesterol acyltransferase; LDL, low density lipoprotein; LRP, low density lipoprotein receptor-related protein.

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birth, after first suckling, at weaning (for general review see ref. 32). Further, we hypothesized that during the suckling period, (when liver morphology and hepatospecific functions begin to mature, with minimal known changes in diet and hormonal pattern) changes in expression of these elements are the result of an intrinsic liver differentiation program.

As a first step in understanding how the elements of cholesterol metabolism are regulated developmentally, we investigated the expression of activities of ACAT, cytosolic and microsomal CEH (acidic, neutral, and bile acid-dependent activities), cholesterol-7α-hydroxylase (both activity and protein level), the LDL receptor, and α2-macroglobulin receptor (LRP) in rat livers from 19-day-old fetuses at time intervals through to 28-day-old animals (1 week post weaning) and compared these values with those in the adult rat liver. The rat was chosen because the developing rat liver provides a good model for studying human hepatic development (33). HMG-CoA reductase activity was used as a developmental reference point in hepatic cholesterol metabolism because the most complete studies to date have been reported for this enzyme (1–6).

EXPERIMENTAL PROCEDURES

Rats

Adult female (264–305 g), male (317–346 g), and timed pregnant (250–395 g) Sprague-Dawley rats (Bantin and Kingman, Newark, CA) were housed under reverse illumination (lights on 3 PM; lights off 3 AM) for at least 1 week prior to experimentation. They were allowed free access to food (Purina Rat Chow) and water at all times. Animals were anesthetized with isoflurane before collection of liver. All livers were harvested at or about 9:00 AM (D6), except for two neonatal (2 h post birth) litters that were collected at 3:40 PM and 6:15 PM. All litters contained between 9 and 14 pups or fetuses. Fetal liver tissue was obtained after hysterectomy, removal of the fetuses, and decapitation. Livers from fetal, neonate, and suckling rats were pooled as necessary to yield at least 2 grams of tissue. Rats were weaned at 21 days old. All protocols were approved by the Animal Studies Subcommittee at the VA Medical Center, San Francisco.

Preparation of liver fractions

Microsomal membranes and cytosolic fractions were prepared as previously described (34). A sinusoidal plasma membrane-enriched fraction also was prepared as previously described (34). All preparations were from freshly isolated livers.

Enzyme assays

Enzyme activities were measured in freshly prepared microsomes or cytosol. No sex differences in enzyme activities, protein content, or lipoprotein receptors were observed in animals 28 days old or younger; therefore, results for each time point and element assayed were pooled. HMG-CoA reductase activity was assayed as previously described (35). Endogenous ACAT activity was measured essentially as described previously (36); the assay volume of 0.2 ml contained 75–300 μg microsomal protein, 135 μl ACAT buffer (0.25 M sucrose, 1 mM EDTA, 0.1 M Tris, pH 7.5), 335 μg bovine serum albumin and 5 nmol [^{14}C]oleoyl-CoA (38,000 dpm/nmol). Assay of the activity at apparent V_{max} was accomplished by addition of exogenous cholesterol in the form of cholesterol:egg phosphatidyl choline (PC) (1:8 by weight) liposomes prepared by sonication in a bath sonicator (Laboratory Supplies Company, Inc., Hicksville, NY). Routinely, 8 μg cholesterol per assay was used. In the absence of exogenous cholesterol the ACAT assay was incubated for 5 min and in the presence of exogenous cholesterol, for 50 min (shown to be optimal) before addition of the [^{14}C]oleoyl CoA substrate. Assay time was 2 min in both assays.

Neutral CEH activities were assayed in both microsomal and cytosolic fractions. Acidic CEH activity was assayed only in the microsomal fraction. The method for CEH assays was based on that described by Brecher et al. (37) and Pittman, Khoo, and Steinberg (38), using cholesterol [^{14}C]oleate:egg PC liposomes (1:50 by weight) prepared by sonication as above. Microsomes were assayed at pH 4.5 (acidic hydrolase) or pH 8.0 (neutral hydrolase) and cytosol, at pH 8.0 in a total assay volume of 0.3 ml buffer containing 60 mM buffer Tris-acetate, pH 4.5, or Tris-HCl, pH 8.0, and 6 mM mercaptoethanol, liposomes (cholesterol [^{14}C]oleate; 4 μg, 330,000 dpm), and microsomal or cytosolic protein (0.5–2.5 mg). The assay for bile salt-dependent CEH activity was that for neutral CEH activity with the addition of 20 mM sodium taurocholate. Assay time for all CEH was 30 min. CEH activities were expressed as pmol cholesterol oleate hydrolyzed per min per mg protein. The concentration of endogenous cholesteryl ester in microsomal or cytosolic fractions was not included in the calculation of activity because the availability of endogenous cholesteryl ester for the CEH reaction is unknown; in most cases, the exogenous concentration of cholesteryl ester greatly exceeded that of the endogenous. All assay conditions were such that measurements were in the linear ranges of activity.

Cholesterol-7α-hydroxylase was assayed essentially as described by Jelinek et al. (39) using [^{14}C]cholesterol as substrate, except that cholesterol was delivered as cholesterol:PC liposomes (1:8 by weight) prepared by sonication as above, and an NADPH-regenerating system (glucose-6-phosphate dehydrogenase, NADP, and glucose-6-phosphate) was included in the assay as a source of NADPH. Cholesterol-7α-hydroxylase protein level was assessed after SDS gel electrophoresis and transfer to nitrocellulose

as described previously (35) by immunoblotting using a rabbit polyclonal antiserum to the C terminal portion of the protein provided by Dr. Roger Davis. In preliminary studies, conditions were determined such that peak heights on densitometric scan were proportional to the amount of protein applied to the gel. Pooled rat liver microsomes from untreated adult rats served as a reference standard run concurrently on the same gel.

Hepatic LDL receptor concentration

LDL receptor protein content was estimated in sinusoidal plasma membrane-enriched fractions by SDS gel electrophoresis under reducing conditions followed by transfer and immunoblotting as described previously (35) using an LDL receptor-specific rabbit polyclonal antiserum provided by Dr. Janet Boyles. In preliminary studies, conditions were determined such that peak heights on densitometric scan for the LDL receptor were proportional to protein concentration applied to the gel.

α2-macroglobulin receptor (LRP) concentration

LRP content in sinusoidal plasma membrane-enriched fractions was estimated by $^{45}Ca^{2+}$ blotting based on the method of Maruyama, Mikawa, and Ebashi (40) after SDS gel electrophoresis under nonreducing conditions on 5% polyacrylamide gels followed by transfer to nitrocellulose. The blots were washed four times in 200 ml of blotting buffer (60 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole, pH 6.8) for 30 min each. They were then incubated in blotting buffer (50 ml containing 50 μCi $^{45}Ca^{2+}$, 37 mCi/mg, NEN) at room temperature for 10 min. The blots were washed by immersing them successively into three separate containers containing 100 ml of 50% ethanol for a 90-sec period in each. The blots were then allowed to air dry. The radioactive bands were visualized by autoradiography and quantified by densitometry. Pooled rat liver microsomes from untreated adult rats served as a reference standard run concurrently on the same gel. The reference standard and each sample were run in triplicate. In preliminary studies, conditions were determined such that densitometric peak heights for LRP were proportional to protein concentration applied to the gel.

Other assays

Liver cytosolic and microsomal total and free cholesterol concentrations were determined as described by Salé et al. (41). Protein was estimated by the biuret method (42) using bovine serum albumin as reference standard.

RESULTS

Hepatic HMG-CoA reductase activity

As the most complete developmental data available for an element of hepatic cholesterol metabolism are those for HMG-CoA reductase (1-6), the developmental pattern for this enzyme activity was determined in livers of animals at 19 days gestation and at time intervals through to the post weaned period in order to provide a frame of reference for the other elements to be assayed in the same livers. The results were similar to those described previously (1-5). At 19 days gestation, HMG-CoA reductase activity was 20% of adult activity (**Fig. 1**). It increased with gestation time and following birth until day 3 of suckling when it reached 55% of adult value ($P < 0.005$). HMG-CoA reductase activity declined steadily thereafter; by days 17 and 18 of suckling, the activity was only 1.2% and 2.3%, respectively, of the adult value. However, by day 19, as the pups began to wean themselves (evident by the presence of solid food in their stomachs), HMG-CoA reductase activity increased, and in livers from 24-h postweaned animals, it was 2.5-fold greater than the adult value ($P < 0.001$). By 28 days (1 week postweaned), HMG-CoA reductase activity level was similar to that of adult.

Hepatic ACAT activity

The developmental pattern of ACAT activity was determined using two different assay conditions. The first assay, which used the endogenous cholesterol as substrate, reflected the level of ACAT activity in vivo, while the

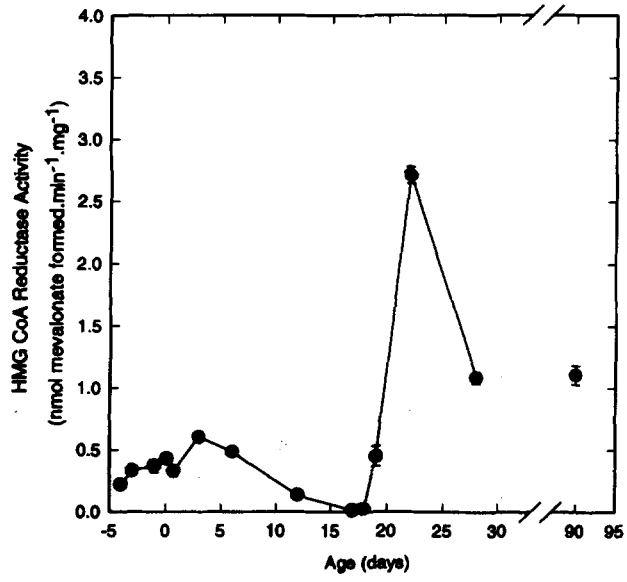

Fig. 1. Developmental pattern of hepatic HMG-CoA reductase activity. Preparation of microsomes and assay of HMG-CoA reductase were as described in Methods. Day 0 refers to birth. Negative ages refer to fetal animals and indicate the number of days before birth. Pups were weaned at 21 days old. The numbers of individual experiments for the various developmental stages were: at day −4, 5; day −3, 1; day −1, 6; birth +2 h, 3; birth +18 h, 6; day +3, 5; day +6, 10; day +12, 8; day +17, 2; day +18, 3; day +19, 9; day +22, 5; day +28, 9; day 90, 12; 6 male and 6 female animals. No statistically significant difference was found between adult male and adult female values; therefore, adult values were pooled. Each point is the mean ± SE.

second assay, in the presence of exogenous cholesterol, measured the activity under apparent V_{max} conditions, and thus approximates the amount of active ACAT enzyme protein present. Previous studies (7, 8) had used only the first assay. When assayed using exogenous cholesterol, V_{max} ACAT activity at 19 days gestation was 3.3% of the adult level, suggesting very low levels of active enzyme protein were present (**Fig. 2**). The activity began to increase by 20 days gestation and reached a peak at 18 h after birth. ACAT activity continued to increase more slowly thereafter, reaching a value at day 17, about 1.5 times that of adult ($P < 0.02$). By days 18 and 19 of suckling, the rats began to wean themselves, and ACAT activity dropped to levels similar to adult. By 24 h ($P < 0.01$) and 1 week postweaned ($P < 0.001$), activity had decreased to levels about 65–70% of adult.

ACAT activity assayed using the endogenous cholesterol substrate pool was almost undetectable at day 19 of gestation but increased to about 50% of adult level ($P < 0.005$) just prior to birth (Fig. 2). By 2 h after birth, ACAT had increased to close to adult level, and after suckling (18 h time point), it had reached a level 3.2-fold that of adult ($P < 0.001$), likely, at least in part, as a response to influx of cholesterol from the mother's milk. Thereafter, levels of activity 2- to 4-fold those found in adult liver were maintained through to day 18 of suckling. By day 19, when the animals had begun to wean to a chow (low fat, low cholesterol) diet, activity had fallen to adult level, and it had decreased further at 24 h postweaned to 65% of adult ($P < 0.05$). However, by 28 days old (1 week postweaning) activity was 148% higher than that of adults ($P < 0.01$), in contrast to the V_{max} results, which were 35% lower than the adult level ($P < 0.001$). This suggested that a change in cholesterol substrate availability rather than a change in amount of active enzyme was responsible for the increase.

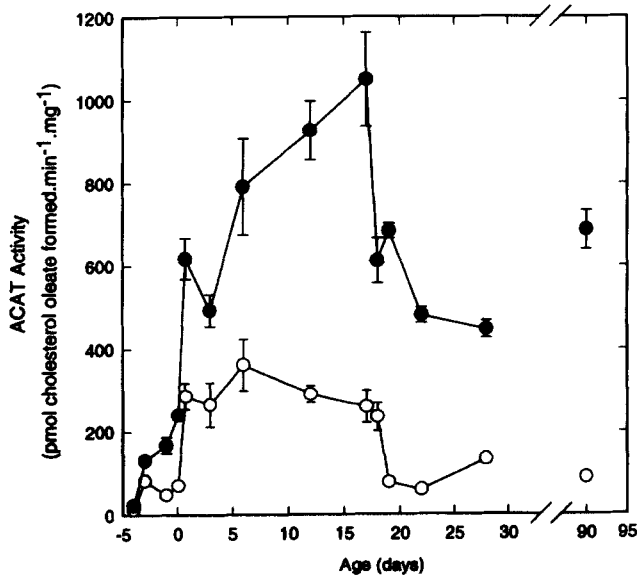

Fig. 2. Developmental pattern of hepatic ACAT activity. Activity was determined by two different assays as described in Methods. Experimental details are the same as for Fig. 1. (○) Activity determined in the absence of exogenous cholesterol (endogenous activity); (●) activity determined in the presence of exogenous cholesterol (apparent V_{max} activity). No statistically significant differences were noted between adult male and adult female values; therefore adult values were pooled. Each point is the mean ± SE.

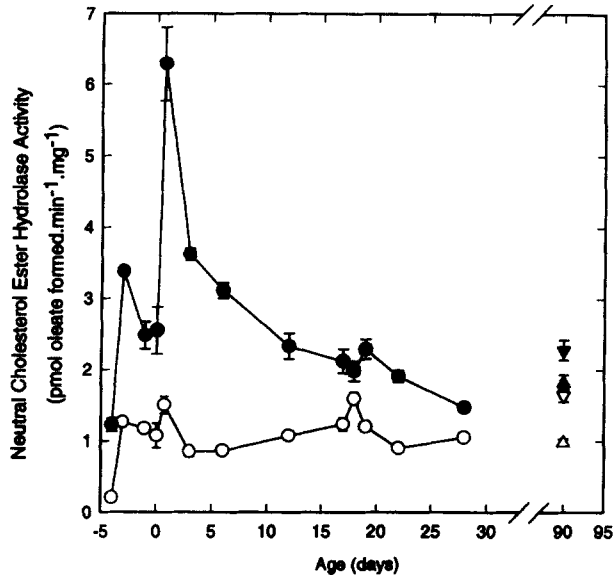

Fig. 3. Developmental pattern of neutral CEH activities in hepatic cytosol and microsomes. Neutral CEH was assayed as described in Methods. Samples analyzed are the same as for Fig. 1. Female adult activity (▽) was different from adult male activity (△) at $P < 0.0001$ for cytosolic CEH (n = 6 in each group) and for the microsomal CEH, at $P < 0.03$ (n = 6 in each group, (▼) female; (▲) male); (○) cytosol; (●) microsomes.

Neutral cholesteryl ester hydrolase activities

The specific activity of neutral CEH was lower in hepatic cytosol compared with hepatic microsomes at all developmental points (**Fig. 3**). The activity of the cytosolic CEH at 19 days gestation was only 16% of adult. The activity then began to increase, reaching adult levels by 22 days gestation. Near adult levels were maintained until postnatal day 3, when the activity in cytosol had dropped to 65% of adult activity ($P < 0.05$). This level then slowly increased to adult levels that were maintained until suckling day 18. After weaning, cytosolic levels first dropped (about 30%, $P < 0.005$) below adult levels then returned to near adult levels by 1 week postweaned.

In contrast to the cytosolic enzyme, the activity in microsomes at 19 days gestation was about 60% that of adult ($P < 0.001$, Fig. 3). It remained at a similar level until after suckling (18 h after birth) when it reached a level 3.1-fold that of adult ($P < 0.001$). By 3 days suckling it had dropped to a level 1.8-fold that of adult ($P < 0.001$), and continued to decrease until day 18, at which point microsomal CEH activity was similar to adult levels.

Although a small increase was observed at day 19, after weaning, activity began to decline once again, reaching about 72% that of adult ($P < 0.001$) by 1 week post-weaned.

Acidic cholesteryl ester hydrolase activity

At 19 days gestation, acidic CEH was 17% of adult level in microsomes ($P < 0.001$; **Fig. 4**). It increased with increasing gestation time, reaching a peak at 18 h postnatally (after suckling), 96–98% of adult levels. The activity dropped after the 18 h peak although by day 6 it was once again similar to adult level. This activity was maintained until just before weaning when it appeared to increase somewhat. One week after weaning it was again similar to adult level. By 1 week after weaning the activity had dropped to about 52% of adult ($P < 0.05$).

Bile salt-dependent cholesteryl ester hydrolase activity

The developmental pattern for this activity was different from those of the other CEH activities; in contrast to the acid and neutral CEH activities, the bile salt-dependent specific activity was much higher in cytosol than in microsomes and was much higher throughout the suckling period than in the adult. The pattern of bile salt-dependent CEH in cytosol (**Fig. 5**) showed two peaks of activity; the first, at 2 h postnatally (>500-fold higher than adult). Although the activity reached a low at day 3, it peaked again at day 6 ($P < 0.02$). By day 12 the activity had dropped to >200-fold adult level. This level was maintained until day 17 of suckling after which it dropped continuously, reaching adult level by 24 h post-weaned. Bile salt-dependent microsomal CEH increased at birth and after suckling (Fig. 5). It remained at similar levels of activity until after weaning, when it also decreased to adult levels. This pattern is different from that of the neutral CEH during development (Fig. 3) suggesting that they are different enzymes.

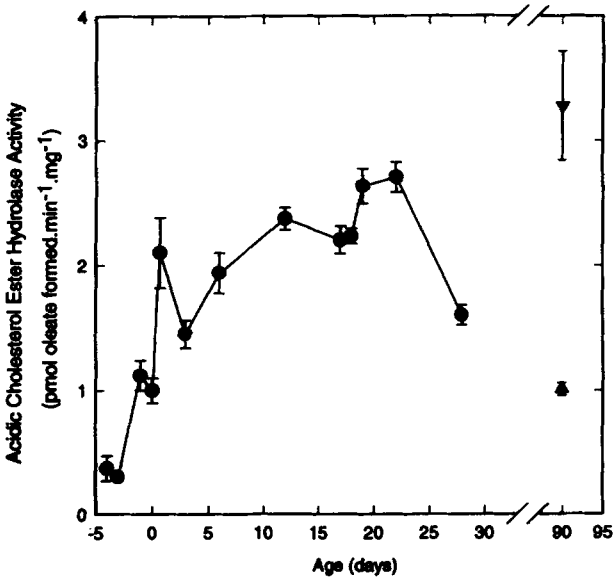

Fig. 4. Developmental pattern of acidic CEH activity in hepatic microsomes. Acidic CEH was assayed as described in Methods. Samples analyzed are the same as for Fig. 1. Female adult activity (▼) was different from adult male activity (▲) at $P < 0.0005$ (n = 6 in each group). Each point is the mean ± SE.

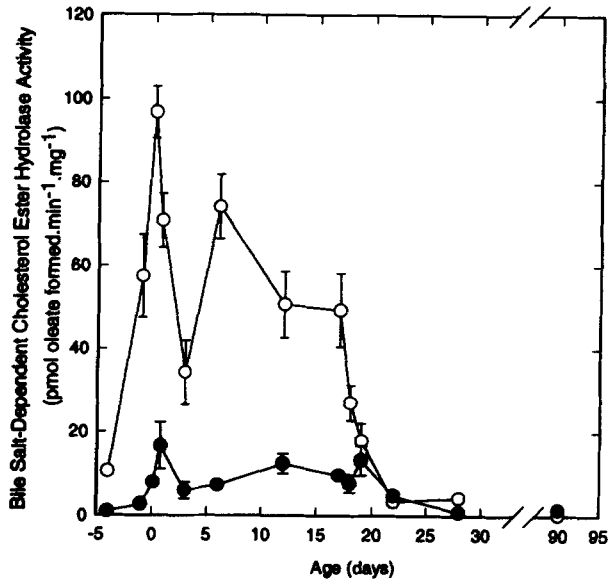

Fig. 5. Developmental pattern of bile salt-dependent CEH activity. The bile salt-dependent CEHs were assayed as described in Methods. Samples analyzed are the same as for Fig. 1. No statistically significant differences were found between adult male and adult female values; therefore, the adult values were pooled. Each point is the mean ± SE; (○) cytosol; (●) microsomes.

Cholesterol-7α-hydroxylase

Cholesterol-7α-hydroxylase activity was near the lower limits of detectability in fetal samples. However, within 18 h of birth (after suckling) it had increased to about 40% of the adult level (**Fig. 6**). By day 3 the activity had fallen to almost undetectable levels and remained at this level until day 12 when the activity began to rise, reaching adult levels by time of weaning. Immunoblotting for cholesterol-7α-hydroxylase protein in the same fractions used for enzyme assay showed that the protein followed a pattern similar to that of the activity (Fig. 6). The ratio of activity to protein units appeared to be different from that in the adult just before birth and at 1 week after weaning.

Hepatic LDL receptors

LDL receptor protein was detectable in liver samples from 19 day fetuses, but the levels were only 19% of those in adult (**Fig. 7**). Receptor concentrations increased thereafter until postnatal day 3 when levels were 2.2-fold above those of fetal ($P < 0.03$). This peak at 3 days suckling occurred coincident with changes in HMG-CoA re-

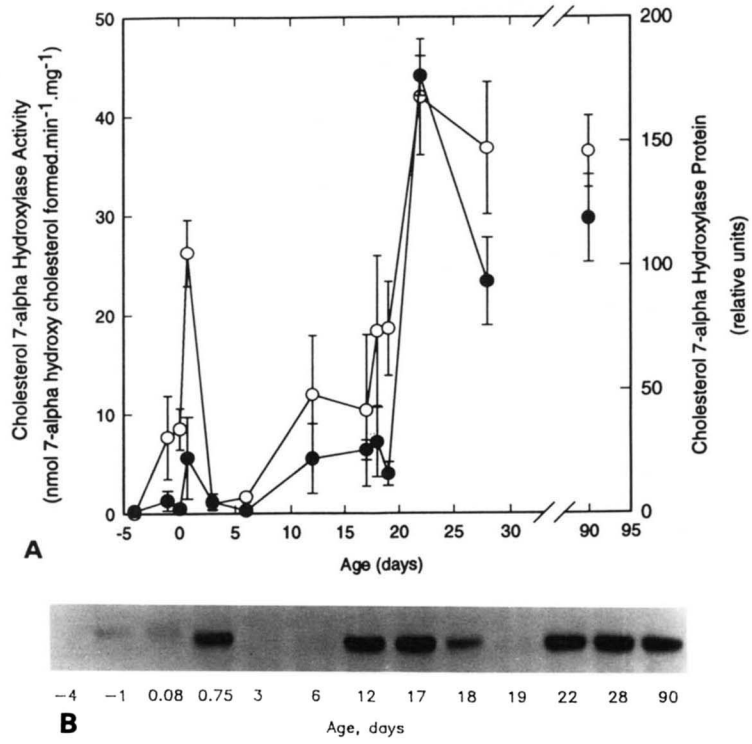

Fig. 6. Developmental pattern of cholesterol-7α-hydroxylase activity and protein. Microsomes were assayed for activity and for protein by immunoblotting as described in Methods. Samples analyzed are the same as for Fig. 1. No statistically significant differences were found between adult male and adult female values; therefore, adult values were pooled. Each point is the mean ± SE. Panel A: (●) activity; (○) protein. Panel B: representative immunoblot pattern.

ductase activity, "V_{max}" ACAT activity, cholesterol-7α-hydroxylase, and the CEHs suggesting a change in intracellular cholesterol trafficking. By 6 days suckling, receptor levels had dropped to 58% of adult level ($P < 0.05$). However, by day 12 LDL receptor protein had increased again to adult levels and remained at this level through day 18. After the pups had begun to wean themselves at day 19, LDL receptor levels increased to levels similar to those of adult. After weaning, levels fell again, and by 1 week post weaned, LDL receptor levels were 40% of adult level ($P < 0.01$).

Hepatic LDL receptor-related protein (α2-macroglobulin receptor)

LRP was almost undetectable at 19 days gestation, only 6% of adult level (**Fig. 8**). By 22 days gestation it had increased 6.9-fold ($P < 0.001$) and it continued to increase thereafter, reaching adult level by day 12 of the suckling period. Levels peaked at day 17 of suckling (125% of adult, $P < 0.05$), but by day 18, they had returned to levels similar to adult, and decreased thereafter to reach a level 74% that of adult by 24 h post weaned. LRP concentrations in 28-day-old (1 week postweaned) rat livers were about half those found in adult.

DISCUSSION

During the course of development, mammals undergo many changes at the physical, metabolic, and molecular levels. With regard to hepatic cholesterol metabolism, a highly integrated function in the adult, few of the elements involved have been studied simultaneously and usually only at limited time points, making it difficult to be certain how the various aspects of cholesterol metabolism are integrated during development. In the current work, a variety of elements (enzymes and lipoprotein receptors) of rat hepatic cholesterol metabolism were studied in the same livers, thus allowing greater overall insight into the mechanism(s) of regulation of cholesterol homeostasis during ontogeny.

The developmental pattern found for hepatic HMG-CoA reductase activity was similar to that reported previously by others (1–5). The relatively high activity during gestation presumably was to provide de novo synthesized cholesterol for growth and development as little cholesterol is provided by the mother (43). HMG-CoA reductase may also be higher just before birth, when its activity appears to be uncoupled from cholesterol synthesis (5) due to a greater demand for nonsterol isopentenoid

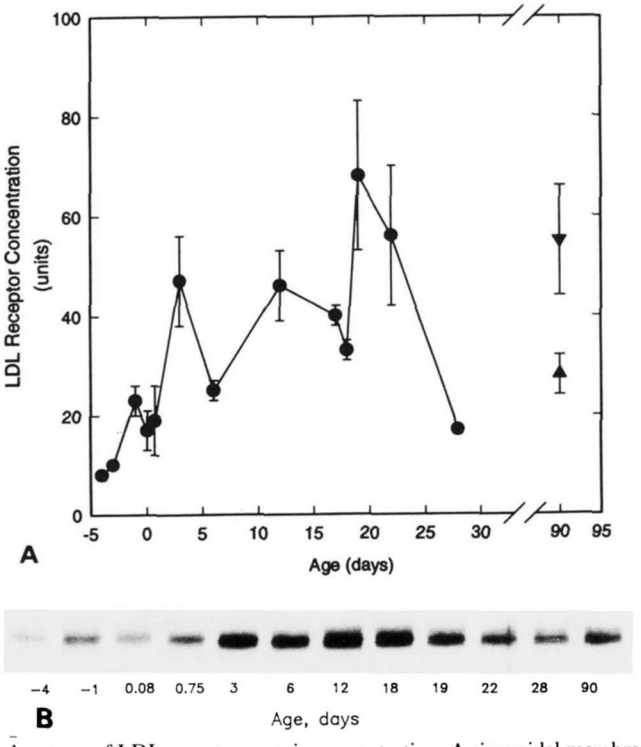

Fig. 7. Developmental pattern of LDL receptor protein concentration. A sinusoidal membrane-enriched fraction of rat liver was prepared and assayed as described in Methods from the same animals as described in Fig. 1. Adult female values (▼) were different from adult male (▲) at $P < 0.05$. Each point is the mean ± SE. Panel A: LDL receptor protein levels as determined by immunoblot. Panel B: representative immunoblot pattern.

products such as ubiquinone and heme *a,* required for mitochondrial maturation necessary for extrauterine life, and, potentially, for specific, yet to be identified prenylated proteins required for extrauterine life.

In contrast to HMG-CoA reductase, LDL receptor protein was expressed at low levels in the fetal livers. These results are in agreement with those of Belknap and Dietschy (43), who showed that although LDL was bound to fetal liver cells, neither apoB nor cholesterol were internalized, and those of Erickson et al. (9), who reported only very low internalization/degradation of β-VLDL by fetal hepatocytes. Thus, delivery of lipoprotein cholesterol to the liver may have been limited by receptor protein expression to allow greater availability of cholesterol to the fetal developing nervous system, adrenal glands, and other tissues.

Hepatic V_{max} ACAT activity increased just prior to and immediately after birth, but before suckling, from very low fetal levels to levels 20- to 30-fold higher, which suggested that hepatic ACAT is among the cluster of enzymes whose expression is essential for extrauterine life (44). This marked increase in V_{max} ACAT activity suggests that the activity increase just prior to birth is due to increased synthesis of active ACAT enzyme protein. These increases may partially reflect cellular needs for activity to cope with increased exogenous cholesterol influx into the liver after birth. The results using endogenous cholesterol as substrate are similar to those reported by others at the time points examined by them (7, 8). Increases in V_{max} activity preceded increases in the endogenous activity.

All CEH activities were very low in the fetal livers, but increased around the time of birth or shortly thereafter, suggesting that these activities are also important for extrauterine hepatic metabolism. A number of hepatic CEH activities have been described in a variety of subcellular compartments. Lysosomes appear in the liver at birth (45), consistent with the developmental pattern of the acidic CEH reported here, suggesting that at least a portion of the acidic activity assayed is of lysosomal origin. Acidic membrane-bound CEH activity similar to that described by Runquist and Havel (46) is also likely included. A neutral CEH activity has been reported in cytosol, microsomes, and plasma membrane (47, 48). That present in plasma membranes (48) may facilitate selective uptake of cholesteryl esters from lipoproteins (49). Bile salts have been shown to stimulate the activity of an hepatic cytosolic CEH at neutral pH in vitro (48, 50).

The developmental pattern of cholesterol-7α-hydroxylase was unusual in that both activity and protein were induced just before birth, both reaching high levels after first suckling. However, both then fall to almost undetectable levels by postnatal day 3. This pattern was unex-

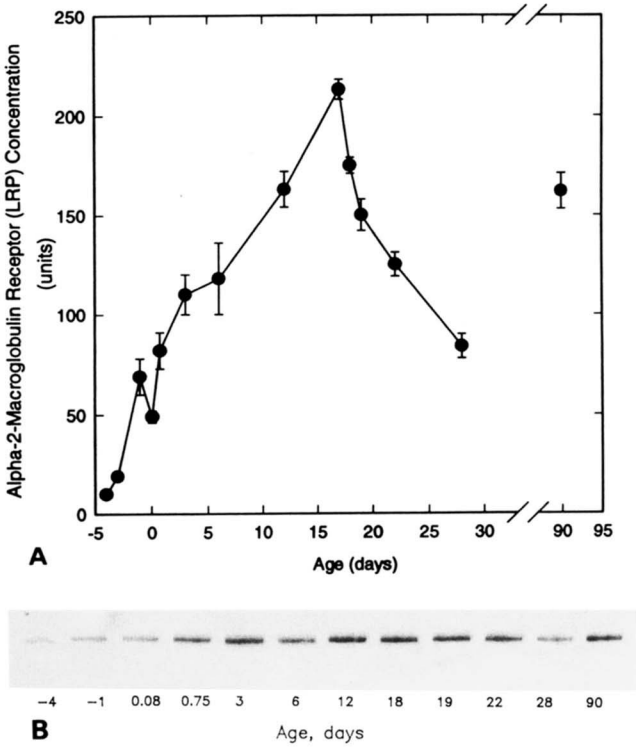

Fig. 8. Developmental pattern of LRP. A sinusoidal membrane-enriched fraction of rat liver was prepared and assayed as described in Methods from the same animals as described in Fig. 1. No statistically significant differences were found between adult male and adult female values; therefore, the adult values were pooled. Each point is the mean ± SE. Panel A: LRP receptor protein levels as determined by Ca^{2+} blot. Panel B: representative blot pattern.

pected and had not been reported previously. The early rise in 7α-hydroxylase is in contrast to other elements of biliary metabolism that are reported to develop more slowly (24–31). It may be in response to outward movement of the bile salt pool present in fetal livers, which begins by fetal day 21 (24). Maternal plasma hormonal increases that occur near the time of birth also might be responsible as, in the adult, 7α-hydroxylase is reported to respond to some of these hormones (51).

LRP or the α_2-macroglobulin receptor is able to bind apoE-containing lipoproteins and has been proposed to play a role in chylomicron remnant uptake (11–13). However, it also binds and internalizes a number of other ligands (52). Hepatic LRP was almost undetectable through the 19 day fetal stage, but it began to rise just prior to birth, reaching adult levels by the mid-suckling period. The developmental pattern for LRP is consistent with a role in chylomicron remnant metabolism because its levels were induced rapidly just before birth. This pattern suggested that hepatic LRP is also among those proteins necessary for extrauterine survival (44). Interestingly, it reached adult levels at a later stage than the hepatospecific asialoglycoprotein receptor, which attained adult levels by day 2 of suckling (53).

After birth, both HMG-CoA reductase activity and LDL receptors increased, peaking at day 3 of the suckling period, probably in response to increased hepatic growth and differentiation demands for both sterol and nonsterol metabolites of mevalonate. A peak of mitosis is reported to occur in this time period (54). Further, acinar metabolic zone formation also has begun (54), and after day 3, the amount of hepatocyte smooth endoplasmic reticulum begins to increase (45, 55, 56), reaching adult levels by day 12.

The decrease in HMG-CoA reductase activity after day 3 of suckling may be explained in part by suppression due to the high fat and cholesterol content of the dam's milk coupled with the increase in hepatic LDL receptors and LRP, allowing more exogenous cholesterol to enter the liver. However, by day 6, when reductase activity was very low, LDL receptors had also dropped considerably below adult levels, probably in response to the high cholesterol diet coupled with very low cholesterol-7α-hydroxylase activity. The peak in plasma LDL level reported at this time point by others (21) suggests that the increased production of hepatic cholesteryl esters via ACAT in this period may reflect an increased requirement for cholesteryl esters for transport in plasma lipoproteins

to satisfy needs of other developing organs for cholesterol, especially the brain and nervous system.

Rat hepatic cholesterol concentrations are essentially constant from birth (cf. refs. 1, 5); thus, from day 6 to just before weaning, when HMG-CoA reductase activity and cholesterol synthesis are very low (5, 57, 58), hepatic cholesterol concentrations are likely maintained by uptake of cholesterol from plasma lipoproteins, especially those of intestinal origin. Consistent with this notion is the observation that increased amounts of hepatic LDL receptor and LRP proteins are found in livers of suckling animals after the day 6 time point. Increased ACAT activity during the suckling period appeared, due in part to an increase in the amount of active ACAT protein as well as possible increase in substrate cholesterol availability.

The observed associations between hepatic LDL receptors and plasma LDL concentration during development suggest that hepatic LDL receptors are not the only determinant of plasma LDL levels nor is the plasma cholesterol level necessarily an indicator of hepatic LDL receptor content. For example, at days 12–15 of suckling, most of the triglyceride in plasma appears to be of intestinal origin, and the livers are reported to secrete only minimal levels of VLDL (23, 59), suggesting that LDL levels were maintained by decreased production of LDL in this time period rather than by changes in hepatic LDL receptor number. In addition to cholesterol, LDL receptors may be regulated by estrogens (60), growth hormone (61), insulin (62), glucocorticoids (63), and triiodothyronine (64). Thus, at some developmental time points, hormonal regulation may overrule cholesterol responses and any primary influence of plasma cholesterol level.

Staels and co-workers (10) measured mRNA levels for the LDL receptor in postnatal rat livers. There appeared to be no consistent correlation at the same time points between reported LDL receptor mRNA levels (10) and those of LDL receptor protein reported in the present work, suggesting that some of the LDL receptor regulation during development may be at the post-translational level.

All the CEH activities remained at a fairly constant level throughout the suckling period, suggesting that most of the cholesteryl ester homeostasis was regulated by ACAT. The drop in ACAT activity in late suckling likely reflects a combination of the decreasing level of milk cholesterol in the diet coupled with decreased VLDL secretion (23, 59), the low cholesterol synthesis rate (5), and increased cholesterol-7α-hydroxylase activity (this work and refs. 29, 31). These changes taken together would be expected to limit the ACAT cholesterol substrate pool and requirements for high levels of active enzyme.

Just prior to weaning, the acidic and neutral CEH activities increased to some extent, coincident with the rises in HMG-CoA reductase, LDL receptors, and cholesterol-7α-hydroxylase and drop in ACAT activity. However, the cytosolic bile acid-dependent CEH behaved very differently; it dropped rapidly from day 17 to a very low and variable adult level. The significance of the bile salt-dependent CEH in the adult is unknown at present, but its activity clearly is much lower than in the suckling animals. Interestingly, its secretion from the perfused adult liver has been reported (65). During the suckling period, pancreatic bile salt-dependent cholesteryl ester hydrolase secretion is very low (66). This suggests that the high level of the liver enzyme during this period may compensate for the lack of the pancreatic enzyme, and that it possibly might be secreted to aid in lipid absorption.

Microsomal neutral CEH specific activity was greater than that of cytosol; however, the total recovered hepatic activity of this CEH at most developmental stages was greater in the cytosolic fraction because of the greater recovery of protein (4- to 6-times) in the cytosol compared with the microsomes at all time points. The bile salt-dependent cytosolic enzyme was also quantitatively more important than the membrane-associated form.

Taken together, the data reported here indicate that no two CEH developmental patterns were precisely the same, suggesting that the activities assayed represent different enzymes, or isozymes subject to different regulation. The data further suggest that both cytosolic and membrane-associated CEHs have physiologically important roles in hepatic cholesteryl ester metabolism.

Cholesterol-7α-hydroxylase began to rise by day 12 in the mid-suckling period as reported also by others (29, 31). Plasma corticosteroid levels begin to rise about day 12 (67), suggesting that this may trigger the rise in hydroxylase. This second increase in 7α-hydroxylase occurs at a time when smooth endoplasmic reticulum has reached adult level (45, 55), apoB mRNA editing has begun (16), apoA-IV mRNA has started to rise (14), and the cytosolic bile acid-binding protein has reached near adult level (26). Thus, cholesterol-7α-hydroxylase in its second developmental phase appears to be part of a late cluster of elements of cholesterol metabolism that have hepatospecific functions required for the mature liver phenotype.

After weaning from milk to solid food (from a high fat, high cholesterol diet to a low fat- low cholesterol, high carbohydrate diet), hepatic HMG-CoA reductase activity rapidly increased, overshooting adult values by about 2-fold (this work and that of others, refs. 1–5). LDL receptors also increased. The change in insulin/glucagon ratio upon weaning (68) suggests that the changes in these two activities may be responding to these hormonal changes rather than solely to dietary changes as both LDL receptors and reductase have been reported to respond to insulin (62, 69). The increases in reductase and LDL receptors also suggest that the liver has an increased requirement for cholesterol in the late suckling and early weaned periods that can be met only by a combination of increases in both reductase and LDL receptors. This might reflect an increased demand for cholesterol re-

quired for cholesterol-7α-hydroxylase which had reached adult level at this time point.

In summary, hepatic cholesterol metabolism undergoes many changes during development. Most of these changes likely reflect growth and differentiation requirements. Although coordinate regulation of cholesterol metabolism similar to that described in human skin fibroblasts (70) appears to be present at some hepatic developmental stages, the elements clearly are uncoupled from this pattern at others. Apparent coordinate regulatory aspects of cholesterol metabolism found at some stages during development are similar to responses observed for normal adult liver, for example, in response to dietary change; others are reminiscent of constellations observed in the premalignant and malignant liver (35).

This investigation of hepatic cholesterol metabolism during development establishes the patterns of a number of its important elements and sets the stage for studies designed to determine whether the observed developmental changes are preprogrammed due to primary, genetically entrained, developmental expression patterns in the liver or whether they reflect those secondary to developmentally entrained hormone or hormone receptor synthesis. At some time points, it is likely that the changes are environmentally induced, for example, in response to change in dietary source and/or content, particularly at the time of birth and at weaning. This work will also provide the basis for future studies designed to determine the modes of regulation of the different elements of cholesterol metabolism during development and how they interact.

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